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GODA NORKUTĖ

CHARACTERIZATION OF POPULATIONS OF INVASIVE PATHOGENS - CAUSAL AGENTS OF THREE MAJOR FOREST TREE DISEASES: ALDER DECLINE, DUTCH ELM DISEASE AND ASH DIEBACK

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VILNIAUS UNIVERSITETAS
GAMTOS TYRIMŲ CENTRAS

GODA NORKUTĖ

TRIS SVARBIAS MIŠKO MEDŽIŲ LIGAS - ALKSNIŲ DŽIŪTĮ, GUOBŲ MARĄ IR UOSIŲ DŽIŪTĮ SUKELIANČIŲ INVAZINIŲ PATOGENŲ POPULIACIJŲ TYRIMAI

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ABBREVIATIONS

AldYp – Alder Yellows phytoplasma
AMEA – Ash leaf malt agar medium
AMOVA – Analysis of Molecular Variance
CA – Carrot agar medium
CAL – protein calmodulin
CTAB – cetyl-trimethylammonium-bromide
CU – protein cerato-ulmin
cu – gene cerato-ulmin
D – corrected Simpson's index
DAMD – Direct Amplification of Minisatellite DNA region
DAPC – Discriminant Analysis of Principal Components
DNA – deoxyribonucleic acid
DED – Dutch Elm Disease
EID – Emerging Infectious Disease
EAN – *Ophiostoma novo-ulmi* ssp. *novo-ulmi*
EAN x NAN – *Ophiostoma novo-ulmi* ssp. *americana* x *Ophiostoma novo-ulmi* ssp. *novo-ulmi*
GDP – Gross Domestic Product
HA – Hagem agar medium
I_A – Index of Association
IAS – Invasive Alien Species
ITS – Internal Transcribed spacer (DNA region)
IFP – Invasive Forest Pathogens
MLG – multilocus genotype
MP1 – multiplex reaction 1
MP2 – multiplex reaction 2
MSN – Minimum Spanning Network
mtDNA – mitochondrial deoxyribonucleic acid
Paa – Phytophthora alni ssp. *alni*
Pam – Phytophthora alni spp. *multiformis*
Pau – Phytophthora alni spp. *uniformis*
PCR – Polymerase Chain Reaction
Pm1 and *Pm2* – unknown origin *Phytophthora* species
P. x *a – Phytophthora* x *alni*
P. x *m – Phytophthora* x *multiformis*
P. u – Phytophthora uniformis
PARPNH-V8 – Medium, containing V8 juice and Ampicillin, Hymexazol, Nystatin, Pentachlornitrobenzene, Pimaricin, Rifampicin antibiotics
PCA – Principal Component Analysis
RAMS – Random Amplified Microsatellites
RAPD – Random Amplified Polymorphic DNA
rBarD – Standardized Index of Association
RFLP – Restriction Fragment Length Polymorphism
SB – Sodium Borate
SCAR – Sequence Characterized Amplified Region
SIS – Susceptible-Infected-Susceptible
s. l. – *sensu lato*

SMM – Stepwise Mutation Model
SNP – Single Nucleotide Polymorphism
s. s. – *sensu stricto*
SSR – Simple Sequence Repeat
vc – vegetative compatibility
VCG – vegetative compatibility groups
WSL – Swiss Federal Institute for Forest, Snow and Landscape Research

INTRODUCTION

Emerging Infectious Diseases (EIDs) are usually associated with biological invasions – introduction of alien pathogenic microorganisms. The main threat of EIDs for many plant populations is the possibility of genetic depletion that could happen due to repeated plant losses and offspring reduction (Santini *et al.*, 2013). Causal agent of EID can be defined as an organism that causes harm and: i) changes the manner in disease development; ii) has more than one host; iii) has been newly recognized, discovered or evolved (Anderson *et al.*, 2004). Human-caused environmental change is one of the main forces in the emergence of plant EIDs that are mainly caused by organisms accidentally introduced to new geographical areas (Daszak *et al.*, 2000). These harmful organisms are commonly referred to as Invasive Alien Species (IAS) (Anonymous, 2018a), while in forest pathology they are usually described as Invasive Forest Pathogens (IFPs) (Santini *et al.*, 2013). Fungi and fungi-like organisms are among the main causal agents of forest plant EIDs (Anderson *et al.*, 2004). North America and Asia are the two continents from where the majority of IFPs are introduced to Europe (Santini *et al.*, 2013). The main IFP introduction pathways are potting media, living plants, timber, wood packing and seeds (Desprez-Loustau *et al.*, 2009). Santini *et al.* (2013) showed that since 1990s, the rate of introduction of oomycetes has grew impressively in Europe, the introduction rate of ascomycetes is continuously increasing, and only basidiomycetes have a constant introduction rate.

The invasion of a pathogen and the severity of the disease it causes are influenced by secondary factors, such as severe weather events, socio-economic development or technological advances in agriculture and the absence of these factors could have an impact on localized or limited pathogen introduction in new area (Anderson *et al.*, 2004). Environmental conditions hindering organisms from dispersing are called dispersal barriers and are one of the most important limitations for species distribution in the world (Holt *et al.*, 2013). Due to increasing global trade, these barriers have started to lose their efficacy

and alien (non-native) species, including plant pathogens, can now more easily enter new environments (Fisher *et al.*, 2013). For example, during last 30 years the number of invasive plant pathogens has doubled in Europe (Santini *et al.*, 2013). In Lithuania, during the past century more than 100 invasive plant pathogens have been identified (Motiejunaite *et al.*, 2017). There are numerous examples of accidental introduction of exotic plant pathogens to European continent followed by disease epidemics, including Dutch elm disease caused by *Ophiostoma novo-ulmi* Brasier (Brasier, 1996a), ash dieback caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (Kowalski, 2006), or chestnut blight caused by *Cryphonectria parasitica* (Murrill) Barr (Rigling & Prospero, 2017).

Usually knowledge about biology, ecological preferences, interaction with other microorganisms, and distribution of a newly to a certain country introduced plant pathogen is limited. The importance of the accumulated data including comparison of invasive pathogens' populations, behavior and caused damage among both neighboring and geographically separated countries is growing.

In case of intensive transit through a certain country, the risk to introduce alien plant pathogens is higher (Santini *et al.*, 2013). Lithuania and Switzerland are countries of similar size and both serve as important European Union transport corridors (European Commission, 2014), thus investigation and comparison of communities and populations of alien organisms that have invaded both countries is highly desired. Invasive microorganisms are already threatening forest ecosystems in both countries, although risk of invasions by new dangerous pathogens persists, and new epidemics may cause devastating consequences.

Probably the best-known examples of forest epidemics caused by IFPs in both Lithuania and Switzerland are Dutch elm disease (DED) caused by *O. novo-ulmi*, ash dieback caused by *H. fraxineus* (Kowalski, 2006) and alder decline caused by *Phytophthora alni* Brasier & S. A. Kirk *sensu lato* (species complex). All three diseases are widely present throughout Europe, of which

DED is the earliest known and probably best investigated; ash dieback epidemic has started more than 20 years ago and is currently devastating European ash stands recalling for major efforts by researchers forest pathologists; while alder decline is known since the beginning of 1990s, and is probably the least studied disease of these three, because its consequences are not that noticeable and tree species the disease affects are less economically important.

Study aim

The main aim of the present study was to characterize populations of invasive pathogens – causal agents of three major forest tree diseases: alder decline **(***Phytophthora alni* species complex – in seven European countries with an emphasis on Lithuania and Switzerland)**,** Dutch elm disease (*Ophiostoma novo-ulmi* – in Lithuania) and ash dieback **(***Hymenoscyphus fraxineus* – in Lithuania and Switzerland).

The main objectives:

1. To investigate communities of oomycetes and fungi that could be recovered from the environment of declining black alder (*Alnus glutinosa*) and grey alder (*A. incana*) trees (tree tissues, rhizosphere and water) in Lithuania and Switzerland;
2. To identify genotypic diversity within populations of two species of *Phytophthora alni* species complex, namely *P.* x *alni* and *P. uniformis* and ascomycetous fungi *Ophiostoma novo-ulmi* and *Hymenoscyphus fraxineus*;
3. To compare results from two different ash inoculation experiments using previously genotyped *H. fraxineus* isolates of Swiss and Lithuanian origin;
4. To investigate relationship between growth rate of *H. fraxineus* mycelium in culture and virulence of the same genotypes of the pathogen.

Theme relevance

All three herein investigated diseases caused by invasive pathogens - alder decline, Dutch elm disease (DED) and ash dieback, are common in Europe and threaten stands of the respective species for decades, yet, despite extensive efforts by scientific community, no effective means of their control have been offered so far. That is why it is important to further accumulate knowledge on disease epidemiology, biological and ecological traits of the disease-causing agents. Advance in molecular research tools helps to investigate genetic properties of the pathogen populations thus enabling to reveal their structure, introduction pathways, formation of hybrids and other properties.

Scientific novelty and practical importance of the research

In Lithuania, the causal agent of alder decline, *Phytophthora alni sensu lato* (species complex), was for the first time identified in 1999 (Jovaišienė, 2002). In Switzerland, it was for the first time identified a bit later - in 2005 (dr. Simone Prospero, personal communication). However, to date detailed investigations into occurrence of different species within *P. alni* species complex and genetic properties of local populations of the pathogen have been carried out in neither country. In both countries, the pathogen is likely spreading, yet it is not adequately monitored. Thus, little is known on distribution of *P. alni* s. l., and the magnitude of the caused damage is likely underestimated. Moreover, the information is lacking on disease distribution, impact, and genotypic diversity of this dangerous pathogen in many parts of Europe.

Communities of oomycete and fungal species in environment of declining alder trees were for the first time investigated both in Lithuania and Switzerland. The study revealed differences of species composition between the two countries, leading to a suggestion that climatic conditions is one of the most important factors determining microorganism diversity and species composition that reside in same substrates. Five *Phytophthora* species, namely *Phytophthora gallica* T. Jung & J. Nechwatal, *Phytophthora gonapodyides* (H.E. Petersen) Buisman, *Phytophthora gregata* T. Jung, M.J.C. Stukely & T. Burgess,

Phytophthora lacustris Brasier, Cacciola, Nechw., Jung & Bakonyi, *Phytophthora* taxon *Oaksoil* were for the first time described in Lithuania. The present study is the first work in Europe describing microbial communities found in rhizosphere of declining alder stands.

Genotypic diversity of *Phytophthora x alni* (Brasier & S.A. Kirk) Husson, Ioos & Marçais and *Phytophthora uniformis* (Brasier & S.A. Kirk) Husson, Ioos & Aguayo was investigated in seven European countries making this study one of the most extensive studies as regards geographical scale. In four countries, Lithuania, Switzerland, Austria and Czech Republic, genotypic diversity of local *P. alni* s. l. populations were investigated for the first time. Microsatellite analysis of *P.* x *alni* population diversity in 7 countries helped to identify 36 multilocus genotypes (MLGs) of the pathogen, of which 24 were identified for the first time (previously unpublished).

In Lithuania, the last study on causal agent of Dutch elm disease, *Ophiostoma ulmi* (Buisman) Melin & Nannf. *sensu lato*, its distribution and impact on Lithuanian elm stands was done in 1960s (Žuklys, 1957). For more than a half of century the information about DED and its causal agent has not been updated. Before the present study there was no information which species is in fact causing the disease (*O. ulmi* s. s. or *Ophiostoma novo-ulmi*); furthermore, no studies have been carried out to investigate population structure of the disease-causing agent. The present study showed that in Lithuania *O. ulmi* s. s. has likely been replaced by a more pathogenic species, *O. novo-ulmi*. Moreover, intraspecific hybrids of *O. novo-ulmi* have been found during the present work (Norkutė, 2013; Motiejūnaitė *et al.*, 2016). These findings suggest that more detailed monitoring of the pathogen and research into formation and distribution of its intraspecific hybrids are required in Lithuania. Vegetative compatibility tests with the collected *O. novo-ulmi* isolates helped to reveal high genotypic diversity in population of this pathogen: only few genets were composed of more than one isolate. In Europe, population genetics of *O. novo-ulmi* was mainly investigated during 1990-2010; later the interest in Dutch elm disease and its causal agent has decreased. The present study suggests that *O.*

novo-ulmi still retains high genetic diversity at least in some parts of Europe and that formation of intraspecific hybrids of pathogen's subspecies originating from different continents (that may be highly pathogenic!), may cause the third wave of Dutch elm disease epidemic in Europe. Formation of highly pathogenic mutants or hybrids may affect so-far survived (tolerant or resistant) *Ulmus* spp. trees, thus aiming to continue selection of the most resistant elm clones it is critically important to test them against the most aggressive pathogen genotypes that could most likely be detected at zones of the most intensive hybridization. Hybrids found during the present study will therefore be tested for their virulence in artificial inoculation experiments.

Since the beginning of ash dieback, that has started in Europe more than 25 years ago, there are still no effective control measures found against its causal agent, *Hymenoscyphus fraxineus*. A study by Pliūra *et al.* (2011) revealed alerting results of the drastic decrease of genetic diversity in devastated populations of common ash (*Fraxinus excelsior* L.), that may affect further survivability of this tree species and sustainability of specific ash-dominated forest ecosystems.

During the present study, a large number of *H. fraxineus* isolates (367) collected at five distant regions of Lithuania, was used to investigate structure and genetic properties of pathogen's population within country. This was the most extensive study of this kind in Eastern Europe. The obtained results constitute an important contribution to studies of *H. fraxineus* population genetics across Europe.

Before the present study, no published results were available about reproducibility of inoculation experiment results where seedlings of common ash were inoculated with the same set of *H. fraxineus* isolates at different time points. Moreover, the present study was the first one aiming to compare growth rate of *H. fraxineus* isolates to their virulence following artificial inoculation into ash seedlings. Both later studies provide more knowledge about pathogen virulence and are of high practical importance.

Defended statements:

- In Lithuania and Switzerland, two species within *Phytophthora alni* species complex (*P.* x *alni* and *P. uniformis*) are commonly found in declining alder stands, while the third species, *P.* x *multiformis*, is uncommon;
- European populations of *P. alni* species complex are characterized by low genotypic diversity;
- In Lithuania, the causal agent of the first wave of Dutch elm disease, *Ophiostoma ulmi sensu stricto*, is replaced by a more aggressive species - *O. novo-ulmi* which populations show high genotypic diversity;
- Lithuanian *O. novo-ulmi* populations consist of two subspecies: *O. novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana*, and formation of hybrids between these subspecies is common;
- Lithuanian populations of ash dieback agent *Hymenoscyphus fraxineus* are characterized by high genotypic and genetic diversities and show clear spatial structure;
- Results of *H. fraxineus* inoculation experiments using the same set of isolates are reproducible (virulence results show high correlation in repeated experiments);
- Growth rate *H. fraxineus* mycelium on agar medium correlates well with virulence of the respective isolates of the pathogen.

Approbation of study results

The dissertation material was reported in 6 international conferences and 2 national conferences. The results of the research were presented in three scientific articles in journals with an impact factor (included into *Clarivate Analytics Web of Science* database) and 8 abstracts of conference presentations.

Volume and structure of the work

The dissertation consists of List of Abbreviations, Introduction, Literature Review, Materials and Methods, Results, Discussion, Conclusions,

List of References, List of Publications and Supplementary data. The results set out in three sections. The list of references includes 286 sources. The dissertation contains 149 pages, including 14 tables and 31 figures. The dissertation is written in English with the summary in Lithuanian.

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1. LITERATURE REVIEW

1.1 Invasive forest pathogen dispersal

Different pathogens have different spreading rates. By them, pathogens are classified to: i) recently introduced, fast spreading pathogens (e.g., *Hymenoscyphus fraxineus*, the causal agent of ash dieback, or *Phytophthora alni sensu lato* – a complex of species responsible for alder decline); ii) rare pathogens that are spreading at low rates (e.g., *Inonotus rickii* (Pat.) Reid, the causal agent of cankers and decay in several ornamental urban trees in Europe); this group also includes long-established pathogens like *Phytophthora cambivora* (Petri) Buisman; and iii) pathogens that are spreading at slower rate compared to organisms from the first group (e.g., the causal agent of Dutch elm disease, *Ophiostoma novo-ulmi*) (Santini *et al.*, 2013). Compared to native pathogens, invasive organisms or newly formed pathogenic hybrids tend to have higher spread rates (Santini *et al.*, 2013). This could be due to plant immune system incapacity to recognize pathogen-encoded effectors (Jones & Dangl, 2006), the new associations with endemic insect vectors (Webber, 2004), or to infect hosts from the same genera as in its native area (Santini *et al.*, 2013). In Europe, IFPs mainly cause cankers, foliar diseases, root and stem rots. During the last few decades, incidence of root and stem rots has increased, while incidence of foliar diseases and cankers has decreased (Santini *et al.*, 2013).

Socio-economical determinants such as the amount of imports (Desprez-Loustau *et al.*, 2009), value of gross domestic product (GDP) (Santini *et al.*, 2013), or total human population and population density (Desprez-Loustau *et al.*, 2009; Santini *et al.*, 2013) also have a certain effect on occurrence of IFPs.

For the last thirty years (since the fall of Iron Curtain in 1991s) spread of EIDs in Lithuania could be associated with increased international trade or the fact that Lithuania is a transit country with an intensive transportation of goods. Till 2017, a total of 125 fungal plant pathogen species were reported in Lithuania (Motiejunaite *et al.*, 2017). As regards numbers of alien oomycete species in Lithuania, the situation is less clear. Due to a lack of plant pathologists that work

with oomycetes the knowledge accumulates very slowly and is mostly sporadic. More information is collected on the most dangerous oomycetes that cause the largest losses to agriculture horticulture and forestry, such as *Phytophthora cactorum* (Lebert & Cohn) J. Schröt (Jovaisiene & Lane, 2006), *Phytophthora cinnamomi* Rands (Jovaišienė, 2004); *Phytophthora infestans* (Mont.) de Bary, the causal agent of potato late blight; *Phytophthora fragariae* Hickman (Jovaisiene, 2004), the causal agent of red stele disease; or *Phytophthora ramorum* Werres, De Cock & Man in't Veld, the causal agent of sudden oak death and root rot on many other plants (Anonymous, 2007). To date, only a few *Pythium* sp. species are known in Lithuania (Stankeviciene & Lugauskas, 2003).

1.2. Hybridization and occurrence of new EIDs

Hybridization plays an important role in fungal and oomycete plant-pathogen evolution (Arnold, 2004; Kroon 2012, Gibson *et al.*, 2014), as newly formed hybrid species may show a better adaptation to the environment and may have a greater impact on natural ecosystems than the parental species (Brasier, 2000, Stukenbrock and McDonald 2008, Gibson et al. 2014). In fungi (kingdom *Mycota*) and fungus-like oomycetes (kingdom *Stramenopila*), hybridization can be defined as a process of genome fusion between non-conspecific individuals, which occurs both sexually and asexually and generates offspring of mixed ancestry (Abbott et al. 2013, Schardl & Craven, 2003, Stukenbrock, 2016). When the ploidy level of the hybrid sums that of the two parental species, the process is called allopolyploid speciation, whereas hybridization without change in chromosome number is called homoploid speciation (Giraud *et al.*, 2008).

Until 1990s, only a few hybrid plant pathogens, mainly causing diseases to agricultural crops, were known (Olson & Stenlid, 2002): e.g. *Tilletia caries* (D.C.) Tul. & C. Tul. x *Tilletia laevis* J.G. Kühn hybrid (Flor, 1932) or *Ustilago hordei* (Pers.) Lagerh. x *Ustilago nuda* (J.L. Jensen) Rostr. hybrid (Fullerton & Nielsen, 1974). At that time it was mainly thought that fungal hybrids are

relatively rare in nature (Barton, 2001). Successively, thanks to the development and application of new DNA-based techniques (Cooke *et al.*, 2000), it became evident that hybridization events and formation of hybrid species are more frequent than previously supposed (Schardl & Craven, 2003; Gibson *et al.*, 2014). Currently, various molecular markers and methods are used to characterize hybrids and their populations, including simple sequence repeats (SSRs) (Hegarty & Hiscock, 2005), single nucleotide polymorphism (SNPs), and mitochondrial DNA (mtDNA) analyses (Burgess, 2015).

Hybridization success strongly depends on the type of isolation (i.e. geographical or reproductive) between the involved species. In sympatric fungal species (i.e. co-existing in the same geographic area), interspecific hybridization is extremely rare because of the presence of reinforced reproductive barriers (Stukenbrock, 2016). Hence, hybridization is more likely to occur between geographically isolated (i.e. allopatric), but not necessarily reproductively isolated, species (Stukenbrock and McDonald 2008, Restrepo *et al.*, 2014). In recent years, international plant trade has led to numerous introductions of microorganisms to new geographic areas (Fisher *et al.*, 2012; Garbelotto & Gonthier, 2013; Callaghan & Guest, 2015), thereby considerably increasing the opportunities for new interspecific hybridization events to occur. Because of this ample movement of species around the globe, it is not uncommon that information about parental species of hybrids is lacking. A well-known example is the oilseed rape pathogen *Verticillium longisporum* (C. Stark) Karapapa, Bainbr. & Heale, whose parental species are of unknown origin (Inderbitzin *et al.*, 2011; Depotter *et al.*, 2016).

Recent studies showed that interspecific hybridization is quite common in oomycetes – e.g., between *Phytophthora* species (Brasier *et al.*, 1999; Kroon *et al.*, 2012; Bertier *et al.*, 2013). Besides being successfully created under laboratory conditions, *Phytophthora* hybrids are commonly found in nature. Specifically, naturally formed hybrids were identified in several *Phytophthora* ITS-clades, including clades 1 (Man In 'T Veld *et al.*, 2007; Goss *et al.*, 2011), 6 (Burgess, 2015), 7 (Brasier *et al.*, 2004b), and 8 (Bertier *et al.*, 2013). Thus, in

the genus *Phytophthora*, interspecific hybridization is increasingly considered as an important process for the generation of new pathogens (Bertier et al. 2013, Burgess, 2015). One of the recently emerged infectious forest tree diseases – alder decline, is also known to be caused by hybrid species within *Phytophthora alni* s. l. species complex (Delcán & Brasier, 2001; Brasier *et al.*, 2003).

Another good example of fungal hybridization is interspecific hybrid of *Ophiostoma ulmi* x *Ophiostoma novo-ulmi* (Brasier *et al.*, 1998) and intraspecific hybrid of *Ophiostoma novo-ulmi* ssp. *americana* Brasier & S. A. Kirk x *Ophiostoma novo-ulmi* ssp. *novo-ulmi* Brasier & S. A. Kirk (Brasier & Kirk, 2010). Unlike interspecific *O. ulmi* x *O. novo-ulmi* hybrids that have lower fitness and are outcompeted by the parental species (Brasier *et al.*, 1998), intraspecific *O. novo-ulmi* ssp. *americana* x *O. novo-ulmi* ssp. *novo-ulmi* hybrids hybridize freely, allowing rapid evolution of the pathogen (Brasier & Buck, 2001; Brasier & Kirk, 2010).

1.3. Alder decline, Dutch elm disease and ash dieback – three major diseases of deciduous forest trees in Europe, caused by invasive pathogens

1.3.1. Alder decline: history, causing agents and distribution of the disease

1.3.1.1. Introduction to European alder species

Genus *Alnus* Mill. belongs to the family of woody plants *Betulaceae.* Across the world this genus includes more than 30 species of trees and shrubs (Savill, 2013). Four alder species are native to Europe: black alder (*Alnus glutinisa* (L.) Gaertn) (Durrant *et al.*, 2016), grey alder (*Alnus incana* (L.)

Moench) (Houston Durrant *et al.*, 2016), European green alder (*Alnus viridis*

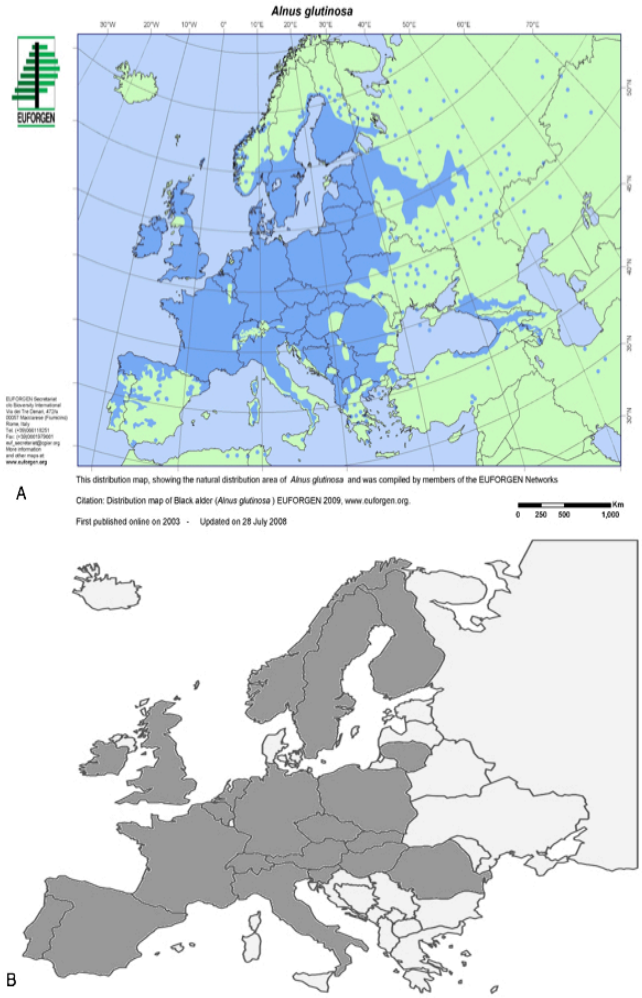

Fig. 1 Distribution of *Alnus glutinosa* and the causal agent of alder decline, *Phytophthora alni* s. l. in Europe: A, distribution map of *A. glutinosa* (blue colour) based on EUFORGEN (www.euforgen.org); B, distribution map of *P. alni* s. l. (dark grey colour).

(Chaix.) D. C.) (Mauri & Caudullo, 2016) and Italian alder (*Alnus cordata* (L.) Duby) (Caudullo & Mauri, 2016). European green alder and Italian alder are mainly found in southern parts of Europe, *A. viridis* is found in mountainous areas (Mauri & Caudullo, 2016), whereas *A. cordata* is an endemic species, found in Italy (Caudullo & Mauri, 2016). Another two species (black and grey alder) are widespread across Europe (Durrant *et al.*, 2016; Houston Durrant *et al.*, 2016). Distribution of *A. glutinosa* in Europe is presented in Fig. 1 A. Both

species prefer moist sites: marshy waterlogged sites, riversides or lake shores (McVean, 1956). All species within genus *Alnus* have rather small importance to the forestry sector in Europe, yet their ecological importance is widely recognized (Cech, 1998). Alder roots form symbiotic relationships with atmospheric nitrogen-fixing bacteria *Frankia alni* Woronin, and this makes these trees important pioneer plants (Savill, 2013).

1.3.1.2. First records of alder decline in Europe

Table 1 First reported isolations of *Phytophthora alni* s. l.

Country	Year of isolation (first record)	Reference
Austria	1996	Brasier *et al.* (2004)
Belgium	2000	Brasier *et al.* (2004)
Czech Republic	2001	Černy *et al.* (2003)
France	1996	Streito *et al.* (2002)
Finland	2015	Poimala *et al.* (2018)
Germany	1995	Brasier *et al.* (2004)
Hungary	1999	Szabó *et al.* (2000)
Ireland	1999	Streito (2003)
Italy	2000	Santini *et al.* (2001); Brasier *et al.* (2004)
Lithuania	1999	Jovaišienė (2002)
The Netherlands	1992	Streito (2003)
Norway	2012	Strømeng *et al.* (2012)
Poland	2003	Orlikowski *et al.* (2003)
Portugal	2014	Kanoun-Boulé *et al.* (2016)
Spain	2009	Solla *et al.* (2010)
Sweden	1996	Redondo *et al.* (2015)
Switzerland	2005	Dr. Simone Prospero (personal communication)
United Kingdom	1993	Brasier *et al.* (1995)

The sudden mortality of alder species (*Alnus* spp.) was observed in the beginning of 1990s in Southern Britain; the previously unknown disease has been called “alder decline” (Brasier *et al.*, 1995; Gibbs, 1995). Some years later, researchers in the United Kingdom determined that the alder decline they had observed in recent years was a disease caused by a hybrid oomycetous pathogen, *Phytophthora alni* s. l., which is highly specific to alder (Brasier *et al.*, 2004b). Since the first report in Great Britain, the pathogen has been found in other countries across Europe (Fig. 1 B & Table 1) (Streito *et al.*, 2002b; Brasier *et*

al., 2004b; Jung & Blaschke, 2004) and in Northern parts of the United States of America (USA) (Adams *et al.*, 2009). Until 2018, *P. alni* s. l. has been reported in 18 European countries (Table 1 & Fig. 1B).

1.3.1.3. Symptoms of alder decline

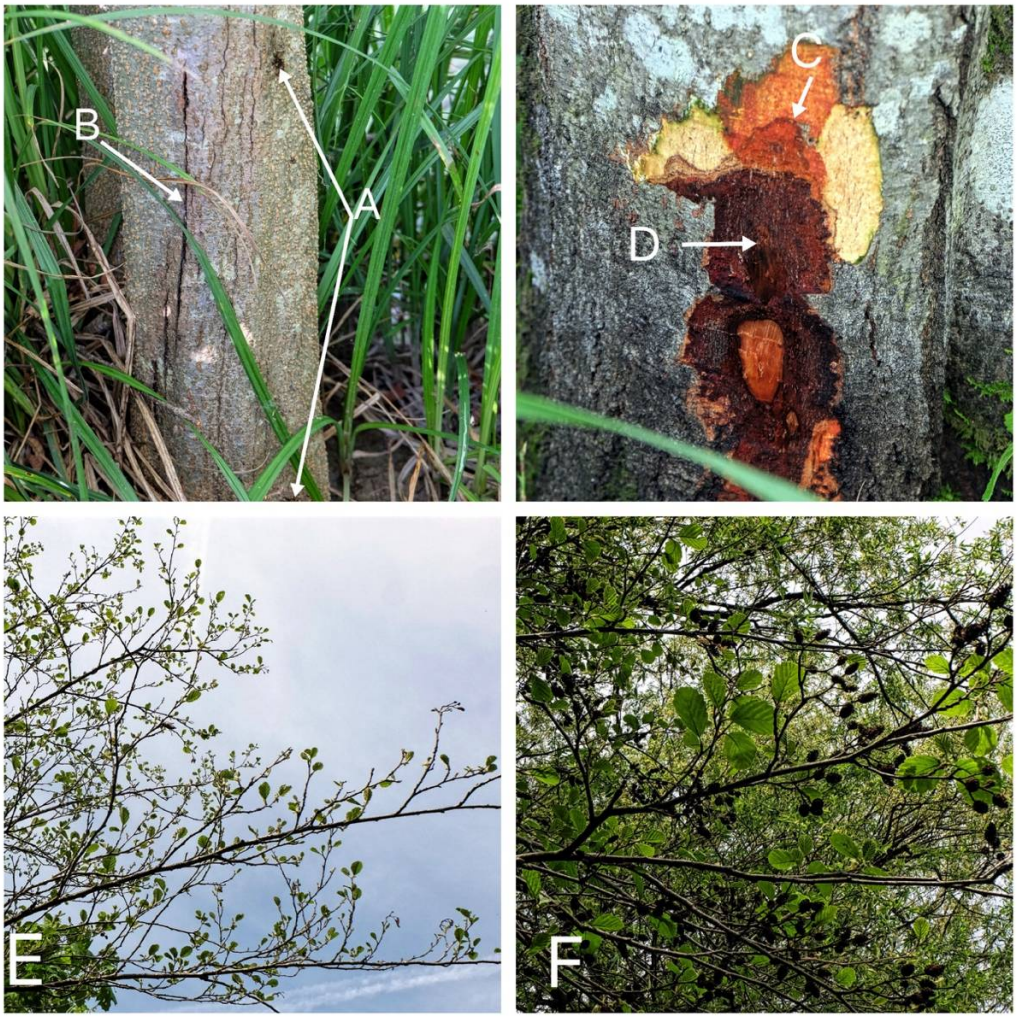

Fig. 2 Typical symptoms of *Phytophthora alni* s. l. infection on alder trees. A - rusty coloured exudate spots on bark of *Alnus glutinosa* stem; B - crack in the bark of *A. glutinosa*; C - tongue-shaped canker under the bark of *A. glutinosa*; D – infection by some secondary pathogen (-s); E - sparse foliage and leaves of reduced size in *A. glutinosa*; F - excessive seed production in a crown of *A. glutinosa*

The main symptoms of alder decline include: rusty (black) coloured exudate spots on lower stem bark (Fig. 2A), cracks in the bark (Fig. 2B), tongue-

shaped cankers (Fig. 2C), reduced-size leaves and sparse foliage with premature leaf fall (Fig. 2E), and excessive seed production (Fig. 2F).

1.3.1.4. Epidemiology of alder decline in Europe

The magnitude of alder decline depends on where trees grows: the rates of the disease are significantly higher in riparian areas - near rivers or other water basins (Jung & Blaschke, 2004; Štěpánková *et al.*, 2013; Redondo *et al.*, 2015). In 2000, Streito *et al.* (2001) surveyed Moselle river in northeast of France and found that about 20 % of alders were diseased or dead. Redondo *et al.* (2015) investigated alder decline in Sweden and found that 28 % of the investigated black alder stands showed symptoms typical to *Phytophthora* infection. The impact assessment showed that 31 % of the investigated stands had 50 % of the trees with symptoms of decline. In Bavaria (Southern Germany), alder decline was even more severe, with more than 50 % of the investigated riparian stands showing disease symptoms (Jung & Blaschke, 2004). Among European countries, only United Kingdom has monitored the progress of alder decline from its start: in 1994, the disease was observed in 4 % of the monitored alder stands, and by 2003 its rate has increased to 15 % (Webber *et al.*, 2004).

Gibbs *et al.* (1999) investigated susceptibility of alder populations to the pathogen, and came to a conclusion that trees growing within 1 m from the river bank are more prone to *P. alni* s. l. infection than trees growing further away. This study also showed that occurrence and rate of the disease are influenced by many factors, such as growth rate of the pathogen, size of a host stem, location of the infection on stem, etc. Jung & Blaschke (2004) demonstrated that the pathogen has poor survivability in soil: without presence of the host its chlamydospores can survive only for a few months.

Štochlová *et al.* (2015) has proposed two factors that may be essential for occurrence of decline symptoms on a tree: 1) geographical origin of the host and it's genotype; and 2) pathogen genotype. Chandelier *et al.* (2016) conducted virulence tests using different *P.* x *alni* inoculation methods to evaluate the

resistance of riparian alders to the disease, and to screen alder genotypes for resistance. Aguayo *et al.* (2014) developed Susceptible-Infected-Susceptible (SIS) model to estimate the dynamics of alder decline. This model showed connection between the average annual temperature and incidence of the disease.

1.3.1.5. Evolution of *Phytophthora alni* s. l. species complex

The sudden emergence of *P. alni* s. l., a previously unknown disease agent, caused a lot of questions about its origin. Already in early 1990's Brasier *et al.* (1995) noticed morphological similarities between alder *Phytophthora* and *P. cambivora*. Further investigations brought the authors to an assumption that alder *Phytophthora* originated through interspecific hybridization, and that *P. cambivora* and *P. fragariae* could be its progenitors (Brasier *et al.* 1999). Later it was shown that alder *Phytophthora* (i.e., *P. alni* s. l.) is not a homogenous species, but in fact it represents a range of species hybrids (Delcán & Brasier, 2001; Brasier *et al.*, 2003). Following identification of the causal agent of alder decline, Brasier *et al.* (2004) has divided *P. alni* s.l. into three subspecies: *P. alni* subsp. *alni* Brasier & S. A. Kirk (also referred to *Paa*) (previously known as 'standard' variant hybrid), *P. alni* subsp. *multiformis* Brasier & S. A. Kirk (also referred to *Pam*) (previously known as 'Dutch', 'UK' and 'German' variant types), and *P. alni* subsp. *uniformis* Brasier & S. A. Kirk (also referred to *Pau*) (previously known as 'Swedish' variant hybrid).

Ioos *et al.* (2006) conducted thorough analyses of nuclear and mitochondrial DNA of the pathogen. Investigations of the mitochondrial DNA revealed that subspecies of *P. alni* s. l. could be divided into six groups: M, M' M", U, U' and U". *Pam* could only display M, M' and M" patterns, *Pau* could only display U, U' and U" patterns, but *Paa* could have all six patterns. Research on four nuclear gene similarities ASF-like, GPA1, Ras-Ypt and TRP1 affirmed that three different alleles were present in the *Paa* genome: two alleles, PAM1 and PAM2, were the same as alleles found in *Pam* isolates, and one PAU allele

was the same as found in *Pau* genes. This study confirmed that *Pau* is a near-diploid organism with a high phenotypic and genetic uniformity. It was also shown that four nuclear gene alleles of *Pam*, PAM1 and PAM2, are diverse, and that this organism could be near-tetraploid or diploid taxon resulting from a homoploid speciation. The mitochondrial DNA features combined with different nuclear allelic patterns led Ioos *et al.* (2006) to a conclusion that *Pau* and *Pam* are the progenitor organisms of *Paa.*

More recent study conducted by Husson *et al.* (2015) determined ploidy in *P. alni* s. l. using Real-Time PCR for three different single-copy genes (ASF-like, GPA1 and RAS-YPT). Researchers revealed that compared to *Pau* and *Pam, Paa* has half of number of copies of a given allele. This led to a suggestion that *Paa* is a hybrid, containing half of the genome of each parent. The hypothetical interspecific hybridization scenario has been suggested (Husson *et al.*, 2015) (Fig. 3): *Pam* could have originated after interspecific hybridization event between two unknown *Phytophthora* species (*Pm*1 and *Pm*2). It could have happened from genome duplication following by a fusion of normally reduced gametes or after a fusion of unreduced (2n) gametes of both *Pm*1 and *Pm*2. As suggested by Ioos *et al.* (2006), *Paa* originated following an event of hybridization between *Pau* and *Pam*. *Paa* should therefore be regarded as a homoploid hybrid. There could be two ways of *Paa* evolution: 1) *Paa* could backcross with *Pau* or selfing, producing allotetraploid offspring; and 2) genome duplication and formation of an allohexaploid organism (Husson *et al.*, 2015).

Based on different ploidy level of *Paa, Pam* and *Pau*, Husson *et al.* (2015) proposed to change subspecies status to species: *Phytophthora x alni* (Brasier & S.A. Kirk) Husson, Ioos & Marçais, nothosop. nov., basionym *Phytophthora alni* subsp. *alni* Brasier & S.A. Kirk; *Phytophthora uniformis* (Brasier & S.A. Kirk) Husson, Ioos & Marçais, nothosop. nov., basionym *Phytophthora alni* subsp. *uniformis* Brasier & S.A. Kirk; *Phytophthora x multiformis* (Brasier & S.A. Kirk) Husson, Ioos & Marçais, nothosop. nov., basionym *Phytophthora alni* subsp. *multiformis* Brasier & S.A. Kirk.

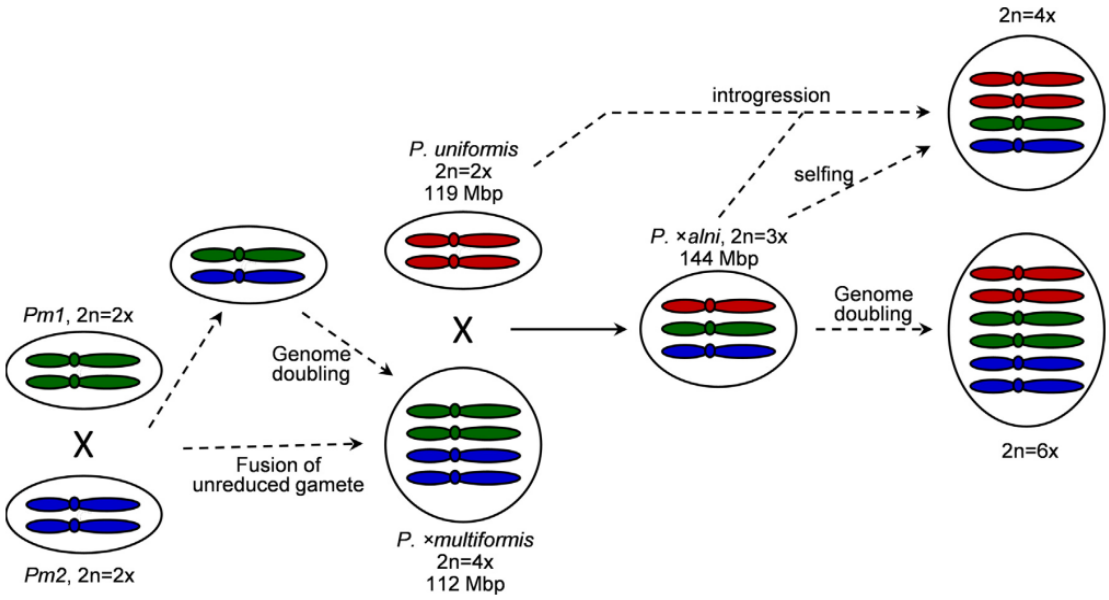

Fig. 3 Hypothetical interspecific hybridization scenario events in evolution of *Phytophthora* x *alni* hybrid (reproduced from Husson et al. (2015)).

1.3.1.6. Life cycle and infection strategies of *Phytophthora* spp.

The life cycle in phytophthoras is complex and involves several "scenarios". Up to three asexual spore types may be produced: chlamydospores (ensure long-term survival in the absence of a host plant), zoospores (ensure plant infection via aquatic environment), sporangia (ensure disease transfer to other plants), and a sexual spore form – oospores (resting structures) (Fig. 4).

Asexual reproduction consists of two phases: 1) sporangiogenesis (formation of multinucleate sporangia); and 2) zoosporogenesis (formation of uninucleate, motile zoospores). Sporangiogenesis can be induced at relatively low temperatures (12-20 °C) and high humidity (91-100 %). Depending on species and environmental conditions, sporangia can remain attached to the hyphal tip or can become detached (becoming a dispersive propagule). Sporangia can germinate directly (forming a hyphae) or indirectly (formation and release of zoospores – zoosporogenesis). Some oomycetes can form asexual thick-walled resting spores – chlamydospores; which, depending on species, may be formed on a hyphal tip (terminaly) or grow intercalary (Fig. 4).

Majority of *Phytophthora* spp. produce sporangia and release zoospores (Erwin & Ribeiro, 1996). After zoospores are released from sporangia, they start swimming using anterior and posterior flagella (Judelson & Blanco, 2005). Zoospores detect host tissue, and then they encyst and start "germination". The produced mycelium penetrates into plant tissues.

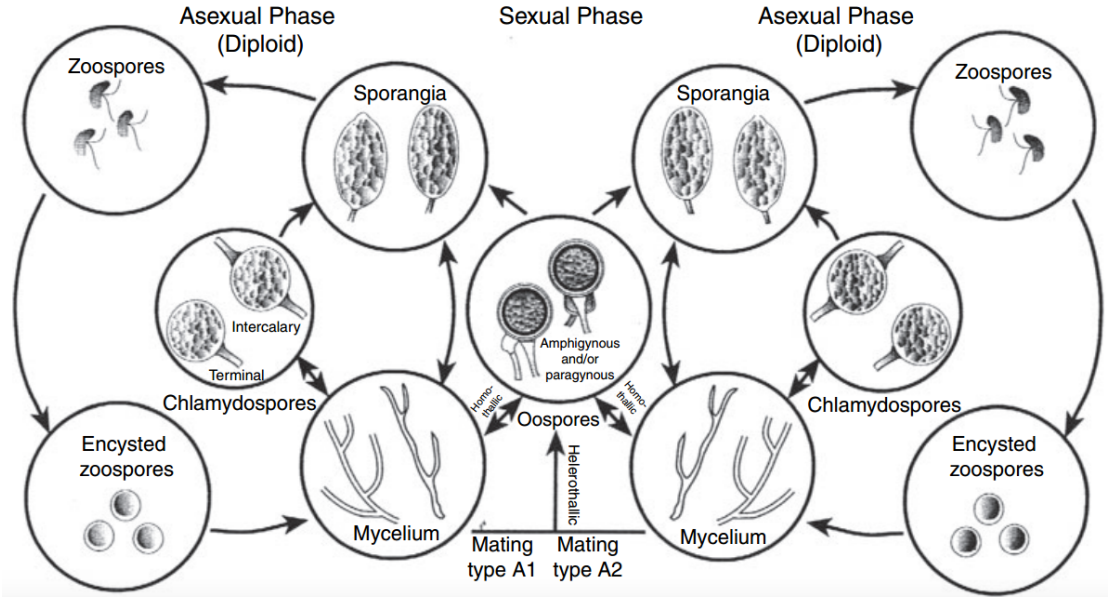

Fig. 4 Representative life cycle of *Phytophthora* spp. (reproduced from Lamour (2013))

Zoospores can detect host plant tissues by several mechanisms. Plant roots secret to soil chemoattractants (amino acids, isoflavones) which attract zoospores that have a positive chemotaxis to these compounds. Plant root tips and tissue wounds form concentration gradient of attractants that are favoured by phytophthoras, so the infection usually takes place via root tips and mechanical wounds on roots or lower stem parts. Many species within genus *Phytophthora* exhibit autoaggregation, which can increase a probability of infection (Tyler, 2002).

Oomycetes have homothallic (sexual reproduction takes place within a single organism) and heterothallic (sexual reproduction requires distinct sexual compatibility types) mating types (Judelson & Blanco, 2005). Sexual phase starts with the formation and development of female (oogonia) and male (antheridium) gametangia, which later fuse and form oospores. Matured

oospores germinate and produce hyphal tube or a germ sporangium (Judelson & Blanco, 2005).

Phytophthoras can infect plants as foliar, bark or root pathogens (Hansen *et al.*, 2012). For host tissue infection phytophthoras have evolved complicated mechanisms (Reeser *et al.*, 2011) (Oßwald *et al.*, 2014). Most of *Phytophthora* species are hemibiotrophic pathogens - they have properties of both biotrophic and necrotrophic organisms (Horbach *et al.*, 2011; Oßwald *et al.*, 2014). As biotrophic organisms, they can establish a close association with a host plant through formation of haustoria (Mendgen & Hahn, 2002). As necrotrophic organisms, they can break through host defensive system by producing toxins and enzymes that kill plant cells (Horbach *et al.*, 2011).

Penetration into plant host tissues involves a range of enzymes that digest and degrade plant cell wall (Hardham, 2005). First, hyphae penetrate to cortex, then they grow further, damaging phloem and to a lesser extent xylem (Davison, 2011). This causes structural changes in plant tissues leading to reduced ability to absorb and conduct water (Maurel *et al.*, 2001). Following severe damage of cortex and phloem, chlamydospores and oospores (resting spores) are formed. Under favorable conditions secondary sporangia formation may occur and this may cause multi-cycling infections to the plant. Penetration of hyphae from root tissues to other parts of the host plant is based on few factors – host ability to restrict the pathogen to roots and/or environmental conditions (drought or flood) that weaken a plant (Oßwald *et al.*, 2014). Phytophthoras can infect host plant directly via stem lenticels, adventitious roots or wounds. Many environmental and ontogenetic factors determine where the infection will start (Oßwald *et al.*, 2014).

1.3.1.7. Detection, identification and population genetics of *Phytophthora alni* s. l. species complex

Since the first records of alder decline it has attracted much attention in Europe, and much effort was directed towards development of efficient methods

for pathogen detection and identification. Three groups of researchers at the same time have developed primer pairs for rapid identification of species within *P. alni* s. l. species complex using Polymerase Chain Reaction (PCR)-based approach:

1) Ioos *et al.* (2005) has designed three primer pairs - PA (PA-F/R), PAM (PAM-F/R) and PAU (PAU-F/R), derived from Sequence Characterized Amplified Regions (SCARs). The first primer pair, PA, can aid in detection/identification of all three species - *P. x alni, P. uniformis* and *P. x multiformis*; the second primer pair, PAM, was designed for *P. x alni* and *P. x multiformis*, and the third primer pair, PAU, was designed for *P. x alni* and *P. uniformis*;

2) De Merlier *et al.* (2005) has designed primer pair D16 (D16-F/R) using the same SCAR-based method. This primer pair can identify two species: *P. x alni* and *P. uniformis*;

3) Bakonyi *et al.* (2006) has designed two primer pairs - SAP (SAP1/SAP2) and SWAP (SWAP1/SWAP2), derived from Randomly Amplified Polymorphic DNA (RAPD) fragments, unique to various complex species. Primer pair SAP was designed for detection of *P. x alni* and *P. x multiformis*, and SWAP - for *P. x alni* and *P. uniformis.*

4) In 2006, Ioos with coworkers designed primers that were intended to investigate hybrid status of *P. alni* s. l. (Ioos *et al.*, 2006). The study was based on high polymorphism level of uncoding DNA sequence part – intron, that could possibly reveal parental *P. alni* organisms (Brasier *et al.*, 2004b). Four genes were selected: Ras-Ypt, TRP1, GPA1 and ASF-like gene (Ioos *et al.*, 2006).

Currently, primer pairs designed by Ioos *et al.* (2006) are used in most studies for detection/identification of *P. alni* s. l. species complex. Up to date, three studies on *P. alni* s. l. population genetics have been performed using microsatellite markers. Ioos *et al.* (2007) developed nine polymorphic microsatellite markers to distinguish *P. alni* s. l. genotypes, and showed that all species had different ploidy level.

P. uniformis was isolated not only in Europe, but also in North America (Adams *et al.*, 2009). Aguayo *et al.* (2013) compared *P. uniformis* genotypes between Europe and North America using five microsatellite markers. This study revealed that *P. uniformis* is much more genetically variable in North America than in Europe. This led to a suggestion that *P. uniformis* is an alien species to Europe and could be native to North America. Aguayo *et al.* (2016) investigated genotype variability of all three species of *P. alni* s.l. complex in Europe. The study addressed a few keynotes: 1) interspecific hybrid population genetic structure could be assessed by the determination of parental subgenomes; 2) It could also reveal some genetic characteristics of the parental species. The study evidenced a low polymorphism in *P.* x *alni*, with European populations (mainly from France, Germany and Hungary) dominated by a single multilocus genotype (Pxa-1). However, since mtDNA patterns of both parental species were found in *P.* x *alni* isolates belonging to the same genotype, the authors concluded that multiple hybridization events occurred independently in several European regions, i.e. Pxa-1 is not a true clone that colonized Europe. Noteworthy, the incidence of Pxa-1 in local populations seemed to increase over time. The population of the parental species, *P. uniformis*, was also dominated by a single multilocus genotype and was less diverse than expected based on the *P. uniformis* subgenomes present in the *P.* x *alni* population (Aguayo *et al.* 2016).

1.3.1.8. Characterization and distribution of *Phytophthora* species, responsible for alder decline

As mentioned in section 1.3.1.2, *P. alni* s. l. was reported in 18 European countries. *P.* x *multiformis* is the rarest species, found only in few countries (Aguayo *et al.,* 2016), and *P.* x *alni* is the most widely distributed species (Brasier & Kirk, 2001a; Aguayo *et al.*, 2016). *P. uniformis* is also rather widely distributed in Europe, and it is the only species within the complex that has been found to be established in another continent (Adams *et al.*, 2009; Sims *et al.*,

2015). All three species cause root and collar rot to *A. cordata*, *A. incana* and *A. glutinosa* (Brasier *et al.*, 2004b), but their pathogenicity differ (Brasier & Kirk, 2001a). Of all species within *P. alni* s. l. complex, *P.* x *alni* is regarded to be the most pathogenic one, *P.* x *multiformis* is less pathogenic, but it is more pathogenic than *P. uniformis* (Brasier & Kirk, 2001a).

P. x *alni* is sensitive to low winter temperatures (Černý & Strnadová, 2012), *P. uniformis* is not that sensitive (Redondo *et al.*, 2015), while information on sensitivity of *P.* x *multiformis* is lacking.

1.3.1.9. Other pathogens causing alder diseases

Zamora-Ballesteros *et al.* (2016) suggested that severity of alder decline may depend not only on presence and aggressiveness of *P. alni* s.l., but also on infections by other pathogens such as e.g. *Phytophthora plurivora* T. Jung & T.I. Burgess. The pathogen is considered to be native to Europe (Schoebel *et al.*, 2014) and has a wide-range of hosts (Jung & Burgess, 2009). *P. plurivora* has been already isolated from declining *A. glutinosa* trees in Spain (Haque *et al.*, 2014).

Two most common alder species in Europe and Lithuania, *A. glutinosa* and *A. incana*, are also susceptible to Alder Yellows phytoplasma (AldYp). Infected trees exhibit symptoms such as defoliation, yellowing and smaller than usual leaves, although sometimes trees may remain symptomless (Lederer & Seemuller, 1991).

"Alder bracket" basidiomycete *Mensularia radiata* (Sowerby) Lázaro Ibiza is a frequent pathogen of *Alnus* spp. causing a heart rot. A widely distributed silverleaf fungus *Chondrostereum purpureum* (Pers.) Pouzar causes symptoms similar to heart rots (Arhipova *et al.*, 2011, 2012).

Fungi of genus *Taphrina* Fr. cause "leaf curl", "witches brooms" or "tongue-like outgrowth" on alder leaves (Bacigálová *et al.*, 2003).

Alder anthracnose is caused by ascomycetes *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *Colletotrichum acutatum* J. H. Simmonds (Bełka & Harrington, 2013).

Infection by an ascomycete *Ciboria alni* (O. Rostr.) N. F. Buchw. precludes alder seeds from germination (Bełka & Harrington, 2013).

However, none of the above-mentioned species have been shown to cause significant (epidemic) decline or mortality to alder as do species within *P. alni* s. l. species complex. Further investigations on pathogenicity and virulence of various alder pathogens and their different combinations are required to shed more light on causes of the present alder decline phenomenon.

1.3.2. Dutch elm disease: history, causes and distribution

1.3.2.1. Distribution of *Ulmus spp.* in Europe and Lithuania

Around the globe, genus *Ulmus* L. (family *Ulmaceae*) includes around 40 species of perennial woody plants (trees and shrubs). The northern hemisphere is the main area of their distribution. Elms are characterized by wood of good quality, resistance to unfavorable environmental conditions; they are important components of specific slope ecosystems forming on fertile sites (Malá *et al.*, 2007). In Europe, there are three native elm species: *Ulmus glabra* Huds. (wych elm), *Ulmus laevis* Pall. (white elm) (Fig. 5) and *Ulmus minor* Mill. (field elm) (Hollingsworth *et al.*, 2000).

U. glabra is common throughout Europe. This species is relatively cold-resistant and therefore is common in mountainous areas and in northern Europe (Hollingsworth *et al.*, 2000). *U. laevis* is widespread across central and eastern Europe (Fig 5). Longitudinally, the area of *U. leavis* distribution ranges from Ural Mountains in the East to eastern parts of France in the West. Lattitudinally, the species ranges from the southern Finland in the North to Crimea Mountains in the South (Hollingsworth *et al.*, 2000). *U. minor* due to its intolerance to cold

temperatures is more common in southern Europe; the northernmost populations occur in the Baltic Sea region (Hollingsworth *et al.*, 2000).

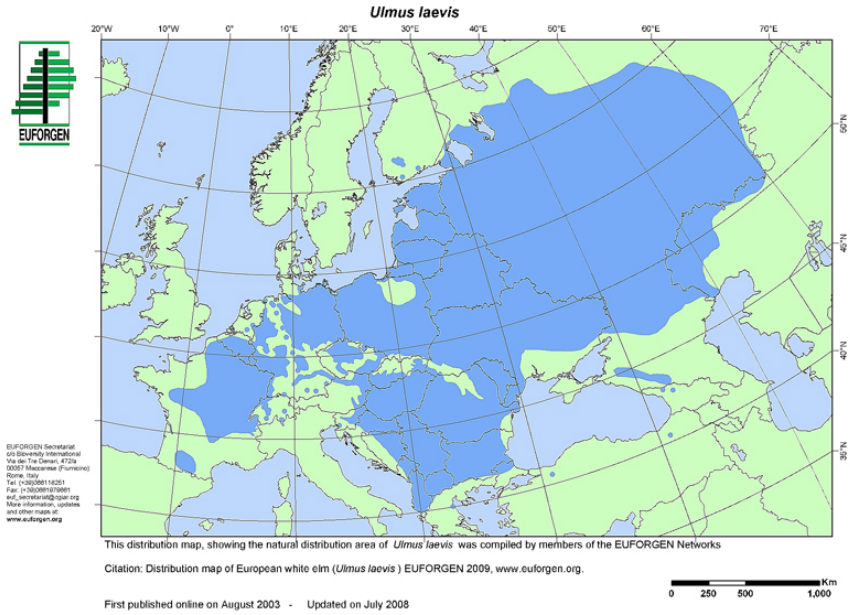

Fig. 5 Distribution map of *Ulmus laevis* (blue colour) (based on EUFORGEN (www.euforgen.org))

All three *Ulmus* species can be found In Lithuania. *U. glabra* grows in mixed forests with other broadleaf trees and spruce and never forms pure stands. This species is more common in Middle Lithuanian Lowland (Navasaitis *et al.*, 2003). *U. laevis* grows in Middle Lithuanian and Coastal Lowland regions. This species is relatively rare in Lithuania (Navasaitis *et al.*, 2003). *U. minor* is mostly distributed in southwest parts of Lithuania and in Middle Lithuanian Lowland (Navasaitis *et al.*, 2003).

1.3.2.2. History of Dutch elm disease epidemics

In 1918-1921 (at the end of First World War), a sudden elm dieback was observed. This was a new threatening phenomenon. The new disease was called Dutch elm disease (DED). First the disease was spotted in France, Germany and the Netherlands (Gibbs, 1978); later (around 1927) it was found in the United

Kingdom and Austria (1928) (Gibbs, 1978). In Italy, Bulgaria, Romania, Ukraine and Moldova, the first DED symptoms were observed in 1930; in Czech Republic and Slovakia – in 1931, in Uzbekistan – in 1939, in Sweden – in 1940. In northwest Europe, the disease was more prominent during 1946-1947 period, but in Norway and Finland it was found only in 1963 (Fig. 6 & Fig. 7 a). In Asia, DED has not been observed until 1960's, where it occurred only in few regions: Turkey, Uzbekistan and Kashmir region of India (Gibbs, 1978) (Fig 7 a). It is believed that to North America DED was introduced from Europe in 1930's (Fig. 7 a) (May, 1934).

It is not known from where and when the disease came to Lithuania. The first reports of DED in Lithuania date back to 1941-1942, so there is a hypothesis that the disease could spread during the Second World War from German-occupied territories. This hypothesis is supported by the fact that the first signs of disease were observed in western and southern parts of Lithuania and only much later - in northern regions of the country (Žuklys, 1957).

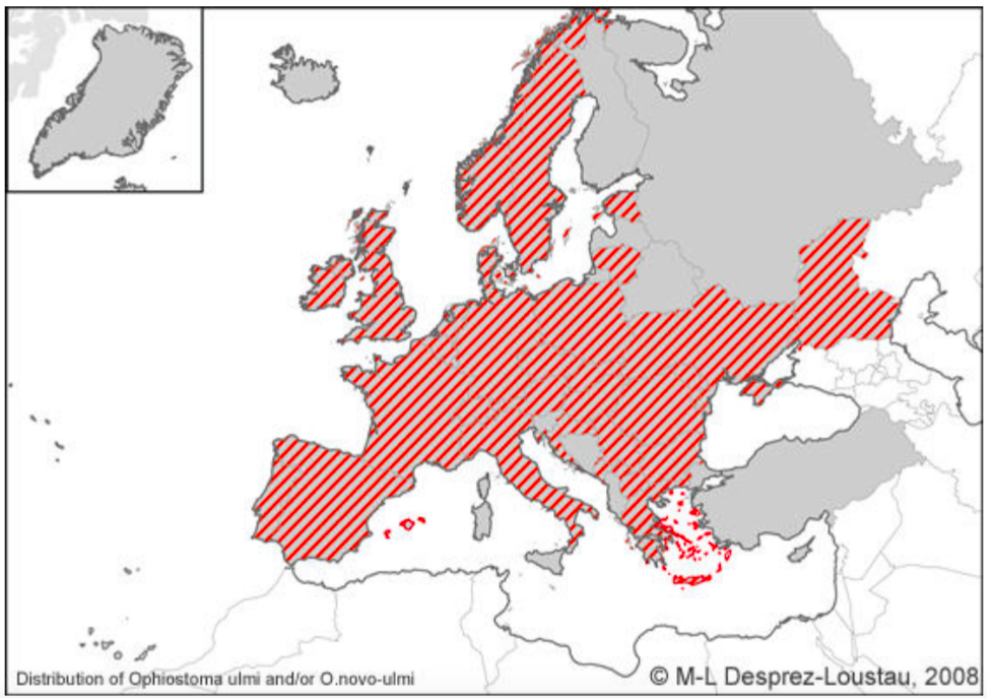

Fig. 6 Distribution map of *Ophiostoma novo* or/and *ulmi Ophiostoma ulmi* s. s. (red colour stripes) (reproduced from Desprez-Loustau (2008))

The causal agent of a first wave of DED, which has rolled across Europe before the Second World War, was a microscopic ascomycetous fungus *O. ulmi* s. s. (Hoog & Scheffer, 1984). However, the harm caused by this pathogen to

elms wasn't big: the population of healthy trees, even in severely damaged regions, remained higher than that of diseased ones (Went, 1954).

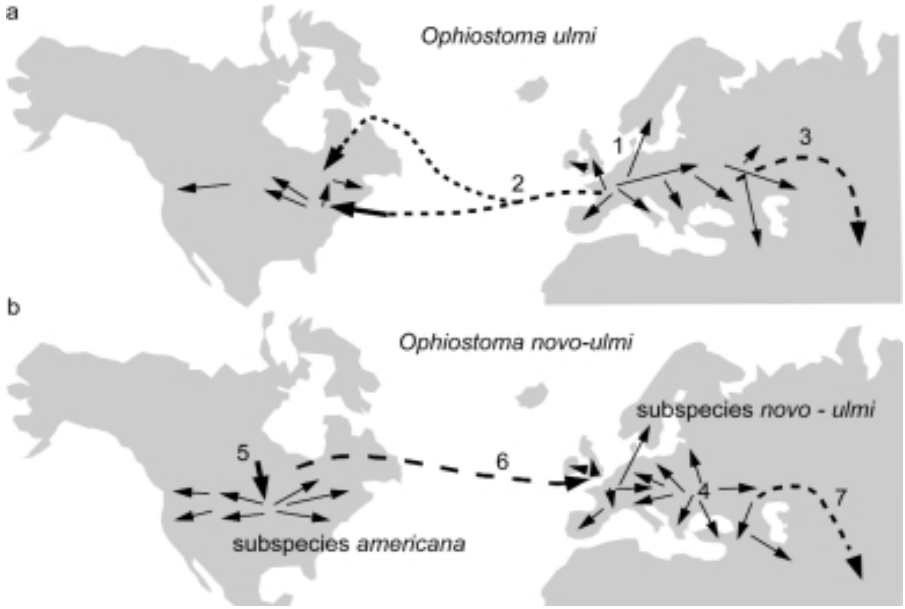

Fig. 7 Dispersal of Dutch elm disease agents between and within North American and Eurasian continents: a – *Ophiostoma ulmi* s.l.; b – *O. novo-ulmi* (Brasier, 1990). Solid arrows show natural spreading, dashed arrows show pathogen secondary spreading via international trade. The numbers show pathogen transmission progress (adapted from Brasier & Buck, 2001)

Around 1940, a second wave of DED epidemic has stroke Europe; the new epidemic was caused by *O. novo-ulmi*, a close relative of *O. ulmi* s. s. The damage caused by this new pathogen far exceeded the damage caused by its predecessor, *O. ulmi* s. s. In North America, *O. novo-ulmi* was found at about the same time as in Europe (Brasier *et al.*, 2004a) (Fig. 7 b). In several years, rapidly spreading *O. novo-ulmi* has outcompeted and replaced *O. ulmi* s. s. in its habitat (at a rate of 10 % of the population per year) (Mitchell, 1988) (Fig. 6 & Fig. 7). This was likely related to different pathogenicity of the two species: *O. novo-ulmi* is regarded to be more aggressive (pathogenic) to European elms compared to *O. ulmi* s. s. (Brasier & Mehrotra, 1995).

1.3.2.3. Theories behind the origin of causal agents of Dutch elm disease

There are a few theories about the origin of *O. ulmi* s. l. Currently, the main theory suggests that the progenitor of *O. ulmi* s. l. could have originated in

a geographically isolated region, such as Himalayan Mountains (Brasier, 1990), from where, due to globalization, it could have been introduced to other parts of the world. This hypothesis is supported by a large variety of DED-resistant elm species in Himalayan region (Fu, 1980) which indicates the formation of "neutral" relationships between the pathogen and the host-plant (Brasier, 1990); for example, *Ulmus pumila* L. and *Ulmus parvifolia* Jacq. are far less susceptible to the causal agents of DED compared to European elm species (Brasier, 1990). In 1995, in the region of Himachal Pradesh (North India), a previously unknown *Ophiostoma* species similar to *O. ulmi* s. l. has been isolated from wood of *Ulmus wallichiana* Planch. The fungus was named *Ophiostoma himal-ulmi* Brasier & M.D. Mehrotra (Brasier & Mehrotra, 1995). This organism causes symptoms similar to that of DED. To date, *O. himal-ulmi* was found only in two regions: Kashmir and Himachal Pradesh. There is another theory hypothesizing that *O. himal-ulmi* and *O. ulmi* s. l. have a common ancestor which has originated from the Himalayan Mountains (Brasier & Mehrotra, 1995).

O. novo-ulmi has been divided into two subspecies which have different regions of origin (Brasier & Kirk, 2001b): *O. novo-ulmi* ssp. *americana* that was first found in North America and here became established, and *O. novo-ulmi* ssp. *novo-ulmi* which has likely originated from eastern Europe (Ukraine or Moldova). The later subspecies has rapidly spread towards Western Europe, and around 1970s the pathogen was found in the Netherlands, France, Germany, Italy and Switzerland. Around 1960s, *O. novo-ulmi* ssp. *americana* has been introduced to Europe. At present, both subspecies have overlapping distribution ranges in Europe (Brasier, 1990, 1996b; Brasier & Buck, 2001).

1.3.2.4. Hybrids between *Ophiostoma ulmi* s. s. and *Ophiostoma novo-ulmi*

Interspecific hybridization may occur when organisms of two species colonize the same host-plant and interact in the same tissues (Kile & Brasier, 1990). In nature, *O. ulmi* s. s. and *O. novo-ulmi* sometimes form hybrids, which due to competition with parental species are usually short-lived (Brasier *et al.*,

1998). The main benefits of hybrids may be gained through gene flow, as both species may receive new genes from each other (Brasier, 2000a). The rapid changes in genetic diversity observed in local *O. novo-ulmi* populations show likely gene flow between *O. ulmi* s. s. and *O. novo-ulmi* as the number of vegetative compatibility groups in *O. novo-ulmi* continuously increases (Brasier *et al.*, 1998). This hypothesis is further supported by findings of increased genotypic diversity of *O. novo-ulmi* over few years following pathogen's introduction to Europe (Brasier, 2000b). The study of *O. novo-ulmi* genotypes in North America showed similar results, although in New Zealand, where only *O. novo-ulmi* has been introduced, genotypic diversity of this species is low (Brasier, 2000b).

1.3.2.5. Subspecies and hybrids of *Ophiostoma novo-ulmi*

Until 2000, *O. novo-ulmi* was divided into two continental populations (races) – Eurasian (further referred to as EAN) and North American (further referred to as NAN) (Brasier, 1991). Later, Brasier & Kirk (2001) suggested that these populations should be regarded as different subspecies: *O. novo-ulmi* ssp. *novo-ulmi* (EAN) and *O. novo-ulmi* ssp. *americana* (NAN). EAN is common in Eurasia, its distribution ranges from United Kingdom to Uzbekistan. NAN is widespread in North America and is also found in Western Europe. Pathogenicity tests (artificial inoculation of elm trees) showed that NAN is more aggressive than EAN (Brasier & Kirk, 2001b).

Both *O. novo-ulmi* subspecies due to partial reproductive barrier can freely hybridize with each other. In Europe, EAN and NAN hybrids occur where both subspecies are found together (Brasier & Buck, 2001). To form a fertile perithecium, EAN shall act as a recipient and NAN - as a donor. When NAN is a recipient and EAN is a donor, the probability of formation of a fertile perithecium decreases by 10 % (Brasier, 1986a).

Both subspecies, EAN and NAN, are found in Europe. In some countries such as Belgium, Italy and Netherlands their distribution range overlaps, and

there EAN x NAN hybrids are common; in other countries only one subspecies is present and overlap zones could be formed by sudden second subspecies transfer to a new territory (taken by the first subspecies) (Brasier & Kirk, 2010).

A replacement of "pure" *O. novo-ulmi* subspecies by their hybrids was observed in the Netherlands (Limburg) and Italy (Orviet), and this may further lead to formation of new races or subspecies (Brasier & Kirk, 2010). It is very likely that a new step in evolution of DED is currently taking place in Europe. In North America, both subspecies (EAN and NAN) are separated by geographic barriers, but there is also a possibility of hybrid formation (Brasier & Buck, 2001).

In Lithuania, some information on distribution of *O. ulmi* s. l. may be found in a study by Žuklys (1958), and since this study no detailed information on distribution and consequences of DED in Lithuania or neighboring countries has been collected. Research on DED was renewed in 2010 by Norkutė (2013) – the most important findings are presented in this thesis.

1.3.2.6. Biology of *Ophiostoma ulmi* s. l.

O. ulmi s. l. is a heterothallic ascomycetous fungus and has both sexual and asexual life cycles. Asexually it can reproduce in two ways: i) by producing asexual spores (conidia); and ii) vegetatively - through a process known as budding (the same reproduction strategy as in yeasts). Formation of sexual structures (perithecia) is relatively rare in *O. ulmi* s. l.; nevertheless, it is assumed to play an important role in life cycle of the fungus due to occurring intra- and interspecific hybridization events and in formation of the heterogeneous population structure.

Brasier (1991) has separated *O. ulmi* s. l. into two species - *O. ulmi* s. s. and *O. novo-ulmi* by mycelium growth rate *in vitro*, optimal growth temperature, pathogenicity, mitochondrial DNA (mtDNA) size and some morphological differences of fungal cultures. The main difference between the two species is that *O. novo-ulmi* is much more pathogenic to elms compared to *O. ulmi* s. s.

The main reason for this is production of larger amounts of toxin cerato-ulmin in *O. novo-ulmi* (Bowden *et al.*, 1994).

Cerato-ulmin

Cerato-ulmin (CU) is a protein of low molecular mass (10 kDa) belonging to hydrophobin protein family (Takai, 1974; Wessels, 1994). It has been suggested by Bowden *et al.* (1994)that CU is a toxic metabolite of *O. ulmi* s. l. and that it is mainly responsible for elm wilt, because this protein is secreted directly into plant xylem cells with a free movement in all plant aqueous system. Takai (1974) showed that the most intense CU secretion occurs during a first phase of infection by the fungus. The CU was associated with pathogenicity of DED agents following finding that *O. novo-ulmi* secrets more of this protein compared to *O. ulmi* s. s. (Brasier, 1990). The mechanism how CU causes elm wilting is still unknown; it has been suggested that wilting is associated with differences in water potential in extracellular space or function disorder in cell membranes (Van Alfen, 1989).

However, it is still not clear if CU is solely responsible for pathogenicity of the DED agents. Brasier (1995) isolated aggressive *O. novo-ulmi* strains that did not have high CU synthesis activity. Bowden *et al.* (1996) interrupted CU gene expression in *O. novo-ulmi* and the pathogenicity of the fungus didn't change. These findings suggest that CU is not the only to be blamed for pathogenicity of *O. ulmi* s. l. Nevertheless, *cu* gene is acknowledged to be an important marker in identification of *O. novo-ulmi* subspecies and their hybrids (Konrad *et al.*, 2002).

Enzymatic activity

Pathogenicity enzymes are known to be highly important in an infection process because they act in dissolving cell walls (Keon *et al.*, 1990; Annis & Goodwin, 1997). First reports on enzymatic activity in *O. ulmi* s. l. appeared in a middle of the last century, when Beckman (1956) revealed celullase activity in isolates of *O. ulmi* s. l. Later, Elgersma (1976) demonstrated polygalacturonic

acid activity in mycelium of the pathogen. Przybyl *et al.* (2006) revealed exoglycanase, endoglucanase and β-glucosidase activity in *O. ulmi* s. l. The fungus also produces lactase, yet function of this enzyme is still unclear. Mayer & Staples (2002) suggested that lactase is important in pathogen protection from phytoalexins and tannins secreted by a host-plant following infection.

1.2.2.7. Symptoms of Dutch elm disease

DED is a vascular wilt disease of elms. There are both external and internal symptoms of the disease (Fig. 8 & Fig 9).

Main external disease symptoms:

External early-stage DED symptoms appear as wilting of a few branches in the upper crown. Leaves on infected branches start to change color from dark green to light green or yellow, later they turn dark brown and curl up, but remain attached to the branches (Fig. 8). Time between the occurrence of the first symptoms of disease and tree death depends both on virulence of the pathogen genotype and susceptibility of a host tree. It may take from a few months to a decade to kill a tree (Žuklys, 1957).

Early symptoms of the disease may be observed already in June. The leaves on one or more branches start to wilt, curl and droop. Wilted leaves become brown and usually remain attached to the branches. If secondary infection by the pathogen occurs during summer, the leaves will turn yellow and will drop prematurely. Trees that were infected in late summer or early autumn do not show typical DED symptoms: the foliage exhibits a general yellowing. The symptoms appear next year during spring as a sudden wilt. If a plant is infected through root contacts with an infected tree, the symptoms appear only within next year (Žuklys, 1957).

Fig. 8 External symptoms of Dutch elm disease on *Ulmus glabra* – wilting, yellowing and curled up leaves (photo: V. Lygis)

Internal disease symptoms:

Internal symptoms include discoloration of elm sapwood with light to dark brown streaks or patches. If the disease is in its aggressive form, one full dark brown ring appears on a cross-section of a stem, branch or twig (Fig. 9 A & B) (Žuklys, 1957).

Fig. 9 Internal symptoms of Dutch elm disease. A – Infected branches cross-section show discoloured ring of patches on elm sapwood; B – brown streaks on elm sapwood are visible after bark removal (adapted from http://www.dutchelmdisease.org; photo: H. Kaljee))

1.3.2.8. Spreading pathways of *Ophiostoma ulmi* s. l.

Larger European elm bark beetle (*Scolytus scolytus* Fabricius) and smaller European elm bark beetle (*Scolytus multistriatus* Marsham) are the main vectors of the pathogen in Europe. The larvae of these beetle species feed exclusively on elm wood (Faccoli & Santini, 2016). When adult beetle emerges from the brood wood, its body is covered with spores of *O. ulmi* s. l. The spores are also present in a digestive tract of the beetle. In this way insects ensure spread of the fungus in longer distances (Faccoli & Santini, 2016).

Different trees respond to infection differently: in some cases, a larger number of spores is needed to successfully infect the tree (Heimann *et al.*, 1997). Other insects such as weevils (*Curculionidae*) or long-horned beetles (*Cerambycidae*) can also transmit *O. ulmi* s. l. spores (Žuklys, 1957).

Insect transmission is not the only pathway of DED dispersal. Adult elm trees have large root systems that can form root grafts with other elms growing nearby. The pathogen inoculum is present in a vascular system of the infected tree and can move via root contacts (grafts) into adjacent healthy trees (Heimann *et al.*, 1997). This way of spreading may be noticed in parks where elm trees are planted in alleyways and disease symptoms are clearly visible. Sometimes elms can be infected through mechanical wounds on stems and roots (Žuklys, 1957).

1.2.2.9. Investigations into population genetics of *Ophiostoma ulmi* s. l. in Europe

In his study, Brasier (1988) demonstrated that initially (at the start of the epidemic) *O. novo-ulmi* had a clonal spreading in Europe, but as the epidemic progressed the pathogen underwent rapid genetic changes, shifting from the clonal to a genetically heterogenous organism (Mitchell & Brasier, 1994). The changes were associated with hypervariability in *vic* and *mat* loci obtained from *O. ulmi* s. s. during interspecific hybridization (Brasier *et al.*, 2004a).

To study genetic properties of Swiss *O. novo-ulmi* populations, Hoegger *et al.* (1996) used Random Amplified Polymorphic DNA polymerase chain reaction (RAPD-PCR). The results of this study showed presence of both subspecies and interspecific hybrids in Switzerland. *O. novo-ulmi* ssp. *americana* occurred more frequently and its populations were more genetically diverse compared to *O. novo-ulmi* ssp. *novo-ulmi*. A clonal pattern of the Swiss *O. novo-ulmi* population was only found only at the disease front, while in post-epidemic sites the fungus showed high genetic diversity (Hoegger *et al.*, 1996).

In Italy, Direct Amplification of Minisatellite DNA region (DAMD-PCR) was done using M13 primer as a core sequence (Stenlid *et al.*, 1994). The analysis revealed a high level of genetic variation in both subspecies, EAN and NAN (Santini *et al.*, 2005). In parallel, a vegetative compatibility test has been carried out which results coincided with the DAMD-PCR results showing a presence of large number of vegetative compatibility (vc) groups (Santini *et al.*, 2005).

In Spain, population of *O. novo-ulmi* was investigated using RAPD-PCR approach. The results of this study were in agreement with studies performed by Hoegger *et al.* (1996) in Switzerland and by Santini *et al.* (2005) in Italy, showing high genetic diversity within pathogen population (Solla *et al.*, 2008).

In Poland, population of *O. novo-ulmi* was investigated using PCR amplification of the internal transcribed spacer (ITS) 1/2 rDNA region. The results showed low genetic diversity in both subspecies, EAN and NAN (Łakomy *et al.*, 2016).

The study performed in Canada by Hintz *et al.* (2013) confirmed results obtained by Mitchell & Brasier (1994) in the United States of America, where *O. novo-ulmi* population showed little genetic diversity, and was dominated only by two clones.

1.3.2.10. Other pathogens causing diseases of *Ulmus* spp.

Botryodiplodia ulmicola (Ellis & Everh.) Buisman (= *Sphaeropsis ulmicola* Ellis & Everh) causes cankers on branches of *U. pumila* trees (Horst, 2008).

Stegophora ulmae (Fries) Sydow & Sydow causes anthracnosis to many tree species within genus *Ulmus*. In spring, during period of leaf sprouting, yellowish spots appear on tree leaves. The fungus can infect not only leaves but also buds. Bud infection can cause withering of young branches (Smith & Cartwright, 2009). *U. glabra* and *U. laevis* are sensitive to this pathogen (EPPO 2005).

Taphrina ulmi (Fuckel) Johanson causes leaf blister galls (Horst, 2008).

Stegophora ulmae (Fr.) Thüm is known to cause elm leaf scab to *Ulmus americana* L., *U. parvifolia* and *Ulmus procera* Salisb. (Horst, 2008).

Mycosphaerella ulmi Kleb., *Coryneum tumoricola* Peck, *Septogloeum profosum* (Ellis & Everh.) Sacc. and *Phyllosticta confertissima* Ellis & Everth also may cause elm leaf spot disease (Horst, 2008).

Dothiorella ulmi Verrall & C. May and *Phomopsis oblongata* (Desm.) Höhn. may cause a dieback of elm trees (Horst, 2008).

P. cactorum, *Botryosphaeria ribis* Grossenb. & Duggar, *Anisogramma apiospora* (Ellis & Everh.) Merezhko, and *Phoma* spp. are known to cause stem and branch cankers to the elm trees (Horst, 2008).

1.3.3. Ash dieback: history, causes and distribution

1.3.3.1. Trees of genus *Fraxinus* in Europe

Genus *Fraxinus* L. (Oleaceae) includes about 40 species of trees and shrubs distributed in the Northern Hemisphere. In Europe, there are three naturally growing *Fraxinus* species: *Fraxinus excelsior* L., *Fraxinus angustifolia* Vahl. and *Fraxinus ornus* L. (Wallander, 2008). Distribution range

of common or European ash (*F. excelsior*) stretches across Europe: from Atlantic coast in the West to Volga river in the East; from central Norway in the North to northern Greece, Turkey and Iran in the South (Fig. 10).

At maturity (90-120 years), common ash tree can reach 20-35 m in height and is considered to be the largest tree in the genus (Douglas *et al.*, 2013). The species prefers rich soil that has pH above 5.5 and shows the best growth on alkaline or neutral soils, but can tolerate as low pH as 4.5. Usually ash trees form unique ecosystems in floodplain forests, slopes and ravines; they can grow also in mountainous areas. Common ash tolerates excess moisture in the soil and can survive flooding and temporary droughts. It may act both as a pioneer species and as a competitor/stress-tolerant competitor able to survive as a permanent forest component. Due to its high quality timber, common ash is the most commercially important tree species in the genus *Fraxinus* (Douglas *et al.*, 2013). Ash trunks support a large diversity of lichen, bryophyte, vascular epiphyte and fungal species (Thomas, 2016).

All three European *Fraxinus* species are susceptible to currently occurring ash dieback disease (Kirisits *et al.*, 2009; Gross *et al.*, 2014b), of which *F. ornus* is the least susceptible (Kowalski *et al.*, 2015). *F. excelsior* of all age classes are susceptible to the dieback irrespective of site conditions (Bakys *et al.*, 2009).

In Lithuania, common ash is the only naturally growing ash species. It forms unique broadleaved ecosystems of mixed wetland forests and occurs mostly in mixed stands (Narbutas, 1975; Navasaitis *et al.*, 2003). The main delimiting factors of common ash natural distribution in Lithuania are their lack of tolerance to prolonged draught, winter cold and late spring frosts; on the other hand, *F. excelsior* is adaptive to a wide range of environmental conditions, exhibiting intermediate properties between a pioneer species and a permanent forest component (Pliūra, 1999; Pliūra & Heuertz, 2003; Dobrowolska *et al.*, 2011). Because of prolific natural regeneration, stands dominated by common ash in Lithuania were usually reforested naturally, but there was some use of ash transplants from tree nurseries in afforestation as well (Pliūra *et al.*, 2017). *F.*

excelsior thrives in humid, nutrient-rich calcareous soils. The species is most common in central and northern Lithuania: the highest concentration of its stands is found in Biržai, Kėdainiai and Panevėžys regions. Although the proportion of common ash in a total growing stock of Lithuanian forests is small, this species is regarded economically important: its wood is used for production of furniture, parquet, interior facing and firewood.

During the XXth century the area of *F. excelsior*-dominated stands in Lithuania has been gradually increasing and reached 52,700 hectares in 2001. However, the situation has changed dramatically due to the ash dieback epidemic which emerged in the country in 1996 (Pliūra *et al.*, 2017). Due to inevitable sanitary fellings, the area of common ash stands in the country has decreased from 52,700 ha (estimation of 2001) to 18.200 ha in 2017, i.e., from 2.7 % to 0,9 % of all forested area (Anonymous, 2017).

1.3.3.2. History of ash dieback epidemics in Europe

The first reports on dieback of *F. excelsior* came from eastern Poland in early 1990's (Kowalski, 2006), and during the last 25 years this disease has emerged in nearly every European country (Solheim & Hietala, 2017) (Fig. 10). Pukacki & Przybyl (2005) suggested that sudden ash dieback could occur due to abiotic factors, mainly drought and frost, however artificial inoculation of *F. excelsior* seedlings with microorganisms isolated from declining ash trees showed that for this dieback is actually responsible a previously unknown fungus (Kowalski & Holdenrieder, 2009). At first the pathogen was identified as new anamorphic ascomycetous species *Chalara fraxinea* T. Kowalski (Kowalski, 2006). In 2011, due to new insights into pathogen ascocarp morphology and deeper DNA analyses, the name was changed to *Hymenoscyphus pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T.N. Sieber & Holdenr (Queloz *et al.*, 2011). In 2014, the pathogen was renamed to *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya; name *H.*

pseudoalbidus became a taxonomic synonym and its anamorphic stage *C. fraxinea* –a basionym (Baral *et al.*, 2014).

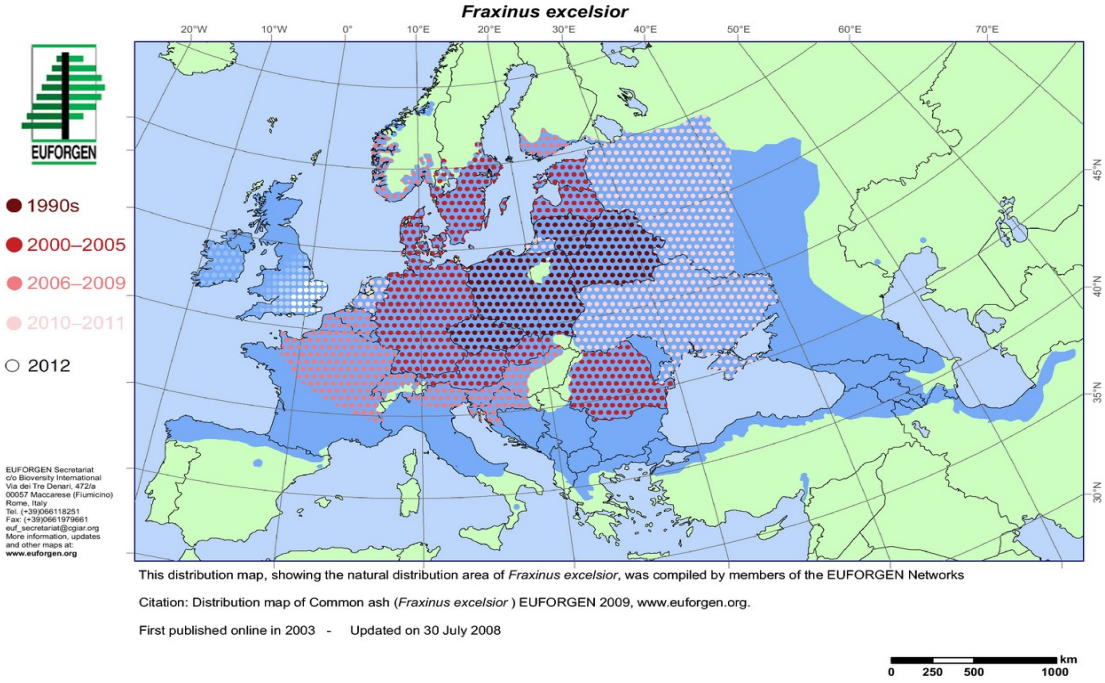

Fig. 10 Distribution map of common ash (*Fraxinus excelsior*) and ash dieback disease in Europe (adopted from McKinney *et al.* 2014): Blue color shows distribution range of *F. excelsior*; dots of different color indicate year of the first record of ash dieback disease in respective areas (see figure legend).

In Lithuania, the dieback of common ash has emerged in 1996 in forests of North-Central Lithuania and soon has spread across the country (Juodvalkis & Vasiliauskas, 2002; Gustienė, 2010). Currently the epidemic is in its chronic phase, large areas of Lithuanian *F. excelsior* stands are already felled by selective sanitary fellings or sanitary clearfellings, and all remaining ash stands are affected to a greater or lesser extent. According to forest monitoring data, health condition of ash stands continues to deteriorate (Pliūra *et al.*, 2011; Lygis *et al.*, 2014).

1.3.3.3. Hypothesis of *Hymenoscyphus fraxineus* origin

The exact area of origin of *H. fraxineus* is still unknown, however most studies suggest that the pathogen has been introduced to Europe from Eastern

Asia (Queloz *et al.*, 2011; Zhao *et al.*, 2012; Han *et al.*, 2014; Zheng & Zhuang, 2014; Cleary *et al.*, 2016). The first to suggest this hypothesis was Queloz *et al.* (2011) following observation of a lower susceptibility of *F. ornus* to *H. fraxineus* compared to other European ash species and genetic similarity of *F. ornus* to Asian ash species. After redefinition of *Lambertella albida* (Gillet) Korf (ascomycete isolated from petioles of *Fraxinus mandshurica* Rupr in Japan) to *H. fraxineus* (Zhao *et al.*, 2012), the origin hypothesis has became more established. Moreover, a study by Zhao *et al.* (2012) revealed higher genetic variation in nucleotides of nuclear ribosomal DNA (ITS), calmodulin (CAL) and translation elongation factor EF1- α (EF1- α) genes in *H. fraxineus* isolates in Japan compared to isolates from Europe. After a few years Zheng & Zhuang (2014) published a report of *H. fraxineus* isolation from *F. mandshurica* leaves in northeast China. During this study the authors also described a new species found on *F. mandshurica* leaves - *Hymenoscyphus albidioides* H.D. Zheng & W.Y. Zhuang. This newly described fungus has close genetic relationship to *H. fraxineus* (Zheng & Zhuang, 2014). South Korea is another country where *H. fraxineus* was isolated from asymptomatic native to Asia *Fraxinus rhynchophylla* Hance tree (Han *et al.*, 2014). Cleary *et al.* (2016) reported *H. fraxineus* isolation from healthy, asymptomatic *F. mandshurica* tree, and suggested that this fungus most likely acts as an endophyte or only a weak pathogen in its native range, causing no harm to locally growing (Asian) ash trees.

1.3.3.4. The main symptoms of ash dieback

The main symptoms of the disease are necrotic spots that occur on ash stems, branches and shoots gradually developing to necrotic lesions (Fig. 11 B & C) that change bark color to orange or brown. Later, cankers develop at the lesion sites (Fig. 11 D). This causes tree crown dieback (Fig. 11 A) that may further be a cause of premature death of the whole tree (Kowalski, 2006). At the initial phase of the disease, symptoms appear at tree top – typical necrotic lesions

on twigs and leaf petioles, causing leaf wilting and premature shedding; later the pathogen gradually affects lower stem parts (Kowalski, 2006; Kräutler & Kirisits, 2012).

Fig. 11 The main symptoms of ash dieback. A – Crown dieback of *Fraxinus excelsior;* B –necrotic lesions of stem and leaves on ash seedling (photos: A. Gustienė) (brown colour); C – necrotic lesion on stem (dark brown colour); D – cankers on ash branches (photos: D. Burokienė).

1.3.3.5. Life cycle of *Hymenoscyphus fraxineus*

H. fraxineus is a heterothallic fungus. During summer-autumn on petioles of fallen ash leaves of the previous year (in leaf debris) the fungus forms fruiting

bodies – apothecia, that release short-lived sexual spores – ascospores (Gross & Holdenrieder, 2013). The ascospores are usually dispersed by wind and can infect healthy ash leaves (Kowalski *et al.*, 2013). The ascospores of *H. fraxineus* are of different mating types (Gross *et al.*, 2012b). A spore germinates on ash leaf surface and the infectious mycelium develops intracellularly, colonizing phloem through pits between parenchymal cells and fiber (Dal Maso *et al.*, 2012). Following successful infection, the pathogen spreads from leaves to twigs and branches where symptoms of the disease develop. On fallen leaves *H. fraxineus* forms opposite mating type structures: spermatia and ascogonium. After fertilization, ascoma (ascocarp) starts to develop and during next summer-autumn new infection cycle starts (Gross *et al.*, 2014a).

Fones *et al.* (2016) showed that in the absence of mating partner the amplification of *H. fraxineus* inoculum may occur also in asexual way. Asexual conidia can germinate and mycelium proliferates in soil, infecting new ash trees through root contacts. It has been suggested that *H. fraxineus* conidia may act as infectious propagules not only in soil but also may be transferred by wind, , implying a higher significance of asexual spores in epidemiology of the disease (Fones *et al.*, 2016).

1.3.3.6. Spreading pathways of the ash dieback pathogen

Airborne ascospores predominantly facilitate the long-range dispersal of *H. fraxineus;* conidia embedded in mucilage-like droplets seem to be far less important (Kowalski & Holdenrieder, 2009; Timmermann *et al.*, 2011) Another important pathway for the long-range dispersal of the pathogen - international trade of diseased nursery seedlings (Kirisits *et al.*, 2010): infected seedlings could carry apothecia in their shoots (Kowalski & Holdenrieder, 2009) or disease could be transported through bare-root container-grown seedlings with infected leaves (Timmermann *et al.*, 2011). It was hypothesized that the fungus has been introduced to Europe from East Asia with infected seedlings of *F.*

mandschurica (Zhao *et al.*, 2012). Moreover, it is believed that the disease has been introduced to the British Isles by nursery plants of *F. excelsior* from the Netherlands (Anonymous, 2018b). A study carried out by Cleary *et al.* (2013) has revealed that *H. fraxineus* can infect ash seeds and thus the inoculum may be transferred over long distances.

1.2.3.7. Investigations of genetic diversity, population structure and virulence of *Hymenoscyphus fraxineus* in Europe

European populations of *H. fraxineus* have been studied using different approaches. Two published studies were based on Random Amplified Microsatellites (RAMS) analyses (Rytkönen *et al.*, 2011; Kraj *et al.*, 2012). Kraj *et al.* (2012) showed genetic variation in Polish *H. fraxineus* population that was explaned by differenes in climatic conditions following elevation gradient.

More recent *H. fraxineus* population genetic studies were based on microsatellite analyses (Bengtsson *et al.*, 2012; Gross *et al.*, 2012a, 2014b; Burokienė *et al.*, 2015; Hañáčková *et al.*, 2015; Cleary *et al.*, 2016; Nguyen *et al.*, 2016). Bengtsson *et al.* (2012) and Gross *et al.* (2012a) were the first that showed low genetic variation among and within *H. fraxineus* populations using SSRs. Gross *et al.* (2014b) compared *H. fraxineus* poulations from Europe and Japan. This study showed that pathogen populations overlap, but *H. fraxineus* isolates from Japan are more genetically diverse. In this work the authors suggested that ash dieback pathogen to Europe was introduced once or in a few separate events (Gross *et al.*, 2014b). Burokiene *et al.* (2015) carried out the study on post-epidemic *H. fraxineus* population in Lithuania and pathogen population in epidemic front in Switzerland and observed no genetic differentiation between the investigated populations (Lithuania vs. Switzerland) and among subpopulations within each country. Cleary *et al.* (2016) demonstrated genetic differentiation between *H. fraxineus* populations in Europe and Eastern Russia. The authors found that Russian *H. fraxineus* population had more allelic diversity compared to the European population.

Nguyen *et al.* (2016) have performed genotyping of *H. fraxineus* DNA after its direct extraction from leaf petioles and came to a conclusion that tree species composition in the sampling site could have an impact to the genotypic diversity of the pathogen. In order to determine *H. fraxineus* population structure in the United Kingdom, Orton *et al.* (2018) combined vegetative compatibility (vc) test results with Single Nucleotide Polymorphism (SNP) analysis results. vc tests and SNPs analysis results showed high local genotypic diversity and little genetic differentiation among continental European populations of the pathogen. Most of the above-mentioned studies showed low allelic diversity but high genotypic diversity in the investigated *H. fraxineus* populations.

Following sequencing of *H. fraxineus* genome Mcmullan *et al.* (2018) suggested that European population of the ash dieback pathogen has been founded by two divergent haploid individuals, that have probably came from the same site or even from a single ascocarp (fruiting body).

For better pathogen biology understanding versatile investigation is must. Pathogen aggressiveness research is as important as its genetic research. At first the inoculation experiments proved *H. fraxineus* pathogenicity to the *F. excelsior* plants (Bakys *et al.*, 2009; Kirisits *et al.*, 2009; Kowalski & Holdenrieder, 2009). Later these experiments helped to verify *H. fraxineus* pathogenicity to other native European *Fraxinus* genus trees (Kirisits *et al.*, 2010; Kräutler & Kirisits, 2012). At the same time, wound inoculation to leaf rachises confirmed that *H. fraxineus* can infect trees through leaf scars and let to better understanding of pathogen infection ways (Kräutler & Kirisits, 2012). Nowadays *H. fraxineus* experiments are done to search resistant or tolerant *Fraxinus* sp. trees (Budde *et al.*, 2016; Landolt *et al.*, 2016; Wohlmuth *et al.*, 2018).

Although there are numerous studies on the genetic structure of *H. fraxineus* in Europe, there still is a lack of studies performed on relationships between pathogen virulence and its genetic structure. The study on pathogen virulence in recently established (Switzerland) and old (Lithuania) populations was done by Lygis *et al.* (2017). During this work, a high variation in virulence

was observed with no significant differences between *H. fraxineus* populations in the two countries.

2. MATERIALS AND METHODS

2.1. Recovery of oomycetes and fungi from declining alder stands and population genetics of *Phytophthora alni* s. l.

2.1.1. Sampling sites (alder Phytophthora)

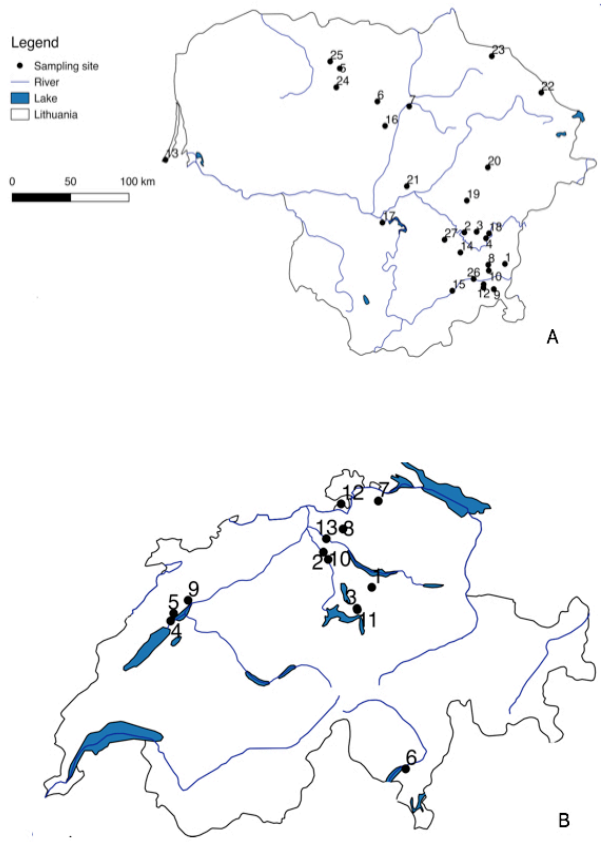

Fig. 12 Sampling locations of symptomatic black alder (*Alnus glutinosa*) and grey alder (*A. incana*) trees, likely infested with alder decline-causing *Phytophthora* species in Lithuania (A) and Switzerland (B). Numbers associated with sampling sites are explained in Table 2.

Alder trees, *A. glutinosa* and *A. incana* were surveyed for symptoms caused by *Phytophthora* spp. in randomly selected regions of Lithuania (Fig. 12 A), and following a survey scheme suggested by French phytopathologist Dr. Claude Husson (personal communication) in Switzerland (Fig. 12 B). In Lithuania, the sampling was carried out in 27 sites during April–October period of years 2014–2016, and in Switzerland – in 14 sites during April–October period of year 2015 (Fig. 12, Table 2). For sampling, selected were alder trees

showing symptoms of bleeding cankers (tarry or rusty spots) on bark at a stem bottom, root and root collar necroses, twig and branch necroses, thin foliage and crown wilting. The most commonly observed symptoms were crown decline (Fig. 2 E) and bleeding tarry spots on bark at the tree base (Fig. 2 A).

2.1.2. Sampling procedures

Three types of samples were collected during the surveys: i) pieces of alder tree tissue (wood); ii) water samples and iii) soil samples taken in the vicinity of symptomatic alder trees. In total, 201 wood, 17 water and 110 soil samples were collected. Information on sampling sites is presented in Table 2.

Alder tree tissue samples for oomycete and fungal isolations were taken using two different approaches:

i) Wood samples (one sample per tree) were cut out from symptomatic stem or root zones (with a typical bleeding canker or necrosis) using a sterilized axe. Using this approach sampled were 130 trees from 19 locations in Lithuania (Table 2). The sampled woody pieces were immediately placed into separate vessels with sterile deionized water and stored in cool conditions for 24 h until processed in the laboratory (Jung, 2009).

ii) Bark has been removed from the symptomatic tree stem area prior to sampling, and 21 infected (showing symptoms typical to *Phytophthora* infections – brownish flame-shaped active front (Redondo *et al.*, 2015) tissue pieces of ca. 1 x 2 mm were taken from each sampled tree using a Jamshidi needle. Using this approach sampled were 10 trees from one location in Lithuania and 61 trees from 14 locations in Switzerland (Table 2). The sampled phloem pieces from each tree were immediately placed into three 90-mm Petri plates with selective PARPNH-V8 juice agar medium (Tsao, 1983) so that each plate contained seven pieces. During fieldwork (but not longer than for 12 hours) the plates were kept cool until transported to the laboratory for further incubation (see subsection "2.1.4. Isolation of oomycetes and fungi from alder wood samples").

Table 2 Information about sampling sites and sample types for recovery of alder *Phytophthora*.

Country	Site	Point on map[a]	Coordinates (WGS84)		Name of waterbody at the sampling site[b]	Sampled *Alnus* species	Forest type[c]	Number of samples taken from a respective substrate[d]		
			Longitude	Latitude				A	S	W
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Lithuania	Ulėnai	1	25.534722	54.5575	Confluent stream to Kena river	*A. glutinosa*	M/ST	1	0	7
	Vaigeliškės	2	25.0025	54.79388	Confluent river to Vaigolė stream	*A. glutinosa*	ST	0	0	3
	Riešė	3	25.165277	54.79388	-	*A. incana*	ST	0	0	6
	Verkių rūmai	4	25.28944	54.7477	Verkė [R]	*A. glutinosa*	UR/R	1	1	5
	Bridai	5	23.3166	56.00277	-	*A. glutinosa*	ST	0	0	21
	Pakalniškiai	6	23.8366	55.76222	Daugyvenė [R]	*A. incana*	R	0	0	4
	Nausodė	7	24.1622	55.4333	Paviesečiai [P]	*A. incana*	UR/R	0	0	3
	Kelmytė	8	25.31277	54.55388	Peteša [R]	*A. glutinosa*	R	0	0	5
	Šalčininkėliai	9	25.38	54.37416	Šalčininkėliai [P]	*A. glutinosa*	R	0	0	5
	Marijampolis	10	25.3188	54.51083	Unnamed stream	*A. glutinosa*	ST	0	0	3
	Dainava	11	25.2472	54.41111	-	*A. glutinosa*	ST	0	0	1
	Kidarai	12	25.2433	54.3875	Upėsė [R]	*A. glutinosa*	ST	0	0	16
	Grobštas	13	20.9711	55.29361	-	*A. glutinosa*	ST	0	10	0
	Trakai	14	24.94527	54.64722	Nerespinka [L]	*A. glutinosa*	UR	1	0	5
	Valkininkai	15	24.8322	54.36777	Geluža [R]	*A. glutinosa*	R	0	2	10
	Rūtakiemis	16	23.94138	55.58083	Unnamed stream	*A. glutinosa*	M	0	0	10
	Freda	17	23.9077	54.87111	Unnamed pond	*A. glutinosa*	UR	1	0	4

Table 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
	Balsiai	18	25.3316	54.78361	Balsys [L]	*A. glutinosa*	UR/R	1	0	5
	Katūniškiai	19	24.938659	54.976084	Unnamed stream	*A. incana*	ST	0	0	5
	Utena	20	25.33195	55.26984	-	*A. incana*	R	0	6	0
	Bukonys	21	24.23559	55.13708	Medukšna [R]	*A. glutinosa* and *A. incana*	ST	0	5	0
	Antazavė	22	25.977934	55.812995	Duburė [R]	*A. glutinosa* and *A. incana*	R	0	5	0
	Panemunėlis	23	25.21236	55.52792	-	*A. incana*	ST	0	5	0
	Tytuvėnai	24	23.12259	55.39769	Šimša [R]	*A. glutinosa* and *A. incana*	R	0	5	0
	Žiogai	25	23.11393	56.02993	-	*A. glutinosa* and *A. incana*	ST	0	5	0
	Baltoji Vokė	26	25.0638	54.2642	Merkys-Vokė [D]	*A. glutinosa* and *A. incana*	R	0	5	17
	Jagėlonys	27	24.34612	54.44033	Spengla [R]	*A. incana*	R	0	5	0
Switzerland	Schwyzerbrugg	1	8.7123	47.15264	Biber [R]	*A. incana*	R	1	0	0
	Eggenwil	2	8.3384	47.36432	Reuss [R]	*A. incana*	R	0	4	4
	Steinerberg	3	8.57789	47.04523	Goldbach [L]	*A. incana*	R	1	2	2
	Le Landeron	4	7.05001	47.0499	Vieille Thielle [D]	*A. glutinosa*	R	1	0	0
	Lignières	5	7.07807	47.09019	Unnamed pond	*A. glutinosa*	R	1	4	4
	Magadino	6	8.8694	46.15581	Ticino [R]	*A. incana*	R	0	6	6
	Nusbaumen	7	8.82237	47.61628	Nussbommersee [L]	*A. incana*	M	0	2	2
	Oberglatt	8	8.51323	47.48062	Glatt [R]	*A. incana*	M	1	2	2
	Orvin	9	7.20246	47.15602	l'Orvine [R]	*A. glutinosa* and *A. incana*	R	2	11	11
	Rottenschwil	10	8.373	47.32264	Reuss [R]	*A. glutinosa* and *A. incana*	R	1	10	7
	Laurez	11	8.58025	47.03799	Chlausenbach [L]	*A. glutinosa* and *A. incana*	R	1	8	8
	Wil	12	8.51377	47.61742	Schwarzbach [R]	*A. glutinosa* and *A. incana*	R	1	2	2
	Würenlos	13	8.37022	47.43536	Limmat [R]	*A. glutinosa* and *A. incana*	R	1	5	5
	Cartigny	14	6.02107	46.18187	Rhône [R]	*A. glutinosa*	R	1	5	5

[a] - Respective sampling points are shown on maps in Fig. 12. [b] - Categories of waterbodies at sampling sites: - no waterbody present at the sampling site; [D] – duct, [L] – lake, [P] – pond, [R] – river. [c]Forest type: M – marsh, R – riparian, ST – forest stand, UR – urban [d]Substrate type: A – water samples, S – soil samples; W – wood samples

Water samples were collected from rivers and lakes, on the banks of which grew symptomatic *A. glutinosa* and/or *A. incana* trees (Table 2). Five water samples were collected in Lithuania and twelve – in Switzerland. At each sampling location, about 1.5 l of water with sediments was sampled into a sterile vessel. The water samples were kept cool for not longer than 12 hours until subjected to baiting tests in the laboratory (Dr. Sabine Werres, personal communication).

Soil samples were collected according to Tedersoo *et al.* (2014). First, at each sampling site, 2–11 trees growing no closer than 8 m from each other were selected. Soil was sampled at a depth of 5–20 cm using a soil core sampler at four cardinal directions at a distance of 0.5–1.0 m from the root collar of each selected tree. All four soil samples (about 1.5 kg each) from one tree were later pooled together and then a representative soil subsample of 500 g was made per tree. A total of 54 soil subsamples were collected in Lithuania and 58 – in Switzerland. The samples were kept cool for not longer than 12 hours until transported to laboratory. In the laboratory, all organic matter and stones were removed from the samples. The soil was sifted using a sieve (mesh opening size 2 mm). From each sample, approximately 150 g of the sifted soil was placed into a sterile plastic bag and deep-frozen for a long-term storage and 200 g was used for baiting tests.

2.1.3. Phytophthora baiting tests (recovery from soil samples)

Phytophthora baiting tests were done for water and soil samples using fresh (green) asymptomatic leaves of rhododendron species *Rhododendron catawbienese* Michx. or rhododendron hybrid 'Cunningham's White'. For baiting tests in water samples, rhododendron leaves were placed into plastic boxes containing 400 ml of the sampled water with sediments from each sampling site. Five leaves of rhododendron hybrid 'Cunningham's White' were used for the baiting tests of water sampled in Lithuania, and four leaves of *R. catawbienese* - for Swiss water samples. Negative controls were prepared in the

same way, but here, instead of sampled water with sediments, 200 ml of peat (pH 4.3±0.1) was mixed with 400 ml of distilled water and 20 ml of sterile CA medium. The prepared baits were incubated at a 16/8-hour light/darkness regime using temperatures of 20 ºC and 15 ºC during light and darkness periods, respectively. The test was regarded positive when typical necrotic spots have appeared on the rhododendron leaves (Fig. 13 A) (Dr. Sabine Werres, personal communication). These leaves were immediately subjected to isolation of target oomycetes (see below).

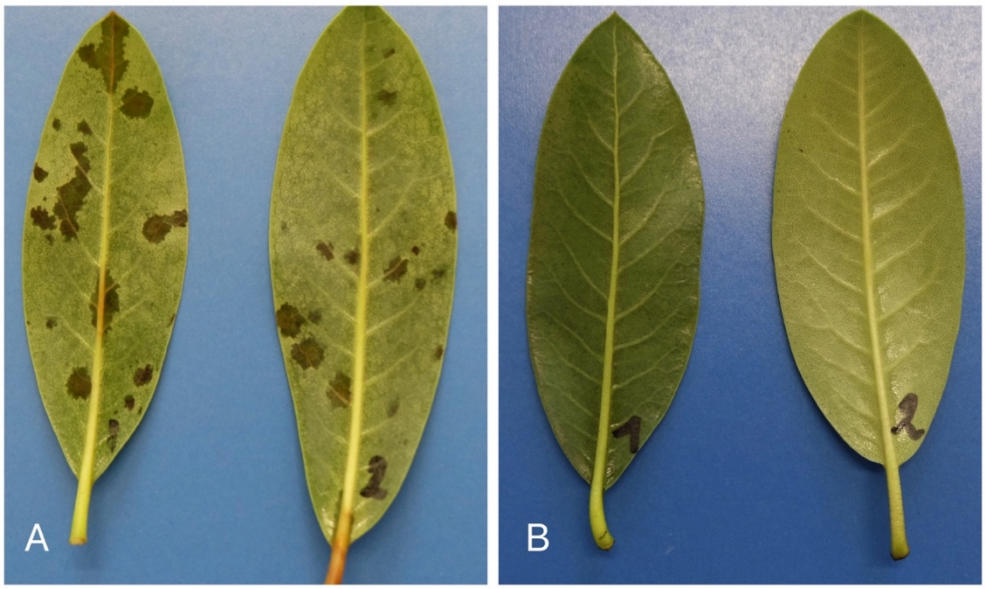

Fig. 13 Baiting-test on rhododendron leaves. A, leaves showing clear symptoms of *Phytophthora* infection (black spots); B, negative control (leaves incubated in sterile water).

Because of failure to collect necessary amount of wound-looking rhododendron leaves in Lithuania, soil bait tests were performed only with soil samples from Switzerland. Each soil sample (200 ml) was mixed with 400 ml of distilled water in a sterile plastic container. Four *R. catawbienese* leaves were used for baiting of each sample. Negative control (Fig.13 B) was prepared in the same way as described above for the water baiting. Incubation regime was the same as in water baiting tests.

2.1.4. Isolation of oomycetes and fungi from alder wood samples

In order to remove excess polyphenols from larger wood samples, the deionized water used to prevent wood samples from drying out was replaced with the fresh deionized water every 6–8 hours (Jung, 2009). After this "washing" procedure (it lasted for three days), 15 small woody pieces (ca 1.0 mm x 1.5 mm in size) were cut out from each sample using a sterile scalpel. The cuttings were made at the margin between symptomatic (discoloured) and wound-looking wood. The small woody pieces were subsequently sterilized by submerging into 0.5 % NaOCl solution for 30 s and briefly rinsing in sterile deionized water (the procedure was repeated twice), then they were blotted up by placing onto sterile filter paper for several seconds, and plated onto selective PARPNH-V8 juice agar medium in 90-mm Petri plates.

All Petri plates with woody pieces were incubated at 22 °C in the dark to induce growth of target oomycetes. The plates were periodically examined for a presence of *Phytophthora*-like hyphae. When hyphal growth was observed, an agar plug from the colony margin was transferred to new Petri plates with CA medium and incubated at 22 °C in the dark to produce pure cultures.

2.1.5. Isolation of oomycetes from rhododendron leaves

Symptomatic rhododendron leaves (with necrotic spots) from baiting tests (Fig. 13 A) were washed in a running tap water. The leaf surface was subsequently sterilized by submerging the leaves for 1 min into 0.5 % NaOCl solution. The leaves were then washed in distilled water two times for 1 min and were allowed to dry out on a sterile filter paper. For *Phytophthora* isolation, five (ca 3 mm x 3 mm in size) tissue samples from around the necrotic spots were cut out from each leaf and placed on separate Petri plates containing CA medium (Themann & Werres, 2000). The plates were incubated at 22 °C in the dark and pure cultures were obtained as described above.

2.1.6. Isolation of oomycetes from soil suspension

Soil samples collected in Lithuania were subjected to oomycete isolation by using soil suspension-plating method. To prepare soil suspension, 50 g of a soil sample was mixed with 100–250 ml of distilled water (amount of water depended on soil type, more water (ca 250-350 ml) was used for organic soil) in 500 ml Erlenmeyer flasks. The soil suspension was incubated for seven days in an incubator shaker Satorius adjusted to 90 rpm and 20 ºC temperature. After the incubation, the flasks were placed at 4 ºC for one hour. Before spreading the suspension onto agar medium, the flasks were gently shaken. Sterile glass spreader was used to spread 1 ml of the soil suspension on selective PARPNH-V8 juice agar medium in 90-mm Petri plates. To induce oomycete growth, the plates were incubated in the dark at 22 °C. After 2–5 days of incubation, the plates were examined using a light microscope for the growth of *Phytophthora*-like hyphae. The occurring hyphal tips were transferred onto CA medium using a sterile inoculation needle.

2.1.7. Genetic analyses

2.1.7.1. DNA isolation

To prepare sample for DNA isolation, an agar plug overgrown with mycelium of the target microorganism was taken from the margin of the actively growing culture and transferred to a new Petri plate containing liquid V8 juice medium. The plates were then incubated for 5–7 days (until plate was ~80 % covered by culture) in the dark at 22 °C. The occurring mycelium was harvested on a filter paper by filtering using a Büchner funnel and a vacuum pump. The harvested mycelium was washed out from the filter paper with sterile distilled water and placed to a freezer at -20 °C until next DNA isolation step. Later collected mycelium was lyophilized. The DNA from the freeze-dried samples was isolated using DNeasy® Plant Mini kit and DNeasy® 96 Plant Kit (Qiagen,

Hilden, Germany) or using CTAB protocol (Rosling, 2003). Prior to DNA extraction, ca. 2 mg of mycelium was homogenized in 2.0-ml microcentrifuge tubes containing about 650 µL of CTAB extraction buffer (3 % cetyltrimethylammonium bromide, 0,5 mM EDTA, 1 M Tris-HCl, 5 M NaCl) with added five sterile 2.5-mm glass beads in FastPrep (FP120) Bio101 (Savant, Qbiogene, Cedex, France) homogenizer of biological samples. The samples were homogenized two times for 45 s at 6,000 rpm. After the homogenisation the tubes were placed on a heating block to incubate at 65 °C for one hour. After centrifugation for 5 min at 8,000 rpm, the supernatant (about 650 µl) was transferred to a new 1.5-ml Eppendorf tube and mixed with equal volume of chloroform. After second centrifugation for 8 min at 10,000 rpm, the supernatant was precipitated with two volumes of ice-cold isopropanol for 30 min at -20 °C, centrifuged at 12,000 rpm for 30 min at 4 °C, washed with 70 % ethanol, centrifuged at 12,000 rpm for 5 min at 4 °C and resuspended in 50 µl of MilliQ water. Concentration of the extracted DNA was determined using spectrophotometer Implen P330 (Implen GmbH, Munich, Germany) and diluted in MiliQ water to concentration of 10 ng/µl.

2.1.7.2. Polymerase chain reaction and DNA sequencing for microorganism identification

Isolates were identified to species by sequencing ribosomal ITS region. PCR amplifications were conducted in a 20-µl reaction volume containing final concentrations of 2x Master mix (JumpStart™ RedTaq® Ready Mix (Sigma-Aldrich, Germany)), 0.625 µM of each ITS6 (Cooke *et al.*, 2000) and ITS4 (White *et al.*, 1990) primers, and 1 µl template DNA. The PCR program consisted of an initial denaturation at 95 °C for 2 min., followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min., and a final extension at 72 °C for 10 min. The PCR products were purified using the Illustra™ ExoProStar™ PCR and Sequence Reaction Clean-Up Kit (Sigma-Aldrich, Germany), according to the manufacturer's instructions. For sequencing, the

BigDye™ Terminator v. 3.1 Cycle Sequencing Kit and BigDye™ x-Terminator Purification Kit (Applied Biosystems, Carlsbad, CA) were used according to the manufacturer's protocol. Sequencing was performed on a 3130xl DNA Analyzer (Applied Biosystems, USA). The obtained sequences were arranged using softaware CLC Main Workbench version 8.0 Beta 4 (QIAGEN Bioinformatics, Denmark) and Geneious version R9.1.5 (Biomatters Ltd, New Zealand). For species identification, sequences were compared with publicly available sequences in the National Center for Biotechnology Information (NCBI) database with the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Two sequences were considered to belong to the same species if they showed at least 99 % similarity.

Table 3 Referential sequences used in neighbor-joining tree

Species	GenBank accession number	Reference
Phytophthora x *alni*	AF139366	Brasier et al. (2004b)
Phytophthora citrophthora	HQ643206	Robideau et al. (2011)
Phytophthora cryptogea	HQ261543	Robideau et al. (2011)
Phytophthora gallica	DQ286726	Jung & Nechwatal (2008)
Phytophthora gonapodyides	HQ643232	Robideau et al. (2011)
Phytophthora gregata	EU301171	Jung & Burgess (2009)
Phytophthora hydropathica	EU583798	Hong et al. (2010)
Phytophthora lacustris	JF714489	Nechwatal et al. (2013)
Phytophthora niederhauseri	HQ261699	Robideau et al. (2011)
Phytophthora pgchlamydospora	HM004224	Reeser et al. (2011)
Phytophthora plurivora	EU263906	Unpublished
Phytophthora pseudosyringae	HQ643326	Robideau et al. (2011)
Phytophthora uniformis	AF139367	Brasier et al. (2004b)
Phytophthora taxon Oaksoil	AF541906	Brasier et al. (2003)
Pythium intermedium	AY598647	Robideau et al. (2011)

The final species identification was made through phylogenetic analysis using Geneious v. R9.1.5 (Biomatters Ltd, New Zealand). Representative DNA sequences of 20 preliminarily identified *Phytophthora* taxa were aligned with 15 sequences from the NCBI GenBank database which included 14

Phytophthora species and one *Pythium* species (*Py. intermedium,* used as an outgroup) (Table 3). Rooted neighbor-joining tree was constructed using Tamura-Nei genetic distance model, with a support threshold of 70 %.

2.1.7.3. Microsatellite analysis of the recovered *Phytophthora* species

For the present study, *P.* x *alni* and *P. uniformis* isolates from seven European countries: Lithuania, Switzerland, Austria, Czech Republic, Hungary, Spain and Sweden was investigated (Appendix 2). Ten polymorphic microsatellite loci were selected for population genetic analyses of the recovered *Phytophthora* species (Table 4): two markers were adopted from Ioos *et al.* (2007), and another ten markers - from Aguayo *et al.* (2013; 2016). A single-tube nested PCR method developed by Schuelke (2000) was used to amplify DNA of the investigated phytophthoras: a sequence-specific forward primer had M13F700 tail attachment at its 5' end, while the fluorescent-labeled primer also had M13F700 tail.

Type-it Microsatellite PCR Kit (Qiagen, Valencia, CA, USA) was used to amplify 1 µl of 10-times diluted DNA following modified manufacturer's protocol. The protocol amendments included i) less of the forward primer was used (0,1 µM) as compared to the reverse primer (0,2 µM) (Schuelke, 2000); and ii) only 3 µl of the Master Mix was used per reaction (Burokienė *et al.*, 2015). PCR conditions were set as follows: initial denaturation for 5 min at 95°C, followed by 28 cycles of the first step: denaturation for 30 s at 95°C, annealing for 90 s at 60°C, extension for 30 s at 72°C, followed by 8 cycles of the second step: denaturation for 30 at 95°C, annealing for 90 s at 55°C, extension for 30 s at 72°C, and a final extension for 30 min at 60°C. PCR amplifications were performed on Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR products were run on ABI 3130 or ABI 3730 xl DNA Analyser with the GeneScan™ 500 LIZ® Size Standard for fragment analysis. All fragments were scored using computer software

GeneMapper v. 3.7 (Applied Biosystems) or Geneious v. R9.1.5 (Biomatters Ltd, New Zealand).

2.1.7.4. Population genetic analyses of phytophthoras (Statistical analyses)

Isolate assignment to multilocus genotypes (clones) was done using GENODIVE v. 2.0b27 software (Meirmans & Van Tienderen, 2004), utilizing Stepwise Mutation Model (SMM) as distance index under the assumption, that allele repeat length differ by ancestry. The threshold was set at zero (0) to set apart individuals with small genetic differences (Meirmans & Van Tienderen, 2004). *P. alni* s. l. genotypic richness, diversity and evenness ($E_{.5}$) were calculated using P_{OPP}R v. 2.6.1 software (Kamvar *et al.*, 2014). Genotypic richness was estimated by the number of observed multilocus genotypes (MLGs) an expected multilocus genotypes (eMLGs) corrected by the smallest sample size based on rarefaction curve. To avoid distorted genotypic diversity values due to uneven sample sizes, corrected Simpson's index (D) was calculated by equation: ((N/N-1)) * lambda, whereas lambda is Simpson's index. Genotype abundance was evaluated using $E_{.5}$ index that is less susceptible to the effect of sample size. Genotype is considered dominant in a population, when $E_{.5}$ value is equal to zero. When $E_{.5}$ value is equal to 1.0, a population has equally abundant genotypes. Clonal richness (R) was calculated as it was proposed by Aguayo *et al.* (2016). Index of association (I_A) and standardized index of association (rBarD) was calculated to test a null hypothesis of panmixia in each country.

To evaluate possible relationships among MLGs of *P.* x *alni*, a Minimum Spanning Network (MSN) has been created. It was based on Bruvo genetic distance (Bruvo *et al.*, 2004), under the assumption of combined and averaged genome addition and loss model. The network was constructed using R package P_{OPP}R v. 2.6.1 software (Kamvar *et al.*, 2014) and visualized using igraph ver. 1.1.2 (Csárdi & Nepusz, 2006), each node represented different MLG, sample sizes were represented by node size.

Table 4 Microsatellites used in the Phytophthora alni species complex population genetic study

Locus	Primer sequences (5'-3')	Repeat motifs	Size (bp)	Alleles		Reference
				P. uniformis	*P. x alni*	
PA17	F-AGCGACAATGCAGGAAGC R-CTGTCTGGGCATTCATGTCG	$(GTC)_4$ (…) $(GC)_4$	336	335	-	Ioos *et al.*, 2007
PA23	F-GGAGATAGCCACGAGACACC R-CAAGCATCGCTGTAAACGAC	$(GAA)_7$	174	-	157/171	
M-PAU3	F-TAAGAGACCTCCGGCAGAGA R-AAAGCGAACACGAAGTCCAC	$(GA)_{10}$	129	127/133	127/133	Aguayo *et al.*, 2013
M-PAU9	F-TCATGGCGCTGATCAAGTAG R-TAGTGGAGACTTACGGGGTT	$(AC)_9$	114	100/109	100/109	
M-PAU32	F-TCAGCTCCTGTATCATCAATCG R-AAGTTGCCGGTGAGTTGG	$(CA)_9$	118	113	107/113	
M-PAU11	F-AGGTGGAGTGCTAGAGGCAA R-GCGACCTTTGAGTGACCAAT	$(CAT)_7$, $C(TTC)_9$	208	173	173/209	Aguayo *et al.*, 2016
M-PAU14	F-GAAGGCTACGTAACTTGCTTTT R-ATCGAACTTCTCTTCCTTCACG	$(GT)_9$	99	89	84/86/89/99	
M-PAU15	F-CCCGTCCTTCATCAACAAAA R-GAGGCTCTGCGATGCAATAG	$(CT)_9$	99	95	92/95	
M-PAU56	F-GCTGGTGGATAATTCGTCGT R-CAAAAGCGATCCTCTTCACC	$(GA)_7$	100	91	91/94/98	
M-PAU72	F-GTTCTCGAGACTCAGCAGCC R-CAGAGGGATACCCGAGTGAA	$(CAA)_7$	165	162	162/174/177	

2.1.7.5. Assessment of population structure of *Phytophthora* x *alni*

P. x *alni* s. l. isolates were assign to country/watershed/river populations. Due to low isolate number of *P.* x *alni* isolates from Lithuania, Switzerland, Austria and Spain, these isolates were artificially assigned to *P.* x *alni* populations of the respective countries. *P. uniformis* were assigned to respective country population, without further division.

To evaluate *P.* x *alni* population differentiations in all selected watersheds of the investigated European countries (Appendix 2), Analysis of Molecular Variation (AMOVA) (Excoffier *et al.*, 1992) was performed. This analysis was done in order to avoid incorrect assessment of heterozygosity, that could lead to overestimation in population differentiation (Meirmans & Van Tienderen, 2013). Estimation of the proportion of genetic variation within and among the watersheds was defined in three ways: i) among watersheds; ii) between river basins within watersheds; iii) within river basins. All calculations were done using POPPR v. 2.6.1 software (Kamvar *et al.*, 2014).

To visualize the genetic relationships among *P.* x *alni* individuals of each MLG, principal component analysis (PCA) was done on the matrix of pairwise Euclidean distances. Calculations for PCA were done using Adegenet v.2.1.1 (Jombart, 2008) and ade4 v.1.7-10 (Dray & Dufour, 2007) packages and visualized with Factoextra (Kassambara, 2015) v. 1.0.5 package. PCA calculation and visualization was performed on RStudio.

2.2. Sampling of elm wood, isolation procedures and population genetics of *Ophiostoma novo-ulmi*

2.2.1. Sampling of diseased elm wood, isolation and identification of Ophiostoma novo-ulmi

During 2011-2012, wood samples were collected from 90 elm trees (47 trees of *Ulmus glabra* and 43 of *U. minor*) from 12 sites in Lithuania (Fig. 23; Table 9). Due to uneven number of symptomatic trees at each location, sample sizes were inconsistent. Branch samples from *U. minor* were collected only in one site in Kėdainiai district, where a total of 35 branch samples were taken. In all other sites, samples were taken exclusively from *U. glabra*, and number of samples taken at one location varied from one to 20. The largest distance between investigated trees in a local population (i.e. in one sampling location) was about 3 km (Paberliai-Gulbinėnai population); elsewhere the samples were collected in more compact areas (the maximum distance between examined trees did not exceed 600 m).

Three branches exhibiting symptoms of Dutch elm disease were taken per tree and stored in cool, damp conditions for 24-48 h until processed in the laboratory. All further work was carried out at the laboratory of Plant Pathology at the Institute of Botany of Nature Research Centre (Vilnius, Lithuania). Fungal isolation and culturing was done following methodology of Brasier (1981) and Bakys et al. (2009). Briefly, bark has been removed from the collected branch samples, and four pieces of wood of approx. 3x7x0.5 mm were cut out from each branch sample using a sterile scalpel close to the border between wound-looking and symptomatic tissues. Woody pieces were sterilized by flaming for 1 s (each side) and placed on Petri plates containing Hagem agar (HA) medium (four woody pieces from the same branch per plate, three plates per tree). All Petri plates were incubated at 20 °C in the dark. The plates were periodically examined for fungal growth from the woody pieces. When hyphal growth was observed, an agar plug from the colony margin was transferred to new Petri plates with HA medium and incubated at 20 °C in the dark to produce pure

cultures. The purified cultures were examined using a light microscope. All isolates morphologically similar to *O. novo-ulmi* (Brasier, 1981) (N=49) were assigned for DNA extraction, and were grown for two weeks on liquid Hagem media in static cultures at a room temperature. The extraction of DNA and PCR amplifications followed established protocols (Kårén *et al.*, 1997), and the ribosomal ITS region was sequenced by Macrogen Inc. (Seoul, Korea) using two primers ITS1F and ITS4 (White et al. 1990). All sequences were checked against the NCBI BLAST database (www.ncbi.nlm.nih.gov). The representative sequence of *O. novo-ulmi* (isolate ON-3, for details see Appendix 4) was deposited at NCBI GenBank database (accession no.: KJ677112).

2.2.2. Polymerase chain reaction using DNA extracted from Ophiostoma novo-ulmi

Polymerase chain reaction (PCR) amplifications were done using primers specific for *O. ulmi* s. l.: cu (Bowden *et al.*, 1994; Jeng *et al.*, 1996; Pipe *et al.*, 1997) and col-1 (Pereira *et al.*, 2000). The PCR was conducted in a 25-µl reaction volume containing 0.8 µM DreamTaq™ Green Buffer (10X) (ThermoFisher Scientific, Lithuania), 0.8 µM dNTP (ThermoFisher Scientific, Lithuania), 0.2 µM $MgCl_2$, 0.16 µM each of the forward and reverse primers, 0.6 U of DreamTaq™ DNA polymerase (ThermoFisher Scientific, Lithuania), 2 µl of the template DNA; water was adjusted to final 25 µl volume. The PCR program consisted of an initial denaturation at 94 °C for 4 min., followed by 30 cycles at 94° C for 15 s, 68° C (with cu primer pair) 58° C (with col-1 primer pair) for 1 min, and 72° C for 2 min, and a final extension at 72° C for 5 min (Konrad *et al.*, 2002). PCR amplification product sizes were determined using 1.5 % gel electrophoresis using Top Vision™ LE GQ agarose (Fermentas, Lithuania), GelGreen stain (Biotium), GeneRuler 100 bp DNA Ladder (ThermoFisher Scientific, Lithuania). Electrophoresis was run at 120 V in 1x sodium borate (SB) buffer for 1 h 30 min.

2.2.3. Restriction Fragment Length Polymorphism (RFLP) for differentiation of Ophiostoma novo-ulmi subspecies and hybrids

Obtained PCR products were digested using rare-cutting restriction endonucleases BfaI (restriction enzyme site 5'...C↓TAG...3') and HphI (restriction enzyme site 5'...GGTGA(N)8↓...3') (Fermentas, Lithuania). Restriction reactions were done according to the manufacturer's protocol. Restriction fragment sizes were determined using 2 % gel electrophoresis, using Top Vision™ LE GQ agarose (Fermentas, Lithuania), GelGreen stain (Biotium), GeneRuler 100 bp DNA Ladder (ThermoFisher Scientific, Lithuania). The electrophoresis was run at 120 V in 1x SB buffer for 1 h 30 min.

2.2.4. Vegetative compatibility tests

To differentiate *O. novo-ulmi* genotypes, all 49 isolates of the fungus were paired in all possible combinations in 90-mm Petri plates containing HA medium (Vasiliauskas & Stenlid, 1998). Agar plugs (approx. 4 x 4 mm in size) from three-week-old *O. novo-ulmi* cultures were transferred to new Petri plates with HA medium. Agar plugs were placed ~2cm apart from each other and incubated at 20 °C in the dark for 2-4 weeks. The results were scored when vegetative incompatibility reactions between the paired cultures have emerged. Three types

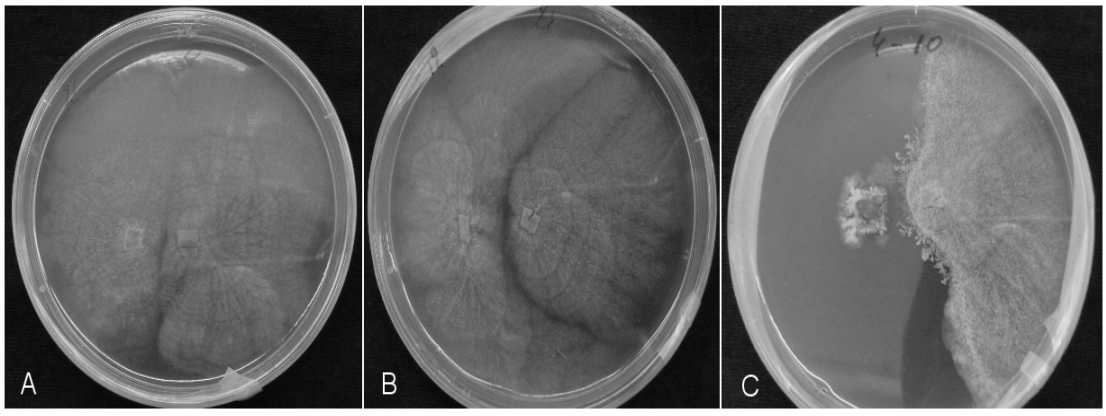

Fig. 14 Vegetative compatibility test reaction types in pairings of *Ophiostoma novo-ulmi* isolates: A, compatible reaction; B, line-gap reaction type; C, incompatible reaction.

of reactions were observed: i) vegetative compatibility (c) (Fig. 14 A); ii) line-gap (lg) (Fig. 14 B); and iii) wide gap (w) reaction type (Fig. 14 C) (Brasier, 1984). Pairings producing unclear results were repeated.

2.3. Sampling of ash wood, isolation procedures and population genetics of *Hymenoscyphus fraxineus*

2.3.1. Sampling and isolation of fungi from wood and leaf petioles of Fraxinus excelsior

Wood samples (bark lesions on shoots, branches or stems) and fallen leaf petioles of symptomatic 1–20-years-old *Fraxinus excelsior* have been collected in five Lithuanian forest stands (Table 5). The distance between the sampling sites ranged from 60 to 260 km. A total of 60 sampling points per site were used to collect *F. excelsior* samples. The minimum distance between two sampling points in a stand was at least 5 m. At each sampling point, from two to four 15–20-cm long pieces of shoots, branches or stems with bark lesions were collected. In addition, ash leaf petioles with pseudosclerotial plates were collected from the forest floor at each sampling point. Fungal isolation from leaf petioles was carried out as described by Kirisits et al. (2013), and isolation from necrotic bark lesions was carried out as described by Schoebel et al. (2014).

Fungal isolation from ash wood was done in the Laboratory of Plant Pathology at the Institute of Botany of Nature Research Centre (Vilnius, Lithuania). Wood samples were sterilized in two steps. During the first step, *F. excelsior* wood cuttings were submerged in NaOCl solution (13 %) for 1 min and dried out on a sterile filter paper. Then the bark has been removed and several smaller pieces (ca. 0.5-1 x 0.2-0.4 cm in size) of phloem from around the necrotic lesion were cut. The second step of wood sterilization included submerging these smaller woody pieces into 96 % ethanol for 1 s and drying out on a filter paper. From each wood sample (shoot, branch or stem), five smaller woody pieces were taken and placed onto 90 mm Petri dishes containing 2 % ash leaf malt extract agar (AMEA; Kirisits *et al.*, 2013). All Petri plates with

woody pieces were incubated at 20 °C in the dark. Following initiation of hyphal growth, pure fungal cultures were placed onto new Petri plates filled with AMEA.

Isolation of fungi from petioles was done in the Department of Forest Protection and Game Management (Institute of Forestry, Lithuanian Research Centre for Agriculture and Forestry, Girionys, Kaunas reg., Lithuania) using methodology of Kirisits *et al.* (2013). Briefly, a 3-5 cm long piece was cut from a middle part of a petiole, sterilized by submerging into 96 % ethanol for 1 min and dried out on sterile filter paper. Then pseudosclerotium was removed and small pieces of a petiole (0.7-1-cm-long) were cut out with a sterile scalpel and placed onto 90-mm Petri plates containing AMEA (five pieces of one petiole per plate). All Petri plates with petiole tissues were incubated at 20 °C in the dark. When hyphal growth was observed, an agar plug from the margin of actively growing fungal colony was transferred to new Petri plates with AMEA medium and incubated at 20 °C in the dark to produce pure cultures. Cultures were examined using a light microscope. Cultures with *Hymenoscyphus fraxineus*-like morphological structures were selected for further work.

Table 5 Sampling locations and period for *Hymenoscyphus fraxineus* isolations

Population	Abbreviation	Longitude	Latitude	Sampling period
Biržai	BIR	55,98980	24,21695	May 2013
Kėdainiai	KED	55,18340	23,76547	November 2012-April 2013
Kretinga	KRE	55,70090	21,48168	May 2013
Ukmergė	UKM	55,27200	24,68208	May 2013
Vilnius	VIL	54,74852	25,28050	November 2012-April 2013

2.3.2. DNA extraction from fungal cultures and species identification

DNA extraction and species identification was carried out by a Swiss team lead by Dr. Daniel Rigling in the Laboratory of Phytopathology of Swiss Federal Institute for Forest, Snow and Landscape Research WSL (Birmensdorf, Switzerland). For DNA extraction from *H. fraxineus*-like cultures, an agar plug of growing mycelium was transferred to new plates containing diamalt agar covered with a cellophane layer (Cellolaire, Switzerland) to separate the mycelium from the agar. After 4–5 weeks of incubation in the dark at room temperature, mycelia were harvested from the cellophane and DNA was extracted following Schoebel et al. (2014) using the Norgen Total RNA Purification Kit (Norgen Biotek Corp., Canada) according to manufacturer's protocol but omitting DNase treatment step.

2.3.3. Microsatellite genotyping of Hymenoscyphus fraxineus isolates

DNA of all *H. fraxineus*-like isolates has been assigned for genotyping. As the genotyping was performed using *H. fraxineus*-specific microsatellite primers (Gross et al. 2012), this allowed correct identification of the collected isolates as *H. fraxineus*. All preliminary selected *H. fraxineus*-like isolates were identified to belong to this species.

H. fraxineus population genetic study was carried out by a Swiss team lead by Dr. Daniel Rigling in the Laboratory of Phytopathology of Swiss Federal Institute for Forest, Snow and Landscape Research WSL (Birmensdorf, Switzerland). Eleven polymorphic microsatellite loci (Gross *et al.*, 2012) have been selected for the population genetic analyses (Burokiene et al., 2015). PCR was performed in two multiplex reactions (MP1 and MP2) using 1 µl of 10 x diluted DNA and the Type-it Microsatellite PCR Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol, except that only 3 µl of the Master Mix were used per reaction (Burokiene et al., 2015). PCR conditions: initial denaturation step for 5 min at 95 ºC, followed by 28 cycles consisting of 30 s at

95 ºC, 90 s at 58 ºC and 30 s at 72 ºC, with a final extension step for 30 min at 60 ºC. PCR amplifications were performed on Veriti® Thermal Cyclers (Applied Biosystems, Foster City, CA, USA). Subsequently, PCR products obtained were analysed on an ABI 3730xl DNA Analyser with the GeneScanTM 500 LIZ® Size Standard (Applied Biosystems). All obtained fragments were scored using GeneMapper software, v. 3.7 (Applied Biosystems).

Computer software POPPR v. 1.1.2 (Kamvar *et al.*, 2014; R Development Core Team 2014) was used to determine the number of multilocus genotypes (MLGs) in each population. Genotypic richness (expected number of genotypes (eMLGs)) and genotypic diversity (Shannon-Wiener index) were calculated using the R package VEGAN (Dixon, 2003; R Development Core Team 2014) corrected by a smallest sample size based on a rarefaction curve. Differences in eMLG between the lesion and petiole isolates were tested for significance by conducting a *t*-test in SPSS Statistics v. 17.0 (SPSS Inc., Chicago). GENEPOP (Rousset, 2008) was used to calculate the total number of alleles (A_M) and the allele frequencies for each locus. Allelic richness (A_R) and incidence of private alleles (A_{PR}) was calculated separately for samples isolated from lesions and from petioles using ADZE computer program (Szpiech *et al.*, 2008), and tested for significance by conducting a *t*-test in SPSS Statistics v. 17.0. The overall genetic differentiation (F_{ST} value with a 95 % confidence interval) among the Lithuanian populations was estimated using FSTAT 2.9.3.2. (Goudet 2001). Pairwise F_{ST} values (Weir & Cockerham, 1984) between isolates from petioles and lesions within each population, as well as between all population pairs (petiole and lesion isolates combined), were calculated using GENEPOP computer software. The existence of a pattern of isolation-by-distance between populations was determined by analyzing the regression of F_{ST} /(1 - F_{ST}) on the natural logarithm of geographic distance (Rousset, 1997). The significance of the regression was determined by performing a Mantel test using the program GENALEX v. 6.41 (Peakall & Smouse, 2006).

Possibilities of *H. fraxineus* random mating (P_{Gen}) and independent sexual reproductive events (P_{Sex}) were calculated using GENCLONE v. 2.0

computer software (Arnaud-Haond & Belkhir, 2007). The hypothesis of random mating within populations was tested using index of association (I_A) statistics (Agapow & Burt, 2001). The index rBarD was calculated using the R package POPPR (Kamvar *et al.*, 2014).

The genetic structure of Lithuanian *H. fraxineus* populations was analyzed by using a Discriminant Analysis of Principal Components (DAPC) (Jombart *et al.*, 2010), as implemented in the R package POPPR (Kamvar *et al.*, 2014) to genetically cluster similar individuals according to their MLG. Analyses were conducted using clone-corrected data sets.

2.3.4. Hymenoscyphus fraxineus virulence test

H. fraxineus isolates for virulence test were selected among the genotyped ones (Burokiene *et al*., 2015). A detailed description of the first *F. excelsior* seedling inoculation experiment (further referred to as "Seedlings 1") is presented in a study by Lygis *et al.* (2017).

The second *F. excelsior* seedling inoculation experiment (further referred to as "Seedlings 2") was carried out during a period stretching between the 20th of July 2015 and the 20th of April 2016. In this experiment, 15 most virulent and 14 least virulent *H. fraxineus* isolates have been selected from the "Seedlings 1" experiment to test reliability of the first (Seedlings 1) experiment. Swiss isolates of *H. fraxineus* used in both inoculation experiments were provided by a Swiss team of forest pathologists lead by Dr. Daniel Rigling (Laboratory of Phytopathology of Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland). For more information on origin of the Swiss isolates see Burokiene *et al.* (2015). During spring of 2014, a total of 500 4-year old *F. excelsior* seedlings from bare-root nursery (Kaunas region, Lithuania) were planted in 5-litre plastic pots filled with a peat substrate and grown outdoors for one vegetation season in a forest nursery of Dubrava Experimental-Educational State Forest Enterprise (Kaunas reg., Lithuania). A total of 145 healthy (without visible symptoms of ash dieback disease) *F.*

excelsior seedlings were selected for further research. Detailed information about the inoculum preparation and seedling inoculation is presented in Lygis *et al.* (2017). The same isolate was inoculated onto five randomly selected seedlings, creating randomly inoculated ash seedling blocks. On the 20th of April 2016, after nine months post inoculation, the experiment was terminated and the final assessment of bark lesion development on seedling stems was made. The evaluation of isolate virulence was done following Lygis *et al.* (2017).

2.3.5. Growth speed test of Hymenoscyphus fraxineus isolates in culture

Growth speed test was done using 190 isolates previously genotyped by Burokiene *et al*. (2015) and tested for virulence by Lygis *et al.* (2017). Agar plugs of three-week-old actively growing *H. fraxineus* mycelia were taken using a cork borer (6 mm in diameter) and placed in the center of the 90-mm Petri plates containing ash leaf malt extract agar (AMEA). Each isolate was used in three replications (i.e. three Petri plates for one isolate). The cultures were incubated at 20 ºC in the dark. After 13 days of incubation (when a fastest-growing culture has reached plate edge), the diameter of the resulting cultures has been measured using a ruler in two perpendicular directions and the average diameter was recorded. Diameter of cultures of each isolate was calculated as an average of two measurements made along perpendicular axes reduced by the diameter of a primary agar plug (6 mm). By dividing this value by two and by the number of days (N = 13), the radial growth rate of each isolate was calculated in mm/day for each replicate plate. A mean radial growth rate of the three replicates was used in the statistical analyses.

2.3.6. Statistical analyses of results of Hymenoscyphus fraxineus virulence and growth speed tests

The significance of differences in lesion lengths between isolates representing different countries, substrates and virulence groups were calculated

using a *t*-test. Pearson's correlation analysis was used to determine the relationship between: i) proximal lesion length in the tested seedlings in experiments Seedlings 1 and Seedlings 2; ii) *in vitro* radial mycelium growth speed of the respective *H. fraxineus* isolates. The significance of differences in the proportion of seedlings with induced lesions between Lithuanian and Swiss samples, and between lesion and petiole isolates were calculated using a two-proportion *z*-test. All statistical tests were performed using SPSS Statistics v. 17.0 (SPSS Inc., Chicago, IL, USA) software.

3. RESULTS

3.1. Oomycetes and fungi from symptomatic alder trees, water and soil – results of recovery, identification, and genetic properties of *Phytophthora alni* s. l.

3.1.1. Results of recovery of oomycetes and fungi from symptomatic alder trees, water and soil

A total of 564 isolates of oomycetes (*Oomycota*) and 110 isolates of fungi were recovered during the present study (Appendix 1). Of oomycetes and fungi, from wood recovered were 37 and 43 isolates, from water – 190 and 2 isolates, and from soil – 337 and 65 isolates, respectively. Molecular tools helped to identify a total of 43 taxa, of which 24 belonged to oomycetes and 19 - to fungi. Most of the isolates (87.1 %) have been identified to species level; from this number, identified to species level were 90.1 % oomycete and 72.6 % fungal isolates (Appendix 1).

Overall success of oomycete isolation (172.0 %) was far better compared to fungi (34.5 %). Among oomycetes, isolates of *Phytophthora* spp. were the most abundant (416 isolates); *Phytopythium* spp. was isolated 102 times, and *Pythium* spp. – 46 times. Among fungi, the most common were zygomycetes (75 isolates; overall success of isolation 22.9 %), while basidiomycetes (24 isolates; overall success of isolation 7.3 %) and ascomycetes were relatively rarely isolated (14 isolates; overall success of isolation 4.3 %) (Appendix 1). Neither of fungal species potentially pathogenic to alder (as e.g., *C. purpureum*, *Heterobasidion* sp. or *Fusarium lateritium* Nees has been isolated at frequencies higher than 1.5 % so their role in the observed alder decline is most likely negligible.

3.1.1.1. Recovery of *Phytophthora* spp. from the collected samples

In both Lithuania and Switzerland, *Phytophthora* spp. were isolated in 23 of the 41 investigated sites (56.1 %). Phytophthoras were recovered from 21 of

201 wood samples (10.4 %), from 40 of 110 (36.4 %) rhizosphere soil samples and from 14 of 17 (82.4 %) water samples. A total of 14 *Phytophthora* species were identified using ITS sequencing. Six *Phytophthora* species were isolated in both countries: *P. x alni*, *Phytophthora. gregata* T. Jung, M.J.C Stukely & T. Burgess*, P. gonapodyides, P. gallica, P. lacustris* and *P.* taxon *Oaksoil* (Fig. 15; Fig. 16 & Appendix 1). *P. uniformis* was isolated only in Lithuania (17 isolates recovered from two sites) (Fig. 15; Appendix 1), while *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *Phytophthora cryptogea* Pethybr. & Laff*., Phytophthora hydropathica* C. Hong & Gallegly*, Phytophthora niederhauserii* Z.G. Abad & J.A. Abad*, Phytophthora chlamydospora* Brasier, C. and E. Hansen*, P. plurivora* and *Phytophthora pseudosyringae* T. Jung & Delatour were isolated only in Switzerland (Fig. 16; Appendix 1). The most abundant species in both countries was *P. lacustris* (147 isolates) recovered from all substrate types, yet the largest number of isolates (n=92; isolation success 541.2 %) was recovered from water samples (Fig. 15; Fig. 16 & Appendix 1). The second most abundant species was *P. plurivora* (142 isolates; isolated only in Switzerland), mostly recovered from soil samples (119 isolates) (Fig. 16 & Appendix 1). Species of *P. alni* s.l. complex were isolated almost exclusively from wood samples: *P. uniformis* has been recovered exclusively in Lithuania from wood (Fig. 15) of *A. glutinosa* (17 isolates, success of isolation from wood 8.5 %) (Appendix 1), while the second species, *P.* x *alni* has been recovered in both countries: nine isolates in Lithuania and eight isolates in Switzerland (Fig. 15; Fig. 16; Appendix 1). Again, most of the isolates originated from wood samples (n=16, success of isolation 8.0 %) and one isolate – from soil (success of isolation 0.9-%-) (Appendix 1).

3.1.1.2. Diversity of possibly alder-associated *Phytopthora* spp.

In Lithuania, *Phytophthora* spp. were found in 8 of the 27 investigated sites (Fig. 15; Appendix 1). Most abundant species was *P. lacustris*, it has been detected in four sites. *P. gonapodyides* was found in three sites, *P.* x *alni*, *P.*

gallica and *P. uniformis* – in two sites, and *P. gregata* and *P.* taxon *Oaksoil* - in one site. *P.* x *alni* and *P. uniformis* were recovered from alder wood, *P. gallica, P. gonapodyides, P. lacustris* and *P.* taxon *Oaksoil* - from water substrate, and *P. gregata* - from soil. Identified *Phytophthora* species belonged to three ITS clades: ITS Clade 6, ITS Clade 7 and ITS Clade 10 (Fig. 15, Appendix 1).

In Switzerland, *Phytophthora* spp. were found in all 14 investigated sites. A total of 281 isolates were obtained. Based on ITS sequencing results, 13 *Phytophthora* species have been identified: *P.* x *alni, P. citrophthora, P. cryptogea, P. gallica, P. gonapodyides, P. gregata, P. hydropathica, P. lacustris, P. niederhauseri, P. pseudosyringae, P. plurivora* and *P.* taxon *Oaksoil.* The most frequently recovered *Phytophthora* species, *P. lacustris* (140 isolates), was detected in 11 of 14 Swiss sites (Fig. 16; Appendix 1). The second most common species – *P. plurivora* (87 isolates) was detected in 9 sites. *P. gonapodyides* was found in 6 sites, *P.* x *alni, P. gallica* and *P. gregata* - in 2 sites, and the rest eight *Phytophthora* species - only in one site (Fig. 16; Appendix 1). *P. cryptogea* and *P. pseudosyringae* were isolated only once. *P. plurivora* was most frequently detected in soil samples (119 isolates), *P. lacustris* was found in water samples (22 isolates) and *P.* x *alni* was isolated from wood (Fig. 16). Only *P. lacustris* was found in all three substrate types (Fig. 16). *P.* x *alni* was detected both in wood and soil samples (Fig. 16). *P. plurivora, P. gallica* and *P. gonapodyides* were found both in soil and water samples (Fig. 16). The rest nine species were recovered from only one substrate: *P. citrophthora* was detected only in water, while *P. chlamydospora, P. cryptogea, P. gregata, P. hydropathica, P. niederhauserii, P. pseudosyringae, P.* taxon *oaksoil* were recovered from soil samples (Fig. 16; Appendix 1). The thirteen identified *Phytophthora* species represented seven ITS clades (Cooke *et al.*, 2000; Kroon *et al.*, 2012). The highest number of species (five) belonged to Clade 6; three species belonged to Clade 7, two species to Clade 2, and only one species belonged to each of the Clades 8, 9 and 10 (Fig. 16).

Both in Lithuania and Switzerland, the proportion of successful recovery attempts of representatives of *P. alni* s. l. species complex (*P.* x *alni* and *P. uniformis*) was low: 13.43 % and 3.98 %, respectively.

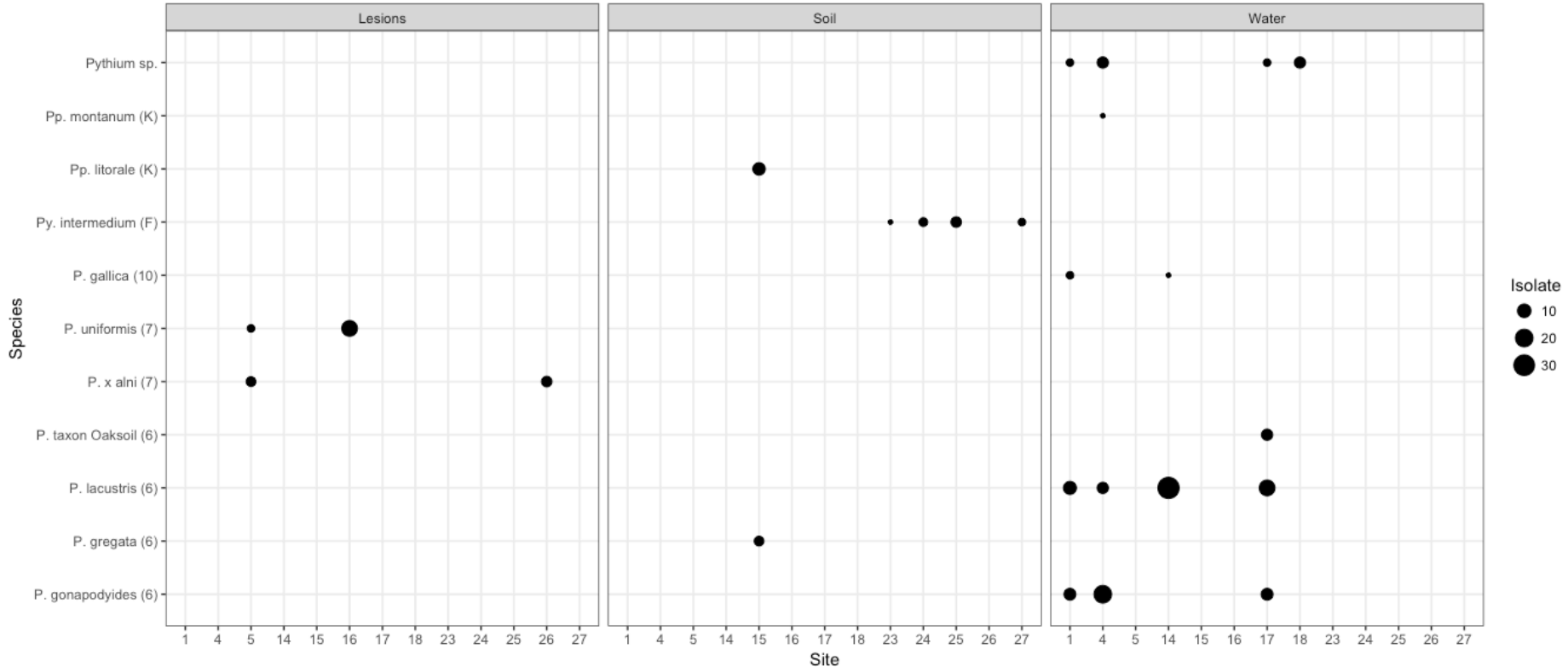

Fig. 15 Diversity of oomycetes recovered in Lithuania from wood of *Alnus glutinosa* and *A. incana*, and recovered from rhizosphere (soil) of the symptomatic alder trees and from water near the symptomatic alder trees. Numbers and letters (in parentheses) after species names indicate representative clade numbers; Site numbers are explained in Fig. 12 A and Table 2. Isolate – dot size indicates a number of isolates recovered in a respective site.

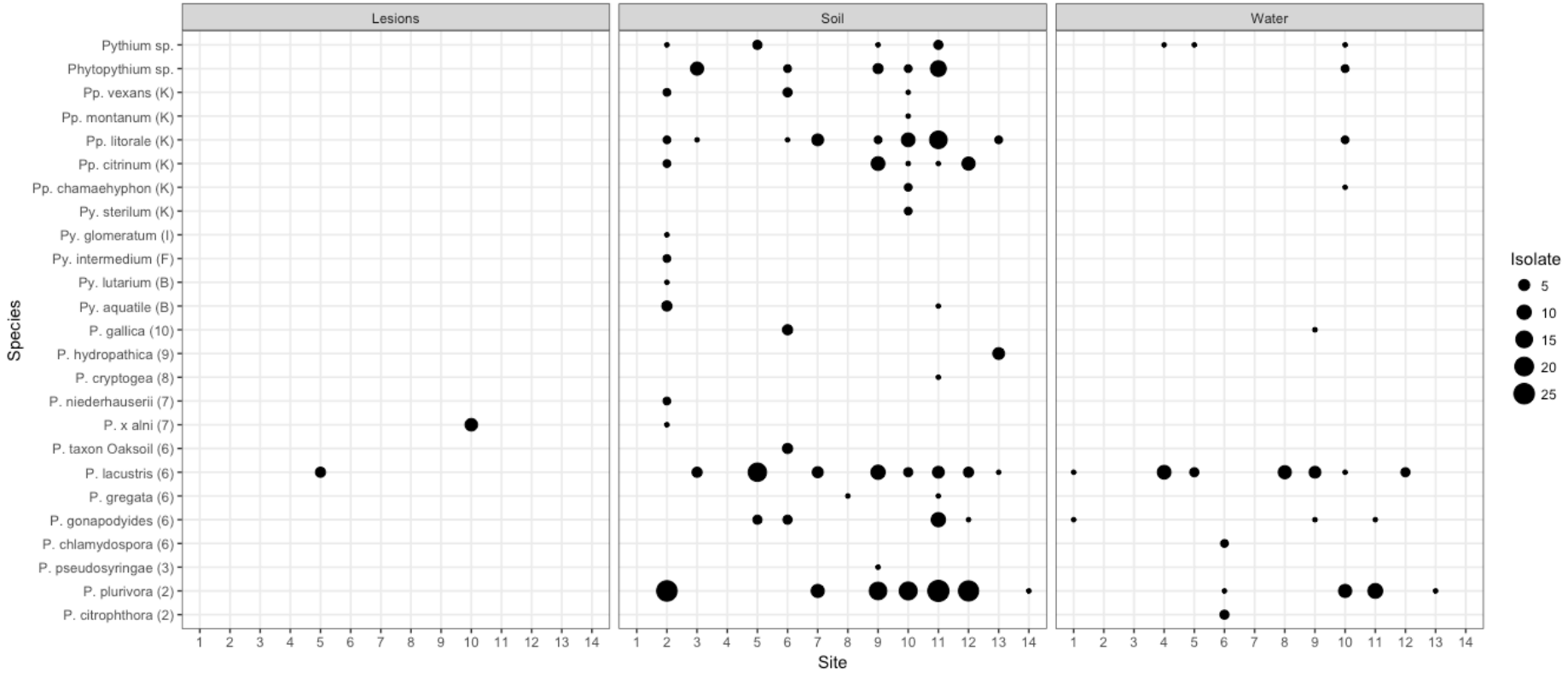

Fig. 16 Diversity of oomycetes recovered in Switzerland from wood of *Alnus glutinosa* and *A. incana*, and recovered from rhizosphere (soil) of the symptomatic alder trees and from water near the symptomatic alder trees. Numbers and letters (in parentheses) after species names indicate representative clade numbers; Site numbers are explained in Fig. 12 B and Table 2. Isolate – dot size indicates a number of isolates recovered in a respective site.

Neighborjoining analysis (Fig. 17) showed that of all *Phytophthora* species isolated during the present study only *P. citrophthora* did not match phylogeny of the reference sequence (data not shown). This group is therefore further referred to as *P. citrophthora*-like oomycetes.

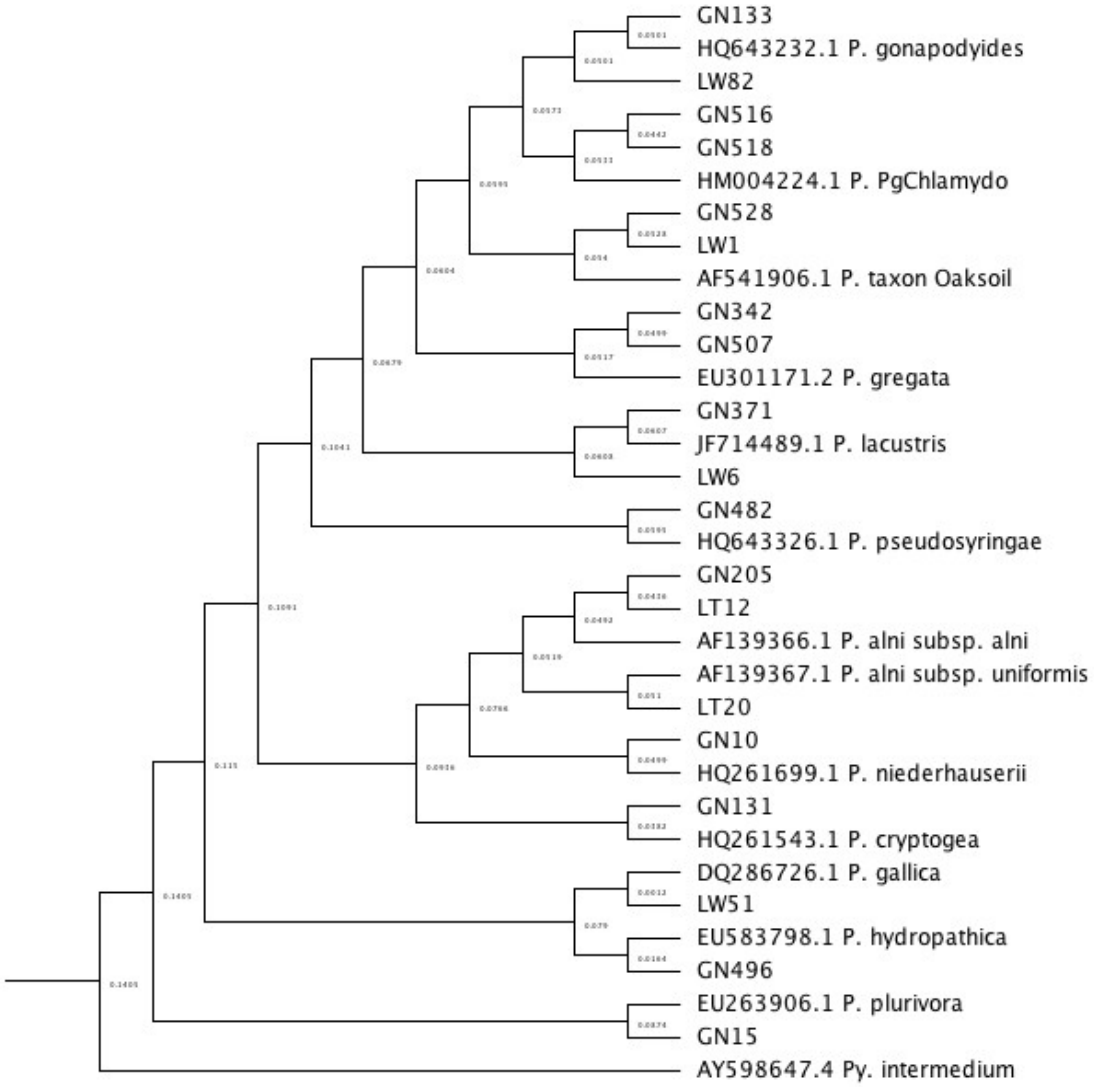

Fig. 17 A phylogenetic tree of recovered *Phytophthora* spp. isolates (rooted neighbor-joining tree was constructed using Tamura-Nei genetic distance model, with support threshold of 70 %)

3.1.1.3. Results of recovery of *Phytopythium* spp.

Representatives of genus *Phytopythium* were found in 11 sites (nine sites in Switzerland (Fig. 16) and two in Lithuania (Fig.15)); these oomycetes were recovered only from rhizosphere soil and water (Fig. 15; Fig. 16; Appendix 1). Out of 110 soil samples, *Phytopythium* spp. were recovered from 25 samples (one from Lithuania (Fig. 15) and 24 from Switzerland (Fig. 16)). These organisms were recovered only from two water samples (one from Switzerland (Fig. 16) and one from Lithuania (Fig. 15)). A total of five *Phytopythium* species were identified using ITS sequencing, although 31 isolates were identified only to genus level (Appendix 1).

In Lithuania, a total of nine *Phytopythium* isolates were recovered; they belonged to two species: *Phytopythium montanum* (Nechw.) Abad, de Cock, Robideau, Lodhi & Lévesque and *Phytopythium litorale* (Nechw.) Abad, de Cock, Robideau, Lodhi & Lévesque. Isolates of each species were recovered from different substrates: *Pp montanum* - from water and *Pp. litorale* - from soil (Fig. 15; Appendix 1). In Switzerland, a total of 208 *Phytopythium* isolates were recovered which belonged to five species (Fig. 16; Appendix 1). The most widespread and most abundant species in Switzerland were *Pp. litorale* (42 isolates) and *Phytopythium citrinum* (B. Paul) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque (21 isolate). Only one species – *Pp. litorale* was recovered from both substrates, while other species were found only in soil samples (Fig. 16; Appendix 1)

3.1.1.4. Results of recovery of *Pythium* spp.

Overall, representatives of *Pythium* spp. were found in 14 sites (in eight Lithuanian sites (Fig. 15) and in six Swiss sites (Fig.16)), and have been recovered from water and soil samples. *Pythium* spp. were recovered from 18 and 5 soil and water samples, respectively. A total of five species and

taxonomically undefined *Pythium* groups were identified. *Pythium. intermedium* de Bary was found in both countries (Appendix 1).

3.1.1.5. Results of recovery of fungi

Fungi were recovered from all three types of substrates, but most frequently - from soil samples (67 isolates). From wood recovered were 43 isolates and from water – four isolates. A total of 23 species (18 in Lithuania and five in Switzerland) and 12 taxonomically undefined groups were identified (Appendix 1). Two species - *Coprinellus micaceus* (Bull.) Vilgalys, Hopple & Jacq. Johnson and *Mucor hiemalis* Wehmer were found in both countries. In Lithuania, a total of 18 species and five taxonomically undefined groups of fungi were found in wood and soil substrates. In Switzerland, five species and 8 taxonomically undefined groups of fungi were found in wood and soil substrates (Appendix 1).

3.1.2. Population genetics of Phytophthora alni s. l. species complex

A total of 258 *P. alni* s. l. isolates (168 isolates of *P.* x *alni* and 90 isolates of *P. uniformis*) originating from seven different countries of Europe have been selected for genetic analyses (Appendix 2). From this number, 245 isolates (158 isolates of *P.* x *alni* and 87 isolates of *P. uniformis*) were successfully genotyped using 10 microsatellite primers developed by Ioos *et al.* (2007) and Aguayo *et al.* (2013; 2016). Depending on species, the number of alleles at each locus varied from one (*P.* x *alni* and *P. uniformis*) to three (*P.* x *alni*). In total, 23 alleles were amplified. In contrast to studies by Ioos *et al.* (2007) and Aguayo *et al.* (2013; 2016), no new alleles were found, but new allele variations between loci were identified.

3.1.2.1. Gentotypic richness ad evenness of *Phytophthora* x *alni* isolates

A total of 36 multilocus genotypes (MLGs) of *P.* x *alni* have been found in the present study, of which 24 were newly described. The most common MLG of *P.* x *alni* across Europe, Pxa-1 (Aguayo *et al.*, 2016), was present in all seven countries and it was detected 86 times. In Austria and Sweden, less than 50 % of *P.* x *alni* isolates belonged to MLG Pxa-1, while in Switzerland all isolates belonged exclusively to this MLG (Table 6). Other MLGs consisted of one to nine isolates (Appendix Table 2). The second most common MLG, Pxa-6 (Aguayo *et al.*, 2016), was found in four countries: Czech Republic, Hungary, Spain and Sweden.

Table 6 Genetic characterization of investigated Austrian, Czech, Hungarian, Lithuanian, Spanish, Swedish and Swiss populations of *Phytophthora x alni*.

Country	**N**	**MLG**	**eMLG (±SE)**	**Pxa-1, %**	**R**	**D**	**E.5**	**I_A (PI_A)**	**rbarD (PrbarD)**
Austria (AU)	9	5	5.00 (±0.00)	33.33	0.4	0.861	0.91	-0.41 (0.792)	-0.13 (1.00)
Czech Republic (CZ)	49	21	5.41 (±1.39)	54	0.04	0.699	0.33	0.52 (0.002)	0.07 (0.001)
Hungary (HU)	39	7	3.19 (±1.00)	74.35	0.02	0.442	0.45	-0.5 (0.967)	-0.08 (0.999)
Lithuania (LT)	9	2	2.00 (±0.00)	88.88	0.03	0.222	0.59	NA	NA
Spain (SP)	4	3	3.00 (±0.00)	50	0.55	0.833	0.91	0.16 (0.33)	0.1 (0.214)
Sweden (SW)	41	9	5.56 (±0.97)	24.39	0.12	0.852	0.89	0.14 (0.262)	0.05 (0.053)
Switzerland (CH)	7	1	1.00 (±0.00)	100	0	0.00	NaN	NA	NA
Total	159	36	5.18 (±1.39)	54.08	0.014	0.695	0.3	-	-

N –number of screened *P. x alni* isolates; MLG – observed number of multilocus genotypes; eMLG – number of expected MLGs at the smallest sample size (N = 4); SE – standard error based on eMLG value; R – clonal richness; D – corrected Simpson's index; $E_{.5}$ – evenness of MLGs; I_A – index of association; PI_A – significance value of I_A; rbarD –standardized index of association; PrbarD – significance value of PrbarD; NaN – imposible value (divided by zero); NA – missing value.

The highest observed genotypic richness was in Czech Republic where all *P.* x *alni* isolates were grouped into 21 MLGs. In other countries, all *P.* x *alni* isolates were grouped into one to nine MLGs. After rarefaction (N = 4), eMLG ranged from 1.00 (± 0.00) in Switzerland to 5.56 (± 0.97) in Sweden; the total eMLG amounted to 5.18 (± 1.39) (Table 6). Total clonal richness was low (R = 0.014), indicating that in all investigated countries one *P.* x *alni* genotype was dominant (Table 6). Genotypic diversity was assessed using a corrected Simpson's index, which showed that genotypic diversity was high in Austria, Spain and Sweden with similar values (Table 6), while Switzerland and Lithuania had the lowest genotypic diversities (Table 6).

The index of evenness $E._5$ allowed evaluation of genotype distribution within *Phytophthora* populations (Grünwald *et al.*, 2003). The total evenness of *P.* x *alni* genotype distribution was low ($E._5 = 0.3$), and most likely this was because, based on our findings, only one genotype is dominant in Europe. Genotypes in Austria, Spain and Sweden were distributed more evenly than in other countries (Table 6). The most uneven distribution of *P.* x *alni* genotypes was observed in Czech Republic, where more than a half of isolates belonged to MLG Pxa-1 and the rest isolates belonged to 20 different MLGs.

The hypothesis of sexual recombination in all investigated *P.* x *alni* populations could not be rejected due to insignificant P value ($P > 0.05$) (Table 6).

The minimum spanning network (MSN) discriminated three groups of genotypes, with two distinct branches (Fig. 18). The first group included *P.* x *alni* genotypes mainly originating from Sweden. This group has a parting at MLG Pxa-9, where all Swedish *P.* x *alni* isolates are differentiated to Eastern and Western watersheds (basins) of Sweden. The second group consisted of Austrian, Czech Republic, Hungarian, Lithuanian and Spanish *P.* x *alni* genotypes. The third group involved MLGs closely related to the dominant MLG, Pxa-1 (Fig. 18)

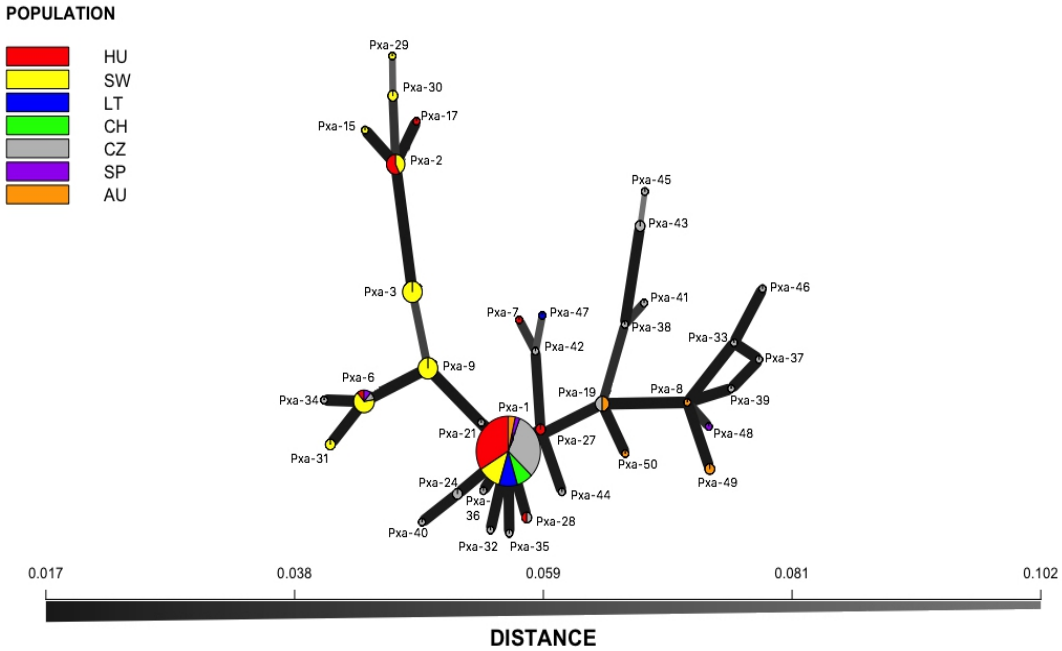

Fig. 18 Minimum Spanning Network (MSN) of *Phytophthora* x *alni* multilocus genotypes across seven investigated European countries (for country name abbreviations see Table 6).

3.1.2.2. Allelic patterns of rare *Phytophthora* x *alni* genotypes

During the present study, a total of 24 new (previously unpublished) *P.* x *alni* MLGs, consisting of one to two isolates, were identified (Appendix 3). A total of 23 MLGs were identified as country specific, and one MLG (Pxa-28) was identified in two countries: Czech Republic and Hungary. The highest abundance of rare genotypes was observed in Czech Republic (16 MLGs), while in other six countries rare MLGs were detected only occasionally.

Apparently, all newly described MLGs have been formed after an allelic loss event. Aguayo *et al.* (2016) assigned *P.* x *alni* alleles to corresponding *P.* x *multiformis* (*P.* x *m*) or *P. uniformis* (*P. u*) subgenomes, by this determination we could link allelic loss to a certain subgenome. Eighteen MLGs have been formed after an allelic loss from *P.* x *multiformis* subgenome, four MLGs - after allelic loss from *P. uniformis* subgenome, and two MLGs - after allelic loss in both subgenomes (Appendix 3). In Hungarian, Lithuanian, Spanish and Swedish *P.* x *alni* populations, alleles were lost from *P.* x *multiformis* subgenome

(Appendix 3). MLGs with allelic loss from *P. uniformis* subgenome were found in Austria and Czech Republic, while MLGs with allelic loss from both subgenomes were identified only in Czech Republic. The dominant MLG, Pxa-1, had the highest number of genetically close rare MLGs: four MLGs have been formed after allelic loss from *P.* x *multiformis* subgenome, and one MLG - after allelic loss from *P. uniformis* subgenome. Nine genotypes were genetically close to other rare genotypes (Appendix 3)

On average, the number of lost alleles within a MLG ranged between one and three. The MLG Pxa-45 showed high allelic loss – it has lost six alleles. M-Pau3 and PA17 were the most stable loci - as they have lost no alleles. In the remaining eight loci, lost were from two to seven alleles. Locus M-Pau56 was the most unstable among all screened loci (seven events of the allelic loss) (Appendix 3).

3.1.2.3. Association of distribution of *Phytophthora* x *alni* MLGs with watersheds in Czech Republic, Hungary and Sweden

Isolates of *P.* x *alni* originating from Czech Republic, Hungary and Sweden were distributed into groups associated with river (or lake) basins and watersheds within each country. In this study, Czech Republic was divided into two river watersheds: of Donau (Dunaj) river and of Elbe-Vltava rivers. Donau watershed includes Dyje, Svratka and Svitava (further these three rivers are referred to as DSS) and Morava basins, while Elbe-Vltava watershed includes Berounka, Elbe, Ohre, Ploučnice, Sazava and Vltava basins (Fig.19). A total of eight *P.* x *alni* isolates were assigned to Donau watershed and 41 isolates - to Elbe-Vltava watershed (Table 7). In each basin, the total number of MLGs ranged between two and five. The MLG Pxa-1 was found in the respective basins: Ploučnice (25 % of the screened isolates), Sazava (25 %), DSS (40 %), Elbe (40 %), and the highest proportion of isolates (76.9 %) belonging to this MLG was found in Vltava watershed (Table 7).

In this study, Hungary was divided into two watersheds: of Donau river and of Balaton lake. Donau watershed covers basins of river Rábca, and Balaton

watershed covers basin of river Zala (Fig. 20). A total of 16 isolates of *P.* x *alni* were designated to Donau watershed, and 23 isolates - to Balaton watershed (Table 7). In both basins (Rábca and Zala), five previously described MLGs were detected. Rábca basin had four previously described MLGs and one new MLG; Zala basin – three previously described and two new MLGs. In both basins, more than a half of *P.* x *alni* isolates belonged to MLG Pxa-1, and in Zala basin as much as 82.6 % of the isolates belonged to this MLG (Table 7).

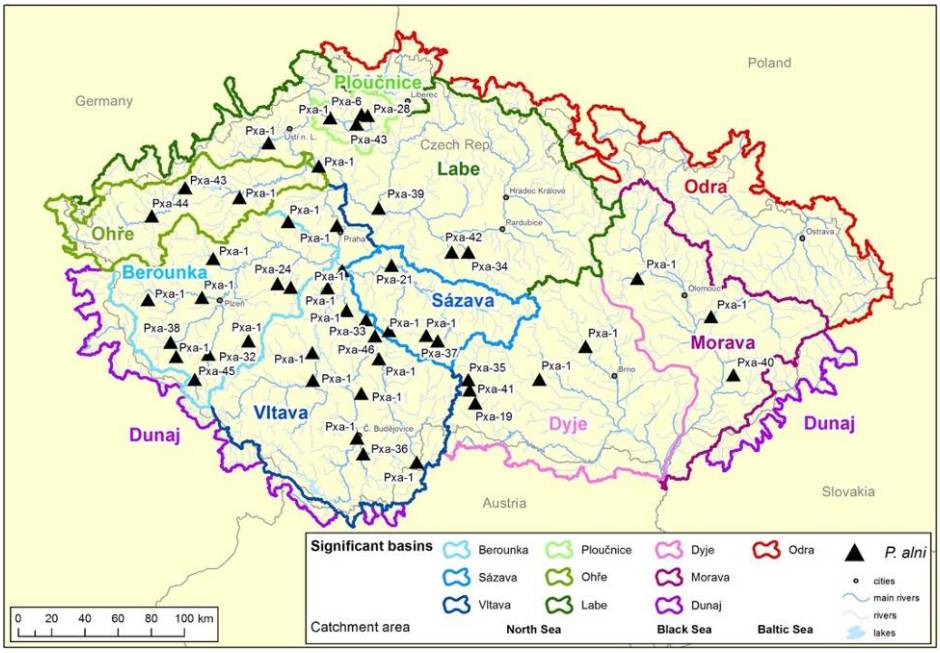

Fig. 19 Distribution of watersheds in Czech Republic and sites of *Phytophthora* x *alni* isolation (black triangles). Labels by each triangle indicate numbers of identified *P.* x *alni* multilocus genotypes (MLGs).

In this study, Sweden was divided into two watersheds: Eastern and Western. Easter watershed includes Helgeå, Lyckebyån and Ronnebyån river/basin systems. Western watershed includes Kävlingeå, Mölndalsån, Ronneå and Viskan river/basin systems (Fig. 21). A total of 16 isolates of *P.* x *alni* were found in Eastern watershed, and 25 isolates - in Western (Table 7) Two MLGs were identified in each of Eastern watersheds basins. The number of MLGs in Western watershed basins ranged between one and four. In Eastern watershed, only one previously undescribed MLG was found (Lyckebyån basin). In Western watershed, three previously undescribed MLGs were found

in Kävlingeå and Viskan basins. The MLG Pxa-1 in Western watershed was detected only once - in Mölndalsån basin, and in Eastern watershed - in all three basins – Helgeå, Lyckebyån and Ronnebyån (Fig. 21).

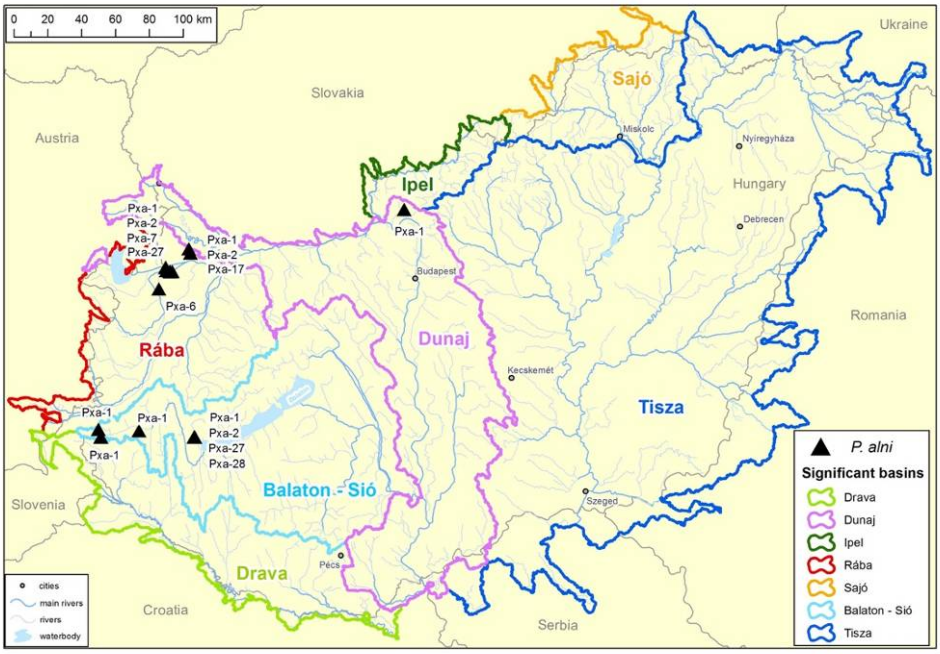

Fig. 20 Distribution of watersheds in Hungary and sites of *Phytophthora* x *alni* isolation (black triangles). Labels by each triangle indicate numbers of identified *P.* x *alni* multilocus genotypes (MLGs).

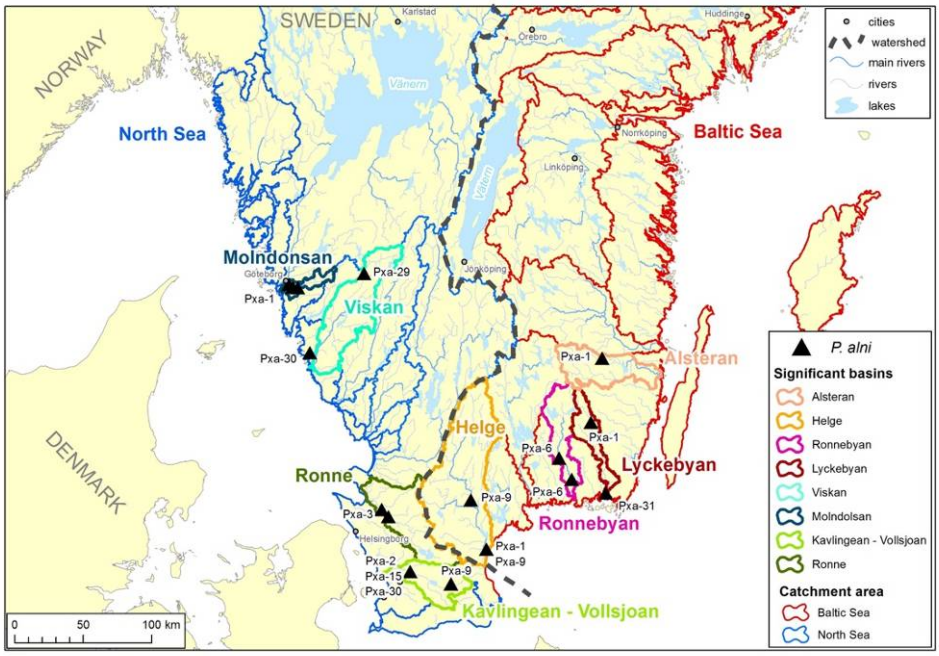

Fig. 21 Distribution of watersheds in Sweden and sites of *Phytophthora* x *alni* isolation (black triangles). Labels by each triangle indicate numbers of identified *P.* x *alni* multilocus genotypes (MLGs).

Table 7 Occurrence of *Phytophthora* x *alni* multilocus genotypes (MLGs) in water basins and watersheds of Sweden, Czech Republic and Hungary.

Country	Watershed	Basin/ River	N[B]	Number of identified MLGs			Number (proportion) of isolates belonging to MLG Pxa-1
				Previously described	New	Total	
Czech Republic (CZ)	Donau	DSS[A]	5	2	2	4	2 (40.0 %)
		Morava	3	1	1	2	2 (66.6 %)
	Elbe-Vltava	Elbe	5	1	3	4	2 (40.0 %)
		Ploučnice	4	2	2	4	1 (25.0 %)
		Ohre	4	2	2	4	2 (50.0 %)
		Sazava	4	3	1	4	1 (25.0 %)
		Berounka	11	2	3	5	7 (63.6 %)
		Vltava	13	1	3	4	10 (76.9 %)
Hungary (HU)	Balaton	Zala	23	3	2	5	19 (82.6 %)
	Donau	Rábca	16	4	1	5	10 (62.5 %)
Sweden (SW)	Eastern	Helgeå	6	2	0	2	1 (16.0 %)
		Lyckebyån	3	1	1	2	1 (33.3 %)
		Ronnebyån	7	2	0	2	1 (14.2 %)
	Western	Kävlingeå	8	3	1	4	0
		Mölndalsån	7	1	0	1	7 (100.0 %)
		Ronneå	8	1	0	1	0
		Viskan	2	0	2	2	0

[A] – Basin composed of three rivers: Dyje, Svitava and Svratka. [B] - Number of observed *Phytophthora* x *alni* isolates;

3.1.2.4. Population structure of *Phytophthora* x *alni*

Analysis of Molecular Variance (AMOVA) showed absence of genetic structure in watersheds between the three investigated countries (Czech Republic, Hungary and Sweden). Moreover, the variance was insignificant, but within watersheds and within basins, a higher (significant) differentiation was observed (Table 8). The 64.9 % of the variation was associated with differences within basins, and 35.95 % of the variation was associated with differences within populations. Fixation indices of "within populations" and "within basins"

had the same values of 0.35. Clone-corrected data showed the lack of genetic structure in between all variables (Table 8) and high insignificance. This indicates that all populations of *P.* x *alni* in Czech Republic, Hungary and Sweden are not differentiated from each other.

The Principal Component Analysis (PCA) was done for all three countries, but only Swedish *P.* x *alni* population showed a certain clustering pattern (Fig. 22). The first two PCA dimensions (axes) explain 62.0 % of the variation in *P.* x *alni* population structure (Fig. 22). The eigenvalues were 0.064 and 0.048 for dimensions 1 and 2, respectively. The PCA plot shows two rather dispersed clusters, where the first cluster is composed exclusively of MLGs found in Western watershed of Sweden, and the second cluster is composed mainly of MLGs detected in Eastern watershed of Sweden. Only two MLGs: Pxa-1 and Pxa-9 were detected in both watersheds (Fig. 22).

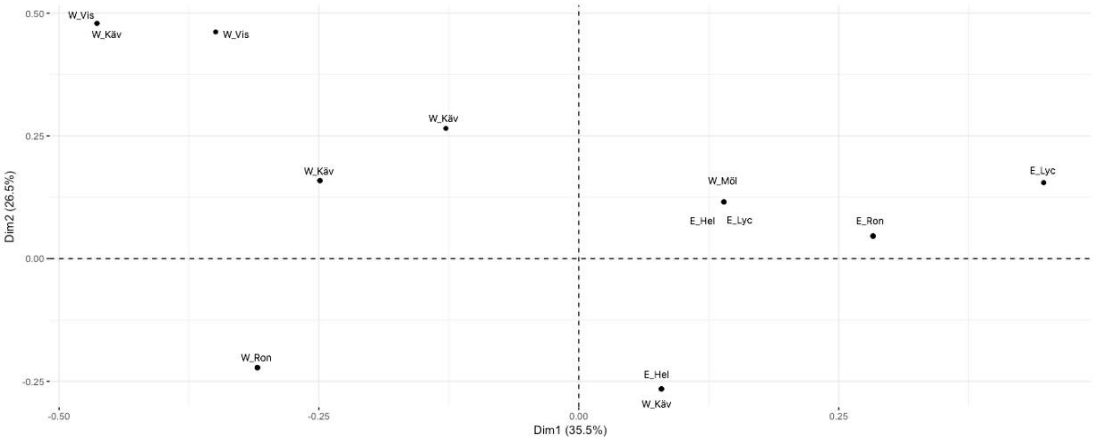

Fig. 22 Principal Component Analysis (PCA) of *Phytophthora* x *alni* multilocus genotypes in Eastern (E) and Western (W) watersheds of Sweden. For watershed and river basin names see Table 6.

3.1.2.5. Population genetics of *Phytophthora uniformis*

A total of 87 *P. uniformis* isolates originating from five countries have been genotyped (Appendix 2). From this number, 68 isolates were identified as MLG Pu-E1 and 20 isolates - as MLG Pu-E2; both MLGs have previously been described by Aguayo *et al.* (2013). The MLG Pu-E1 was found in all five countries and MLG Pu-E2 was found only in Sweden (Appendix 2).

Table 8 Analysis of Molecular Variance (AMOVA) in populations of *Phytophthora* x *alni* in Czech Republic, Hungary and Sweden

Source of variation	Variance components (percentage of total variance)	Fixation index	P-value
Variation among populations (among watersheds)	-0.007 (-0.861 %)	-0.008	0.317
Variation within populations (among basins)	0.293 (35.95 %)	0.35	0.001
Variation within basins (isolates)	0.529 (64.90 %)	0.35	0.001
Source of variation after clone correction			
Variation among populations (among watersheds)	0.0927 (6.75 %)	0.06	0.05
Variation within populations (among basins)	0.017 (1.24 %)	0.01	0.541
Variation within basins (isolates)	1.3711 (92.14 %)	0.07	0.101

3.2. Results of recovery and identification of *Ophiostoma novo-ulmi* from symptomatic elm trees, and genetic properties of *O. novo-ulmi* populations

3.2.1. Results of Ophiostoma novo-ulmi isolation from wood of symptomatic elms

Pure fungal cultures were isolated from a total of 81 (95.2 %) wood samples. According to morphological characteristics, all fungal isolates were distributed into 33 morphological groups. Following ITS sequencing, one of the groups, consisting of 49 isolates, was identified as *O. novo-ulmi*, the causal agent of DED. *O. novo-ulmi* was isolated from declining *U. glabra* and *U. minor* wood samples from eight sites in Lithuania (Table 9; Fig. 23; Appendix 4). No isolates of *O. ulmi* s. s. were identified.

3.2.2. Assignment of Ophiostoma novo-ulmi to subspecies

To assign *O. novo-ulmi* isolates to subspecies, RFLP of nuclear cerato-ulmin gene *cu* and colony type gene *col1* has been performed. During the analysis, both gene fragment sizes (for *cu* and *col1*) were consistent with those described by Konrad *et al.* (2002) and Kirisits & Konrad (2004).

Table 9 Data on results of *Ophiostoma novo-ulmi* isolation from wood of symptomatic elm trees (*Ulmus glabra* and *U. minor*) (More detailed information in Apendix 4)

District of Lithuania	**Site of isolation**	**Host tree**	**Subspecies and hybrids of *O. novo-ulmi* (no. of recovered isolates)**
Biržai	Karajimiškis	*U. glabra*	*O. novo-ulmi* ssp. *novo-ulmi* (1)
Kaunas	Raudondvaris	*U. glabra*	*O. novo-ulmi* ssp. *novo-ulmi* (1)
Kėdainiai	Aristava	*U. minor*	*O. novo-ulmi* ssp. *novo-ulmi* (24); *O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* (5)
Pasvalys	Gulbinėnai, Paberliai	*U. glabra*	*O. novo-ulmi* ssp. *novo-ulmi* (3)
Plungė	Kuliai	*U. glabra*	*O. novo-ulmi* ssp. *novo-ulmi* (4); *O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* (2)
Rokiškis	Dusetos	*U. glabra*	*O. novo-ulmi* ssp. *novo-ulmi* (4)
Vilnius	Dūkštos	*U. glabra*	*O. novo-ulmi* ssp. *novo-ulmi* (3)
Vilnius city	Verkiai	*U. glabra*	*O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* (2)

Amplified fragments of *cu* gene were digested using restriction endonuclease HphI, this endonuclease resulted 101 bp, 161 bp, 262 bp, 672 bp size restriction fragments. Amplified fragments of *col1* gene were digested using restriction endonuclease BfaI, this endonuclease resulted 100 bp, 156 bp, 236 bp, 328 bp size restriction fragments. By combination of both restriction enzymes subspecies of *O. novo-ulmi* was distinguished: *O. novo-ulmi* ssp. *novo-ulmi* was separated by 101 bp, 161 bp and 672 bp size profile of HphI restriction enzyme, and 100 bp and 382 bp size fragments of BfaI (Konrad *et al.*, 2002). The hybrid of both subspecies EAN x NAN was identified by unusual restriction fragment size profile: 101 bp, 161 bp and 672 bp size fragments of HphI, and 100 bp, 156 bp, 236 bp BfaI (Konrad *et al.*, 2002) (Fig. 24). All identified 49 *O. novo-ulmi* isolates were assigned to subspecies: 36 isolates were identified as *O. novo-ulmi* ssp. *novo-ulmi*, and 13 isolates - as *O. novo-ulmi* ssp. *novo-ulmi* ssp.

americana hybrid. The hybrids were found in Plungė, Kėdainiai and Vilnius districts and Vilnius city (Table 9). No hybrids were found in Biržai, Kaunas, Pasvalys and Rokiškis districts. *O. novo-ulmi* ssp. *americana* was not found in Lithuania during the present study.

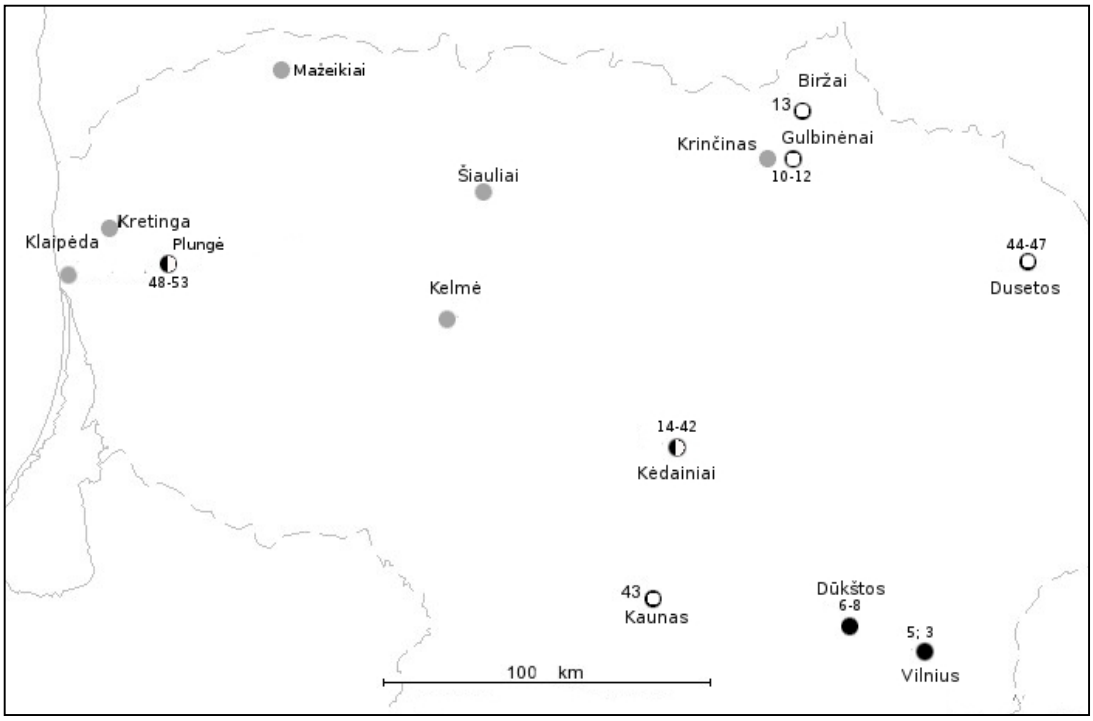

Fig. 23 Distribution of sites in Lithuania where symptomatic elm trees (*Ulmus glabra* and *U. minor*) were subjected to sampling. Grey circles indicate sites with elms showing symptoms of Dutch elm disease from which *Ophiostoma novo-ulmi* has not been isolated; black circles – sites, where hybrids of *O. novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana* were isolated; open circles – sites, where *O. novo-ulmi* ssp. *novo-ulmi* was isolated; half black and half white circles – sites where both subspecies hybrids and *O. novo-ulmi* ssp. *novo-ulmi* were isolated.

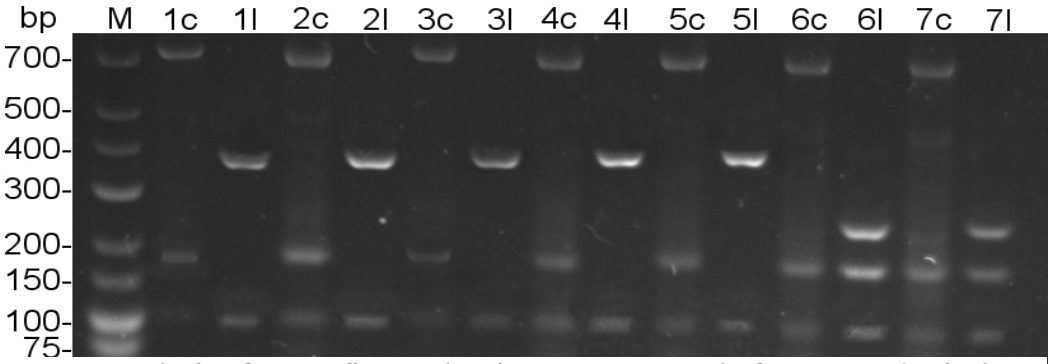

Fig. 24 Analysis of RFLP fingerprints in 2 % agarose gel of *O. novo-ulmi* isolates. 1c – 7c fragments of cu gene; 1l – 7l fragments of col1 gene. M – DNA GeneRuler Low Range DNA Ladder, Ready-to-use 25 to 700 bp (ThermoFisher)

3.2.3. Vegetative compatibility groups of Ophiostoma novo-ulmi isolates

Vegetative compatibility test allowed to grouping of all *O. novo-ulmi* isolates to 38 vegetative compatibility groups (VCGs). Most isolates did not form groups (Fig. 14 C), and all groups consisted of no more than two isolates (Fig. 25). In Aristava population (Kėdainiai district), 29 *O. novo-ulmi* isolates were divided into 24 groups (Fig. 25 A). Here, five VCGs consisted of two isolates. In this population, 23 isolates were identified as *O. novo-ulmi* ssp. *novo-ulmi* and six isolates - as *O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* hybrids. Dusetos population (Rokiškis district) included four *O. novo-ulmi* ssp. *novo-ulmi* isolates that formed three VCGs (Fig. 25 B). Kuliai population (Plungė region) included six *O. novo-ulmi* isolates that were grouped into three VCGs. Here, both VCG I and VCG II consisted of two *O. novo-ulmi* ssp. *novo-ulmi* isolates, and VSG III - of two *O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* hybrid isolates (Fig. 25 C). Verkiai (Vilnius city) population did not form any groups: both isolates were vegetatively incompatible (Fig. 25 D). Gulbinėnai-Paberliai population (Pasvalys district) included three *O. novo-ulmi* ssp. *novo-ulmi* isolates that were grouped into two VCGs. One group was composed of isolates recovered 3 km apart from each other (this was the largest distance within an identified group) (Fig. 25 E). Dūkštos population (Vilnius district) included three *O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* hybrid isolates that were grouped into two VCGs (Fig. 25 F). Only one *O. novo-ulmi* ssp. *novo-ulmi* isolate was obtained from each Kaunas and Biržai sampling sites. (Full results in Appendix 5).

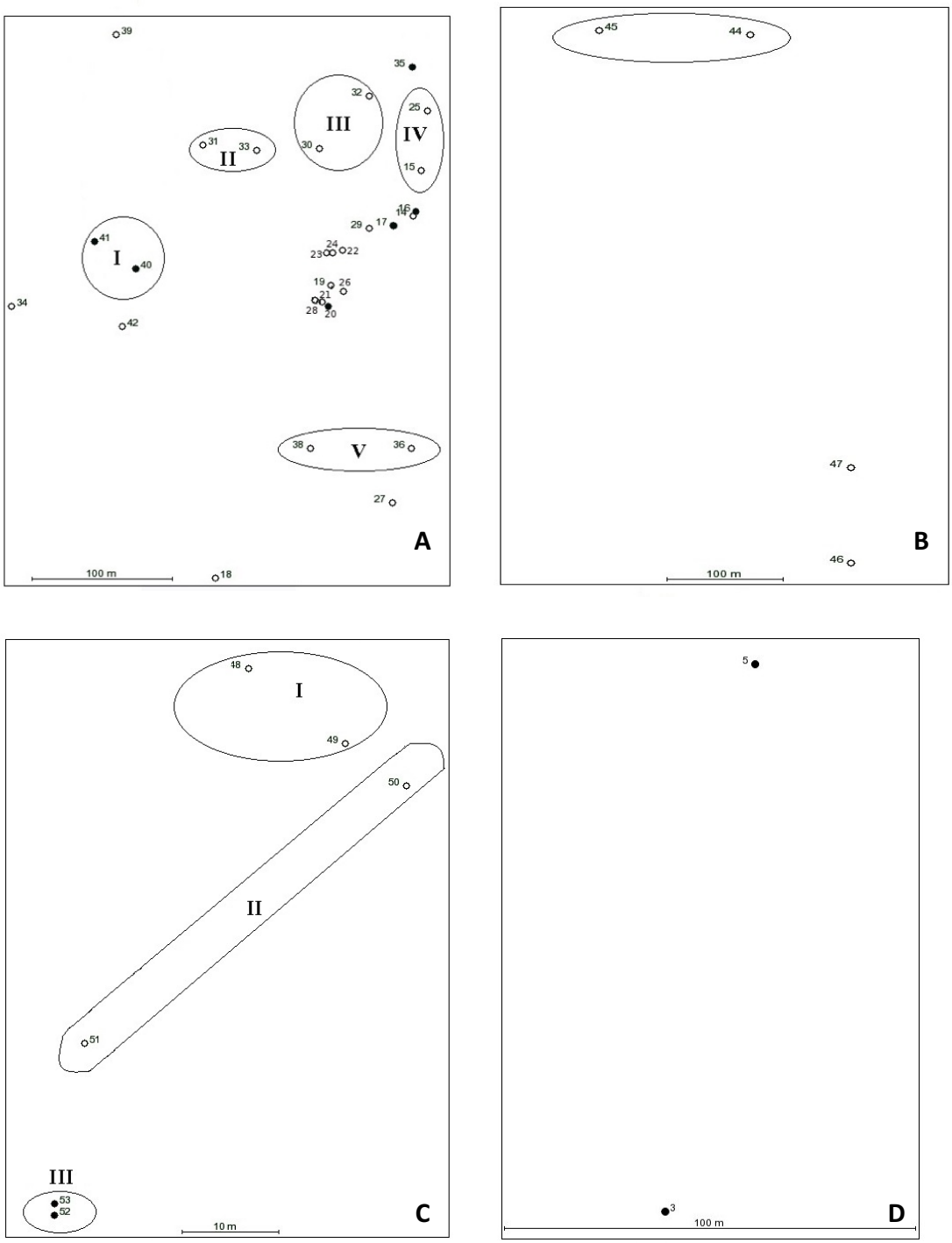

Fig. 25 The structure of local populations of *Ophiostoma novo-ulmi* constructed following results of vegetative compatibility tests on agar plates. Open circles show elm trees from which isolated were *O. novo-ulmi* ssp. *novo-ulmi*, while black circles show trees from which isolated were hybrids of *O. novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana*. Encircled circles show isolates belonging to the same vegetative compatibility group. A, Aristava population (Kėdainiai district); B, Dusetos population (Rokiškis district); C, Kuliai population (Plungė district); D, Verkiai population (Vilnius city); E, Paberliai-Gulbinėnai population (Pasvalys district); F, Dūkštos population (Vilnius district).

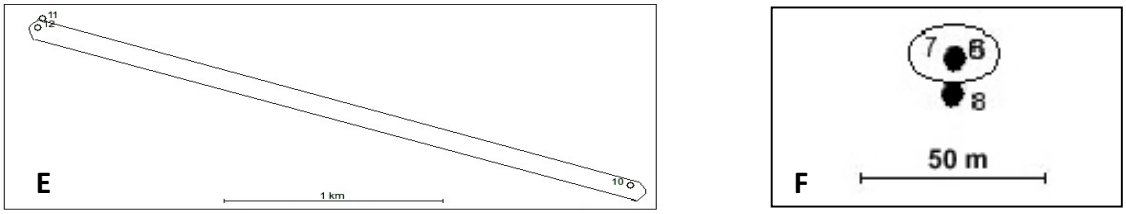

Fig. 25 The structure of local populations of *Ophiostoma novo-ulmi* constructed following results of vegetative compatibility tests on agar plates. Open circles show elm trees from which isolated were *O. novo-ulmi* ssp. *novo-ulmi*, while black circles show trees from which isolated were hybrids of *O. novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana*. Encircled circles show isolates belonging to the same vegetative compatibility group. A, Aristava population (Kėdainiai district); B, Dusetos population (Rokiškis district); C, Kuliai population (Plungė district); D, Verkiai population (Vilnius city); E, Paberliai-Gulbinėnai population (Pasvalys district); F, Dūkštos population (Vilnius district).

3.3. Characterization of Lithuanian *Hymenoscyphus fraxineus* populations

3.3.1. Genetic properties of Hymenoscyphus fraxineus populations

3.3.1.1. Genotypic diversity

All herein investigated *H. fraxineus* isolates have been recovered from five sites, with a sample size ranging from 25 to 46 isolates (Table 10). A total of 367 isolates were genotyped. Of this number, 181 isolates originated from ash leaf petioles and 186 isolates - from lesions on ash twigs and stems. All successfully genotyped isolates were grouped into 244 MLGs. Two of the identified MLGs (0.8 %) consisted of seven isolates (maximum number), nine MLGs (3.6 %) consisted of four isolates, 23 MLGs (9.4 %) - of three isolates, 37 MLGs (15.1 %) – of two isolates and 173 MLGs (70.9 %) included only one isolate (data not shown). A total number of 71 non-private MLGs were found in this study, 49 MLGs consisted of petiole and lesion isolates, 11 MLGs consisted only of petiole isolates and 11 MLGs – only of lesion isolates. The number of MLGs at each site ranged from 56 to 87 (Table 10)

Shannon-Wienner index (H) showed high genotypic diversity in all five sampling sites (populations), with the highest index (3.98) detected in Ukmergė population (Table 10). The highest number of MLGs (87) was identified in Biržai population (Table 10).

3.3.1.2. Genetic diversity

A total of 29 alleles were detected across 11 microsatellite loci. All loci were polymorphic, allele number per locus ranged from two to five. Locus 53 consisted of five alleles (Appendix 6). The mean number of alleles per locus (A_M) showed low genetic variation in all investigated populations of *H. fraxineus*; the value of A_M varied from 1.9 in Kėdainiai population to 2.3 in Biržai population (Table 11). Allelic richness values were similar in all populations and varied between 1.86 and 2.08 (Table 11). Calculation of the number of private alleles (A_{PR}) revealed no endemic traits in all *H. fraxineus* populations. A_{PR} ranged from 0.00 (± 0.00) in Kėdainiai population to 0.09 (±0.01) in Biržai population (Table 11). Values of allelic richness and the number of private alleles were not statistically significant ($P > 0.05$) (data not shown).

Private alleles were present in three MLGs: isolate KR6L (recovered from lesion, Kretinga population) had private allele 197 at locus 15; isolate BU60L (recovered from lesion, Biržai population) had private allele 234 at locus 38; and isolate UKM39P (recovered from petiole, Ukmergė population) had private allele 268 at locus 69 (Appendix 6).

The values of Standardized Index of Association (rbarD) (Appendix 6) showed free recombination with no observed clonality across all investigated populations. All *H. fraxineus* MLGs had the probability of development by a sexual event, P_{Gen} values were less than 0.05 (Table 11). The probability that MLG occurred second time through different random sexual reproductive event (P_{Sex}) was observed only once - in Biržai population (Table 11).

Table 10 Genotypic diversity indicators in the investigated *Hymenoscyphus fraxineus* populations. For population name abbreviations see Table 5.

Population	Sample size (number of isolates)			Number of identified multilocus genotypes (MLGs) and expected number of MLGs (eMLG)					H^a	$A_R{}^b$		$A_{PR}{}^c$		$F_{ST}{}^d$
	Petioles	Lesions	Total	Petioles	eMLG (±SE)	Lesions	eMLG (±SE)	Total	3.94	Petioles	Lesions	Petioles	Lesions	
BIR	43	44	87	43	25.0 (±0.00)	41	23.9 (±0.84)	81	3.77	1.96 (0.08)	2.08 (0.16)	0.01 (0.01)	0.10 (0.06)	-0.006
KED	32	28	60	28	22.6 (±0.89)	25	22.6 (±0.65)	50	3.93	1.78 (0.12)	1.84 (0.09)	0.00 (0.00)	0.00 (0.00)	0.013
KRE	38	43	81	37	24.6 (±0.49)	37	23.0 (±1.05)	70	3.98	1.96 (0.07)	1.96 (0.08)	0.04 (0.03)	0.03 (0.03)	-0.005
UKM	37	46	83	35	24.1 (±0.69)	43	24.1 (±0.76)	75	2.1	1.89 (0.11)	1.89 (0.11)	0.03 (0.03)	0.02 (0.02)	0.009
VIL	31	25	56	28	23.1 (±0.78)	24	24.0 (±0.00)	49	NA	1.87 (0.05)	1.87 (0.05)	0.00 (0.00)	0.03 (0.02)	0.029
Total	181	186	367	148	NA	144	NA	244		NA	NA	NA	NA	-0.001

SE – standard error
NA – not applicable
[a]Shannon-Weiner index, showing genotypic diversity (based on smallest sample size N = 56)
[b]Allelic richness per locus
[c]Number of private alleles per locus
[d]Fixation index, the values calculated among all Lithuanian *H. fraxineus* population

Table 11 Indicators of genetic diversity and mode of reproduction in the investigated *Hymenoscyphus fraxineus* populations. For population name abbreviations see Table 5.

Population	N	Genetic diversity			Mode of reproduction			
		A_M[a]	A_R[b] (SE)	A_{PR}[c] (SE)	P_{Gen}[d]	P_{Sex}[e]	rbarD[f]	P rbarD[g]
BIR	87	2.3	2.08 (0.02)	0.09 (0.01)	81/81	1/5	0.002	0.618
KED	60	1.9	1.86 (0.01)	0.00 (0.00)	50/50	0/9	0.000	0.517
KRE	81	2.1	1.99 (0.02)	0.07 (0.01)	69/69	0/10	0.002	0.660
UKM	83	2.2	1.99 (0.02)	0.04 (0.00)	75/75	0/8	0.004	0.231
VIL	56	2.1	1.99 (0.02)	0.01 (0.00)	49/49	0/6	0.004	0.288
Total	367	NA	NA	NA	243/243	1/71	0.000	0.508

[a]Mean number of alleles observed per locus
[b]Allelic richness per locus
[c]Number of private alleles per locus
[d]Number of MLGs with $P_{Gen} < 0,05$
[e]Number of MLGs with $P_{Sex} < 0,05$
[f]Standardized index of association indicating multilocus linkage disequilibrium
[g]Significance value of rbarD
SE – standard error
NA – not applicable

3.3.1.3. Population structure

The mean F_{ST} value across Lithuanian *H. fraxineus* populations was -0.001 (with 95 % confidence interval). Pairwise comparison of all populations revealed absence of genetic structure: the highest genetic distance among the investigated populations was observed between Biržai and Vilnius ($F_{ST} = 0.014$) and Biržai and Kėdainiai populations ($F_{ST} = 0.006$) (Table 10 & Table 12). In all five herein investigated populations, pairwise differentiation was insignificant ($P > 0.05$). Fixation index values within populations between isolates from petioles and lesions were also low, the highest value was in Vilnius population ($F_{ST} = 0.029$), and the lowest - in Kretinga and Biržai populations ($F_{ST} = -0.005$ and -0.006, respectively) (Table 10 & Table 12).

Table 12 Pairwise differences of fixation indices (F_{ST}) across investigated Hymenoscyphus fraxineus populations. In bold indicated are F_{ST} values showing genetic distances between lesion and petiole isolates within a respective population. For population name abbreviations see Table 5

Population	BIR	KED	KRE	UKM	VIL
BIR	**-0.006**	-	-	-	-
KED	0.006	**0.013**	-	-	-
KRE	-0.003	-0.003	**-0.005**	-	-
UKM	-0.002	-0.007	-0.004	**0.009**	-
VIL	0.014	-0.007	-0.005	-0.003	**0.029**

Discriminant Analysis of Principal Components (DAPC) could not define structure in *H. fraxineus* populations (Fig. 26).

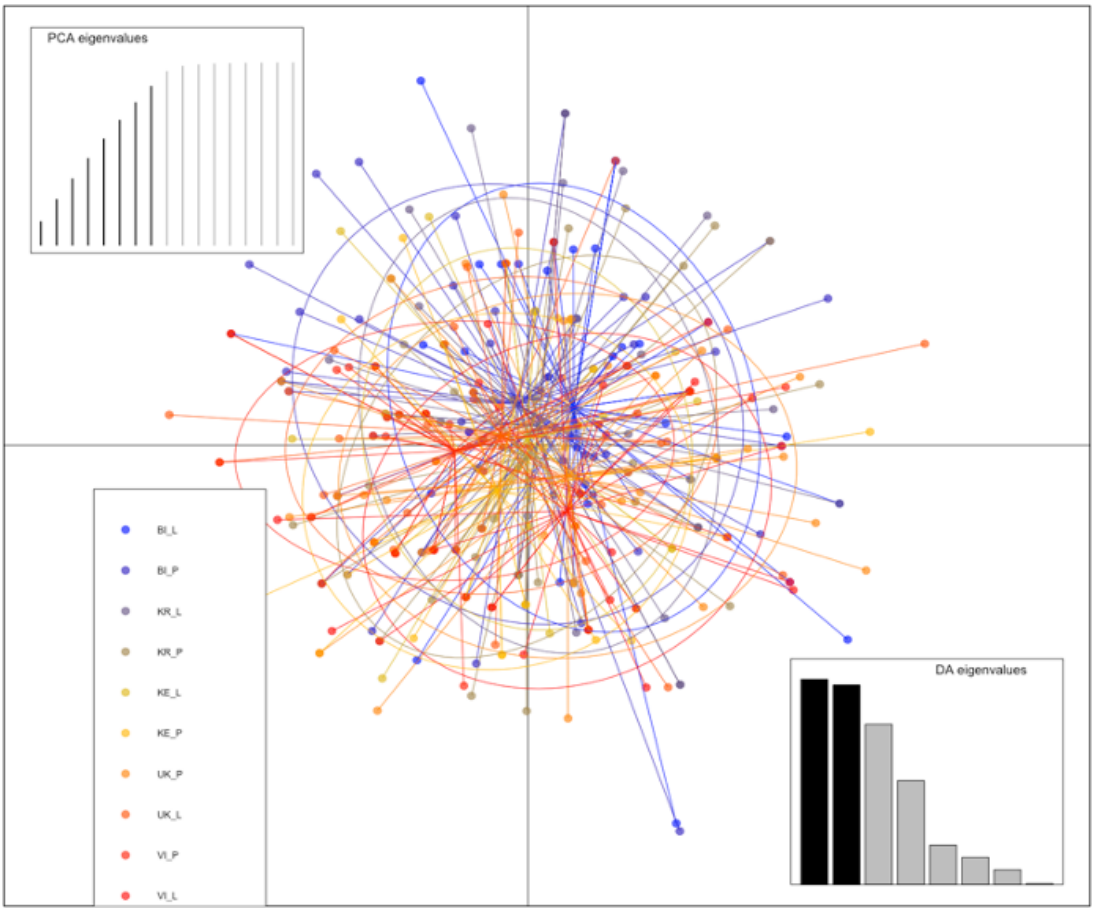

Fig. 26 A scatterplot of the first two axes of DAPC analysis of the investigated *Hymenoscyphus fraxineus* populations

3.3.2. Results of Fraxinus excelsior seedling inoculation experiment with Hymenoscyphus fraxineus isolates

From a total of 145 inoculated *F. excelsior* seedlings in Seedlings 2 experiment, 128 ones were used for further analysis and 17 ones were excluded due to naturally occurring ash dieback symptoms. Out of the 128 inoculated seedlings, bark lesions of variable size developed on 81 (63.3 %) seedlings. This proportion was significantly lower ($P < 0.001$, two-proportion *z*-test) compared to that found in Seedlings 1 experiment, where as much as 90.0 % of the inoculated seedlings had bark lesions (Lygis *et al.*, 2017).

Table 13 Main results of two virulence tests (Seedlings 1 & Seedlings 2, see Materials and Methods section) of *Hymenoscyphus fraxineus* isolates on 3-year-old *Fraxinus excelsior* seedlings following their artificial inoculation. Within columns, pairs of values labelled with different letters are significantly different at $P \leq 0.05$; n.s. – non-significant difference at $P \leq 0.05$. SE = standard error. A *t*-test was applied for comparison of mean necrosis lengths; two proportion *z*-test was applied for comparison of proportions

Isolate origin[a]	**No. of inoculated seedlings[b]**	**No. of isolates tested**	**No. (%) of isolates producing necrosis from the inoculation site**	**No. (%) of seedlings with necrosis development from the inoculation site**	**Mean necrosis length[c], cm ± SE (length range: min-max value, cm)**
1	***2***	***3***	***4***	***5***	***6***
Seedlings 1[d]					
LT population	499	100	98 (98.0) n.s.	459 (92.0) n.s.	6.0±0.3 (0–11.3) n.s.
CH population	500	100	98 (98.0) n.s.	449 (89.8) n.s.	5.7±0.3 (0–12.8) n.s.
P value			1.000	0.230	0.343
lesion isolates	499	100	98 (98.0) n.s.	445 (89.2) n.s.	5.3±0.3 (0–11.3) a
petiole isolates	500	100	98 (98.0) n.s.	463 (92.6) n.s.	6.4±0.3 (0–12.8) b
P value			1.000	0.060	0.004
LT lesion isolates	249	50	49 (98.0) n.s.	224 (90.0) n.s.	5.3±0.4 (0–11.3) a
LT petiole isolates	250	50	49 (98.0) n.s.	235 (94.0) n.s.	6.7±0.3 (0–11.0) b

Table 13 (continuation)

1	*2*	*3*	*4*	*5*	*6*
P value			1.000	0.097	0.007
CH lesion isolates	250	50	49 (98.0) n.s.	221 (88.4) n.s.	5.2±0.4 (0–10.9) n.s.
CH petiole isolates	250	50	49 (98.0) n.s.	228 (91.2) n.s.	6.1±0.4 (0–12.8) n.s.
P value			1.000	0.301	0.149
LT lesion isolates	249	50	49 (98.0) n.s.	224 (90.0) n.s.	5.3±0.4 (0–11.3) n.s.
CH lesion isolates	250	50	49 (98.0) n.s.	221 (88.4) n.s.	5.2±0.4 (0–10.9) n.s.
P value			1.000	0.575	0.875
LT petiole isolates	250	50	49 (98.0) n.s.	235 (94.0) n.s.	6.7±0.3 (0–11.0) n.s.
CH petiole isolates	250	50	49 (98.0) n.s.	228 (91.2) n.s.	6.1±0.4 (0–12.8) n.s.
P value			1.000	0.232	0.212
Mean/total	*999*	*200*	*196 (98.0)*	*908 (90.9)*	*5.8±0.2 (0–12.8)*
Seedlings 2					
LT population	64	14	14 (100) n.s.	39 (60.9) n.s.	2.8±0.7 (0–8.9) n.s.
CH population	64	15	14 (93.3) n.s.	42 (65.6) n.s.	3.3±0.8 (0–9.0) n.s.
P value			0.326	0.582	0.660
lesion isolates	68	16	15 (93.8) n.s.	38 (55.9) n.s.	2.2±0.6 (0–8.7) n.s.
petiole isolates	60	13	13 (100) n.s.	43 (71.7) n.s.	4.1±1.0 (0.3–9.0) n.s.
P value			0.359	0.064	0.086
LT lesion isolates	35	8	8 (100) n.s.	19 (54.3) n.s.	1.7±0.5 (0–3.5) n.s.
LT petiole isolates	29	6	6 (100) n.s.	20 (69.0) n.s.	4.2±1.5 (0.3–8.9) n.s.
P value			1.000	0.231	0.089
CH lesion isolates	33	8	7 (87.5) n.s.	19 (57.6) n.s.	2.7±1.1 (0–8.7) n.s.
CH petiole isolates	31	7	7 (100) n.s.	23 (74.2) n.s.	4.0±1.4 (0.5–9.0) n.s.
P value			0.333	0.162	0.457
LT lesion isolates	35	8	8 (100) n.s.	19 (54.3) n.s.	1.7±0.5 (0–3.5) n.s.
CH lesion isolates	33	8	7 (87.5) n.s.	19 (57.6) n.s.	2.7±1.1 (0–8.7) n.s.
P value			0.302	0.785	0.412
LT petiole isolates	29	6	6 (100) n.s.	20 (69.0) n.s.	4.2±1.5 (0.3–8.9) n.s.

Table 13 (continuation)

1	*2*	*3*	*4*	*5*	*6*
CH petiole isolates	31	7	7 (100) n.s.	23 (74.2) n.s.	4.0±1.4 (0.5–9.0) n.s.
P value			1.000	0.653	0.905
Mean/total	*128*	*29*	*28 (96.6)*	*81 (63.3)*	*3.1±0.6 (0–9.0)*

[a]LT = Lithuania, CH = Switzerland, lesion isolates - *H. fraxineus* isolates recovered from necrotic bark lesions of *F. excelsior*; petiole isolates - *H. fraxineus* isolates recovered from fallen ash leaf petioles. For more information on isolate origin see Burokiene et al. (2015).

[b]number of seedlings in which lesion development has been assessed by end date of the respective experiment.

[c]mean proximal (experiment Seedlings 1) length of bark necroses on inoculated *F. excelsior* seedlings (experiments Seedlings 1 & 2)

[d]results published in Lygis et al. (2017).

Out of 29 *H. fraxineus* isolates used for the inoculations, 26 (89.7 %) induced lesions by the end of the experiment, and this proportion was significantly lower (P = 0.015, two-proportion *z*-test) compared to Seedlings 1 experiment (98.0 %) (Lygis *et al.*, 2017). Virulence of *H. fraxineus* isolates didn't depend on host tissue from which they have been recovered (lesions vs. petioles) (Table 13).

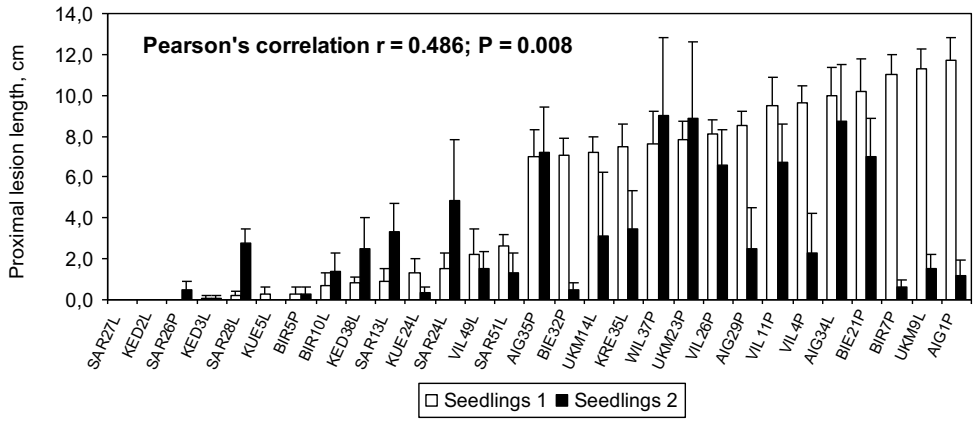

Fig. 27 Comparison of proximal length of external bark necroses on *Fraxinus excelsior* seedlings inoculated with 14 least virulent and 15 most virulent *Hymenoscyphus fraxineus* isolates (as ranked according to the results of a large seedling inoculation experiment Seedlings 1 (Lygis *et al.*, 2017)) between two inoculation experiments, Seedlings 1 and Seedlings 2. The bars are means of 2-5 inoculated seedlings ± standard error. Isolates originate from five Swiss (AIG=Aigle, BIE=Bière, KUE=Kuessnacht, SAR=Sargans and WIL=Wil) and five Lithuanian (BIR=Biržai, KED=Kėdainiai, KRE=Kretinga, UKM=Ukmergė and VIL=Vilnius) *Fraxinus excelsior* stands.

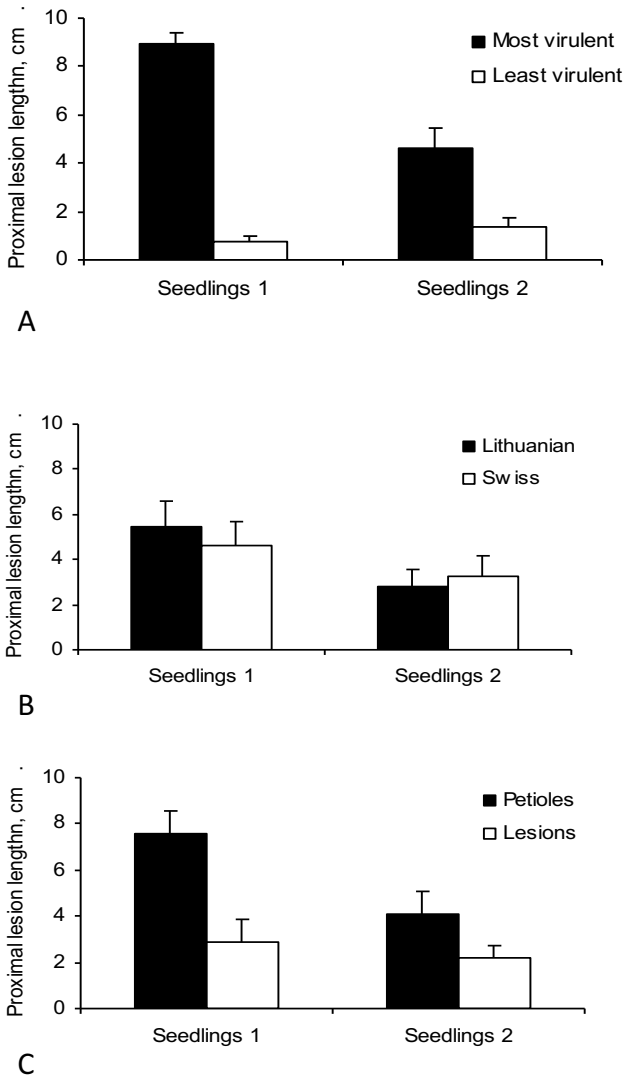

Fig. 28 Comparison of mean proximal length of external bark necroses on *Fraxinus excelsior* seedlings caused by pre-selected 15 most virulent and 14 least virulent *Hymenoscyphus fraxineus* isolates (Lygis et al., 2017) in two inoculation experiments, Seedlings 1 and Seedlings 2. The presented comparisons are between: A, most virulent (N = 15) and least virulent (N = 14) isolates; B, Lithuanian (N = 14) and Swiss (N = 15) isolates; and C, isolates recovered from fallen ash leaf petioles (N = 13) and from necrotic

Overall, the mean proximal lesion length on all seedlings (N = 128) by the end of the experiment Seedlings 2 was 3.1 ± 0.6 cm (Table 13). No significant differences ($P > 0.05$, *t*-test) were found either between seedlings inoculated with Swiss and Lithuanian isolates, or between seedlings inoculated with lesion and petiole isolates (Table 13). The largest lesions were observed on seedlings inoculated with Lithuanian petiole isolates (4.2 ± 1.5 cm, Table 13), while seedlings inoculated with Lithuanian lesion isolates developed the smallest lesions (1.7 ±

0.5 cm, Table 13). The difference in lesion length between lesion and petiole isolates was however non-significant (P > 0.05, *t*-test) within both Lithuanian and Swiss populations (Table 13).

A significant positive correlation (r = 0.418; P = 0.024) was found in proximal lesion length on seedlings between experiments Seedlings 1 and Seedlings 2 caused by the same set of the most and the least virulent *H. fraxineus* isolates (N = 29; as ranked according to the results of virulence test in experiment Seedlings 1 (Lygis *et al.*, 2017)) (Fig. 27; Table 13).

Significant differences in proximal lesion length (P ≤ 0.005; *t*-test) were found between most virulent and least virulent isolates in both inoculation experiments (Fig. 28). Although in the Seedlings 1 experiment, a significant difference in proximal lesion length was found between "petiole" and "lesion" isolates (7.57±1.00 cm vs. 2.91±0.95 cm, respectively; P = 0.002), in the

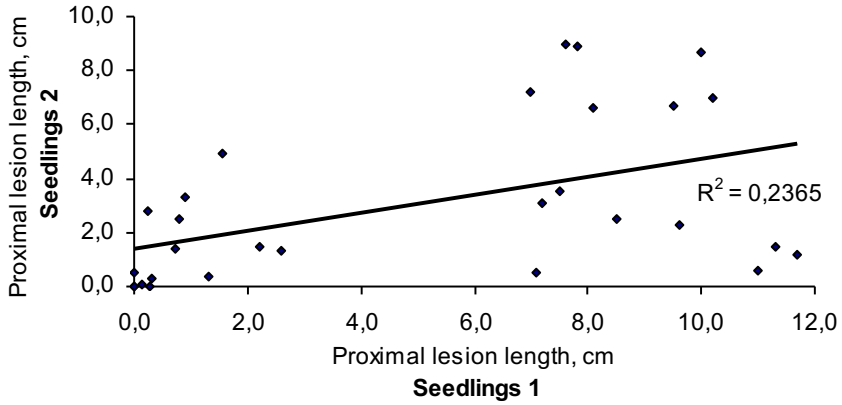

Fig. 29 Correlation between mean proximal length of external bark necroses caused by the same *Hymenoscyphus fraxineus* isolates on *Fraxinus excelsior* seedlings in two inoculation experiments, Seedlings 1 and Seedlings 2. Here, most virulent (N = 15) and least virulent (N = 14) isolates were selected according to ranking made in seedling inoculation experiment Seedlings 1 (Lygis et al., 2017))

Seedlings 2 experiment this difference was non-significant (3.06±0.82 cm vs. 1.43±0.33 cm for "petiole" and "lesion" isolates, respectively; P = 0.057). No significant differences (P > 0.05) were found between Lithuanian and Swiss isolates in either experiment (Fig. 29). The proximal lesion length did not significantly correlate with seedling parameters, namely, height and stem

diameter at the inoculation site in either seedling inoculation experiment (data not shown).

3.3.3. Comparing results of two inoculation experiments (Seedlings 1 & 2) and growth speed of Hymenoscyphus fraxineus in culture

Radial growth speed in culture did not correlate with length of bark lesions produced by respective *H. fraxineus* isolates in either inoculation experiment (Table 14, Figs. 30 & 31). No significant difference in mean radial growth speed in culture was found between the pre-selected most virulent (N = 15; 1.3±0.1 mm/day) and least virulent (N = 14; 1.4±0.1 mm/day) *H. fraxineus* isolates as well (P = 0.570).

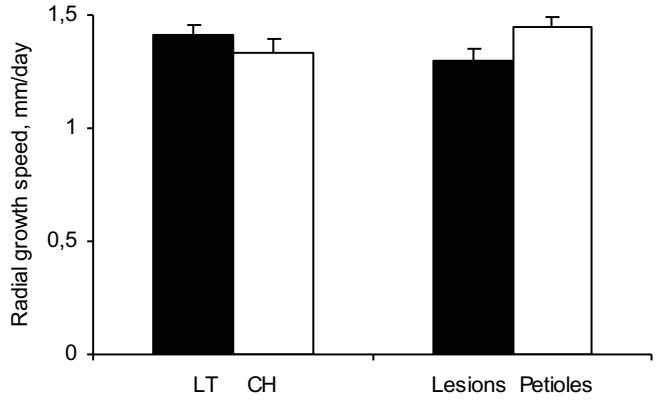

Fig. 30 Comparison of mean radial growth speed of *Hymenoscyphus fraxineus* mycelium over 13 days of growth on agar medium in Petri plates. LT, Lithuanian *H. fraxineus* isolates (N = 97) and CH, Swiss isolates (N = 93); Lesions - isolates recovered from necrotic bark lesions (N = 95); and Petioles - isolates recovered from fallen ash leaf petioles (N = 95). The bars are means ± standard error. Bars labelled with different letters are significantly different from each other at $P \leq 0.05$; n.s., non-significant difference at $P \leq 0.05$.

No significant differences in mean radial growth speed of *H. fraxineus* mycelium over 13 days of growth in culture were found between Swiss and Lithuanian isolates (P = 0.292; *t*-test), while growth speed of "lesion" and "petiole" isolates differed significantly (P = 0.038; *t*-test) (Fig. 30). When culture growth speed was compared between "lesion" and "petiole" isolates in each

country separately, although "petiole" isolates showed generally faster growth, the differences were non-significant at $P \leq 0.05$ (*t*-test).

Table 14 Pearson's correlation between proximal length of *Fraxinus excelsior* seedling bark lesions produced by *Hymenoscyphus fraxineus* isolates in inoculation experiments Seedlings 1 and Seedlings 2 and radial growth speed of respective *H. fraxineus* isolates in in culture. Number (N) of compared isolates in each case is given in brackets.

Pearson's correlation	Proximal lesion length, Seedlings 1	Proximal lesion length, Seedlings 2
Radial growth speed in culture	r = -0.005; P = 0.941 (N = 190)	r = 0.095; P = 0.623 (N = 29)
Proximal lesion length, Seedlings 2	r = 0.418; P = 0.024 (N = 29)	-

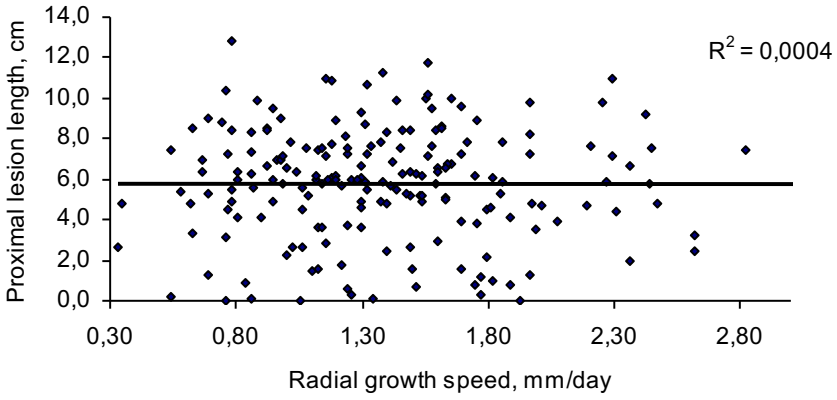

Fig. 31 Correlation between mean radial growth speed of *Hymenoscyphus fraxineus* mycelium over 13 days of growth on agar medium in Petri plates and mean proximal length of external bark necroses caused by the same *H. fraxineus* isolates (N = 190) on *Fraxinus excelsior* seedlings (experiment Seedlings 1).

4. DISCUSSION

4.1. Alder decline

4.1.1. Microorganism communities in Lithuania and Switzerland

Lithuania and Switzerland are two geographically separated and rather distinct countries. The countries are characterized by different mean annual temperatures, mean amounts of precipitation, relief (presence/absence of mountains), soil composition and biodiversity (Central Intelligence Agency, 2016). Therefore, the differences in microorganism communities could also be expected.

Fungal community recovered in declining *Alnus* spp. wood was mainly composed of saprophytes and endophytes with only a few isolates of plant pathogens, indicating their possibly minor influence in alder decline. In comparison, Lithuanian fungal community was more diverse compared to Swiss. This inconsistency could be associated with differences in local environment, as a few studies already showed its importance in fungal community composition (Arnold *et al.*, 2001; Higgins *et al.*, 2007).

In Lithuania, the dominant microorganism group that has been recovered from soil belonged to genus *Mortierella* (kingdom *Fungi*), while in Switzerland, the dominant group consisted of oomycetes, namely - *Phytophthora* spp. (kingdom *Protista*). One of explanations why microbial communities in both countries were so different could be different methodologies of microorganism isolation that have been applied. In Switzerland, microorganisms were recovered from soil by baiting tests using rhododendron leaves, while in Lithuania, due to difficulty in obtaining a sufficient amount of healthy rhododendron leaves, the microbial recovery was made from soil suspension. Baiting tests using rhododendron leaves are commonly used to study oomycete communities as they allow recovering of multiple species (Hahn *et al.*, 1999). Soil suspension is also commonly used for oomycete isolation (e.g., Yang & Hong, 2016), although recovery of *Phytophthora* spp. may sometimes be hampered by so-called

"suppressive soils" (Dignam *et al.*, 2016). There may be several explanations, why *Mortierella* sp. was dominant in Lithuanian soil samples. The main soil types in the present study were clay and humus, both of which are known to have an inhibitory effect on activity of hymexazole – the main antibiotic used against *Mortierella* spp. (Shew, 1982; Kato *et al.*, 1990). It has also been shown that in soil, phytophthoras are rapidly suppressed and outcompeted by *Mortierella* spp. (Cooke *et al.,* 2007).

Not only *P. alni* species complex can cause alder decline. Jung & Burgess (2009) and Haque & Diez (2012) demonstrated that *P. plurivora* can also be responsible for the disease. Another species, *P. lacustris*, was isolated from declining alder trees (Szabó *et al.*, 2013; Sims, 2014; Sárándi-Kovács *et al.*, 2015) that also raises questions about the role of this species in alder decline. In the present study, *P. lacustris* has been isolated from wood of declining *A. incana* in Switzerland, while *P. plurivora* was the most common *Phytophthora* species recovered from soil (rhizosphere of the declining alder trees). Jung & Burgess (2009) demonstrated that if *P. plurivora* infection starts in roots, it remains restricted to the roots with no penetration to other parts of the plant. This could explain why *P. plurivora* was isolated mainly from soil and water, and rarely from wood samples.

Diversity of *Phytophthora* species recovered from water samples was higher in Switzerland than in Lithuania. This difference could be determined by different mean annual temperature which is higher in Switzerland: many *Phytophthora* species are sensitive to low temperatures or large temperature fluctuations (Lamour, 2013).

4.1.2. Occurrence of representatives of Phytophthora alni species complex in Lithuania and Switzerland

Both in Lithuania and Switzerland, the proportion of successful recovery attempts of representatives of *P. alni* s. l. species complex (*P.* x *alni* and *P. uniformis*) was low. This low isolation success rate may be due to our attempts

to isolate phytophthoras from older necroses (alder wood). Streito *et al.* (2002) has demonstrated that *P. alni* s. l. could easily be isolated if the necrosis is fresh (the pathogen is active), but during the present study, usually sampled were older necroses (one-year-old or even older). This may also explain slightly higher isolation rates of saprophytic fungi. To avoid false negative results in isolation of *Phytophthora* spp., Davison & Tay (2005) suggested pathogen screening in different substrates and this suggestion was followed in the present work. Jung & Blaschke (2004) pointed to a poor survival of alder phytophthoras in soil what could explain low rate of *P. alni* s. l. recovery from this substrate (during the present study from soil this pathogen was recovered only once). Another explanation why *P. alni* s. l. was isolated with a relatively low success in Lithuania could be unfavorable environmental conditions for this pathogen (too low winter temperatures). Černý & Strnadová (2012) noticed that survival rate of *P. alni* s. l. decreases rapidly when temperature in winter drops below -5 ºC for several days. In Lithuania, average temperature in January is around -4 ºC, and it is not uncommon to have temperatures below -5 ºC for several days during winter time (http://www.meteo.lt/lt/naujienos//asset_publisher/15W0qASILd6o/content/2016-02-05-2016-m-sausio-men-vidutine-oro-temperatura?inheritRedirect=false).

More recently, Redondo *et al.* (2015) has found limited occurrence of *P.* x *alni* in Sweden that could also be due to low winter temperatures in more northern regions. Distribution of another species within *P. alni* complex, *P. uniformis*, in Sweden was likely less affected by cold winter temperatures and thus occurred more frequently. However, according to Brasier *et al.* (2004), in Europe *P. uniformis* is less frequent than *P.* x *alni*; and *P.* x *alni*, being more competitive species, is replacing *P. uniformis* from sites, where it has been previously established (Štěpánková *et al.*, 2013). Redondo *et al.* (2015) presumed that this replacement has not yet taken place in northern regions of Europe, including Sweden. Results of the present study support both presumptions: in Lithuania, *P. uniformis* was recovered more frequently than *P.*

x *alni* that may be a consequence of more unfavorable climatic conditions to the latter species. In Switzerland, where annual temperature is higher than in Lithuania, no isolates of *P. uniformis* were recovered, while *P.* x *alni* was isolated eight times, indicating that more aggressive *P.* x *alni* is likely replacing or has already replaced *P. uniformis* at least in the sampling sites.

P. x *multiformis* was found in neither of the two countries during the present work. Brasier et al. (2004) describes *P.* x *multiformis* as species that is exclusively present in Central and Western Europe. Although absence of this species in Lithuania should not therefore be surprising, it may also be explained by utilization of locally produced alder planting material for establishment of forest plantations (i.e., there is no seedling import from Central or Western Europe). There could be several reasons why *P.* x *multiformis* has not been isolated in Switzerland: the pathogen could be present but was overlooked, or it could already be replaced by the more aggressive species - *P.* x *alni.*

4.1.3. Population genetics of Phytophthora x alni and P. uniformis in seven European countries

4.1.3.1. Genotypic richness and evenness of recovered *Phytophthora* x *alni* isolates

The present study revealed rather low genotypic diversity of the investigated *P.* x *alni* populations, with one MLG (Pxa-1) dominant across the five countries, where this species has been isolated. Similar results were obtained by Aguayo *et al.* (2016), where out of 26 MLGs, Pxa-1 included 80 % of the isolates; moreover, the authors pointed out a decline of genotypic diversity (clonal richness) in *P.* x *alni* populations in the course of time. The dominance of one MLG in a metapopulation (wide geographic range) may be related to evolutionary novelty, where the main factor for genotype selection is adaptation to environment (Ellstrand & Schierenbeck, 2000). MLG Pxa-1 could therefore have the highest plasticity to adjust to different environmental conditions across

Europe. Another reason for domination of one genotype across large territory could be the reduced *P.* x *alni* fertility as it has been revealed in some populations of swamp loosestrife (*Decodon verticillatus* (L.) Elliot) (Dorken *et al.*, 2004).

Among the five European countries, Czech Republic had the highest genotypic diversity in local population of *P.* x *alni* (21 MLGs). The reason behind this high diversity could be generally earlier sampling years (compared to other countries) and the gradual increase in abundance of MLG Pxa-1 across Europe, as it has already been hypothesized by Aguayo *et al.* (2016). The genotyping was done using Czech *P.* x *alni* isolates collected between years 2006–2014 (although the majority of isolates originated from period 2006–2009). All isolates collected between years 2010–2014 belong to MLG Pxa-1 what further supports the hypothesis of decreasing genotypic (clonal) richness of *P.* x *alni* populations and increasing domination of one genotype in Europe.

In this study, total *P.* x *alni* genotypic evenness was low, but somewhat higher compared to genotypic evenness observed in Aguayo *et al.* (2016) work. Austria, Spain and Sweden had the most evenly distributed MLGs in this work but it's difficult to indicate the variables under this distribution. It could appear that similar *P.* x *alni* collection year is an important indicator, as it can be observed in Swedish and Czech Republic *P.* x *alni* populations.

The spatial structure among the investigated *P.* x *alni* populations was observed only in Sweden. The distribution of Swedish MLGs (PCA, Fig. 22) coincides with locations of the main watersheds in Sweden (Fig. 21). This is not surprising since for longer distances, *P. alni* s. l. mainly spreads by zoospores through waterways (Jung & Blaschke, 2004). Other ways of pathogen spreading are also known - for example, *P. alni* s. l. can be transferred to new territories by import of infected planting stock (Jung & Blaschke, 2004), human activity (e.g., hiking, biking) (Jung *et al.*, 2016), or cattle movement (Redondo *et al.*, 2015), yet the results of the present work do not allow further discussion of importance of the above-presented pathways in the investigated countries. Nevertheless, the obtained results provide some opportunity to learn possible

spreading ways of the identified MLGs of *P.* x *alni*. For example, Aguayo *et al.* (2016) described two MLGs of *P.* x *alni* (Pxa-3 and Pxa-15) that have been found exceptionaly in Germany. During the present study both were detected in Southern Sweden, and Pxa-3 was rather common there. This suggests that two above-mentioned MLGs could have been introduced to Sweden from e.g. Central Europe by infected planting stock. Furthermore, results of the present work allow monitoring of the pathogen evolution; for example, they will complement investigations aiming to reveal whether dominating MLG Pxa-1 will replace other MLGs of *P.* x *alni* across Europe.

4.1.3.2. Occurrence of rare *Phytophthora* x *alni* genotypes

The present study helped to identify a large number of new (previously undescribed) and rare genotypes (MLGs) of *P. x alni*. The new MLGs were found in all investigated countries except Switzerland, and only one genotype (Pxa-28) was found in two countries – Czech Republic and Hungary. The random allele scattering (with no layering and clustering) (Dobrowolski *et al.*, 2003) led to a suggestion that all new *P. x alni* multilocus genotypes have formed separately from each other.

The development of new MLGs was due to allelic loss. There are several reports of new genotype formation due to loss of heterozygosity in genus *Phytophthora*: it was observed in *P.* x *alni* (Aguayo *et al.*, 2016), *P. cinnamomi* (Dobrowolski *et al.*, 2003) and *P. ramorum* (Prospero *et al.*, 2007, 2013). Alleles were lost from both (*P.* x *multiformis* and *P. uniformis*) or one parental subgenome. More genotypes were formed after allelic loss from a subgenome of *P.* x *multiformis*. There is a possibility that alleles from *P.* x *multiformis* subgenome are more responsible for the loss of heterozygosity compared to alleles assigned to *P. uniformis* subgenome. This could happen because duplicated regions are more susceptible to the allelic loss (Garsmeur *et al.*, 2014).

The only way for clonal organism to accumulate genetic variation is through somatic mutation (James & McDougall, 2014). Dobrowolski *et al.* (2003) suggested that the changes (loss of heterozigosity) creating new genotypes are the result of mitotic recombination and/or mitotic crossig over. Population fragmentation is another important factor in formation of new genotypes (Young *et al.*, 1996). It has been demonstrated that population size and availability (gene flow) play a major role in formation of rare genotypes. If a population remains small and isolated for several generations, it starts to lose alleles due to a genetic drift (Young *et al.*, 1996), and new MLGs may develop from one or several founder genotypes as an event of local evolution (Vercauteren *et al.*, 2010). *P.* x *alni* is more aggressive alder pathogen compared to its progenitors (*P.* x *multiformis* and *P. uniformis*) (Brasier *et al.*, 2004b), and its pathogenicity could be responsible for fragmentation of host trees and therefore for isolation of the pathogen itself.

4.1.3.3. Population structure of *Phytophthora* x *alni* in Czech Republic, Hungary and Sweden

Analysis of Molecular Variance (AMOVA) showed absence of genetic structure in *P.* x *alni* populations in three investigated countries (Czech Republic, Hungary and Sweden) with a high insignificance rate in clone-corrected data across all geographic variables (countries, watersheds and basins). Thus, no geographical pattern could be found. All populations of *P.* x *alni* in Czech Republic, Hungary and Sweden are not differentiated from each other. Aguayo *et al.* (2016) received similar results, with no clearly observed geographical pattern. It should be pointed out though that this absence of the genetic (and geographical) structure could be due to different numbers of individuals present in each tested variable.

Interestingly, Principal Component Analysis showed that Swedish *P.* x *alni* population (unlike other countries populations) exhibited a certain clustering

pattern of East and West. There is a possibility that in the near future this clustering will became more pronounced.

4.1.3.4 Population genetics of *Phytophthora uniformis*

Among the investigated countries, the highest genotypic diversity of *P. uniformis* population was found in Sweden, where two MLGs (Pu-E1 and Pu-E2) were identified, while in other four countries, where *P. uniformis* has been isolated, only one, dominant MLG (Pu-E1), was found. Prior to the present study, MLG Pu-E2 was found only three times in geographically separated sites in France, Italy and Sweden (Aguayo *et al.*, 2013). *P. uniformis* isolates used in study by Aguayo *et al.* (2013) were collected during 1997-2000 year period, while isolates used in the present study were collected in 2013. In this work, Pu-E2 MLG included 31.35 % of all Swedish *P. uniformis* isolates and shows successful Pu-E2 spreading in Sweden.

No new (previously unpublished) MLGs of *P. uniformis* were found in the present study.

4.2. Dutch elm disease

4.2.1. Occurrence of DED agents and their intraspecific hybrids in Lithuania

No isolates of *O. ulmi* s. s. have been found during the present study. All ophiostomoid fungi isolated from wood of symptomatic elm trees were identified as *O. novo-ulmi*. Before the present work, no information was available on distribution of *O. ulmi* s. s. or *O. novo-ulmi* in Lithuania. In 1951-1957, Žuklys (1957) has carried out a study on distribution, biological and ecological properties of *O. ulmi* s. l. in Lithuania, yet it is unknown which of the two species or both were investigated at that time. It is possible that *O. novo-ulmi* has already been introduced to the territory of Lithuania in early 1960-ies, and that Žuklys (1957) has investigated both pathogens together as one species.

Based on scientific data, *O. ulmi* s. s. and *O. novo-ulmi* exhibit antagonistic interactions with each other. *O. ulmi* s. s. is considered to be less adaptive species and it is being replaced by the more aggressive *O. novo-ulmi* (Mitchell, 1988). The replacement of *O. ulmi* s. s. by *O. novo-ulmi* was observed in Europe (Gremmen *et al.*, 1976; Brasier, 1983; Hoegger *et al.*, 1996; Dvorák *et al.*, 2007) and North America (Brasier, 1996b). It is possible that *O. ulmi* s. s. has been replaced by *O. novo-ulmi* also in Lithuania, but due to the lack of data this statement remains only hypothetical.

During the present study, *O. novo-ulmi* subspecies EAN was more frequently isolated compared to EAN x NAN hybrid, and subspecies NAN was not found at all. According to Brasier & Kirk (2001, 2010), subspecies NAN was not found (at least before 2010) in countries that have a common border with Lithuania – Poland and Kaliningrad Oblast (Russia). Germany and Sweden are the closest countries in which subspecies NAN has been isolated so far (Brasier & Kirk, 2001, 2010).

In North-Western Poland (Szczecin), EAN x NAN hybrids were detected already in 1980s. This part of Poland was considered to be a possible hybridization border between the two pathogen subspecies (Brasier & Kirk, 2010). This leads to a hypothesis that EAN x NAN hybrids were introduced to Lithuania from North-Eastern Poland. This hypothesis is further supported by the fact that no EAN x NAN hybrids were found in Northern Lithuania. If the hybrid pathogen is slowly spreading from South-Western Europe towards North, as hypothesized by Brasier & Buck (2001), there is a possibility that EAN x NAN is still not common in Northern Lithuania. The location of detection sites of *O. novo-ulmi* also confirms this presumption: in South-Eastern Lithuania (Vilnius district and city) only EAN x NAN hybrids were found.

Because subspecies NAN has not been detected during the present work, it is possible that this subspecies is not common in Lithuania (or has not yet entered Lithuania at all), or that it has already been replaced by EAN x NAN hybrids. Scientific reports indicate that intraspecific hybrids of *O. novo-ulmi* are able to replace the original subspecies of *O. novo-ulmi*. For example, a

comprehensive long-term study of *O. novo-ulmi* population in Limburg (The Netherlands) showed a successful replacement of *O. novo-ulmi* subspecies by EAN x NAN hybrids (Brasier & Kirk, 2010): the observed *O. novo-ulmi* population in 1980s consisted of ~70 % EAN x NAN hybrids, of ~17 % of EAN isolates, and of ~13 % of NAN isolates. After three years, the isolation works were carried out repeatedly and the changed population structure was found: *O. novo-ulmi* population consisted of ~79 % of EAN x NAN hybrids, while isolates of subspecies EAN and NAN together made ~22 % of the investigated population (Brasier & Kirk, 2010).

4.2.2. Population structure of Ophiostoma novo-ulmi in Lithuania

Hybrid isolates of *O. novo-ulmi* originating from Vilnius district showed strong antagonistic reactions against other *O. novo-ulmi* isolates in vegetative compatibility tests, which may indicate possibly higher aggressiveness and competitiveness of EAN x NAN hybrids. Populations of *O. novo-ulmi* in middle (Aristava population, Kėdainiai district) and Western (Kuliai population, Plungė district) Lithuania were mixed – both subspecies EAN and EAN x NAN hybrids were found. The recent expansion of EAN x NAN hybrid and hypothesis of EAN replacement can not be excluded in these populations.

Vegetative compatibility tests showed that Lithuanian *O. novo-ulmi* populations are composed of a large number of small vegetative compatibility groups (VCGs), that shows high genotypic diversity and high level of recombination. High genotypic diversity of *O. novo-ulmi* populations have also been reported previously (Mitchell & Brasier, 1994; Santini *et al.*, 2005). Caten, (1972) and Brasier (1986) pointed out that high diversity of genets could be linked to d-factors (mycoviruses) of *O. novo-ulmi* that may be responsible for high mutation rate in the host fungus. The authors suggest that high genetic diversity acts as effective protection against harmful mycoviruses.

During the present study, the same genotype was isolated from trees that grew separated by relatively large distance (3 km, see Fig. 25E). This may

explained by effective asexual reproduction and way of spreading with the aid of insect vectors: genetically identical spores are transmitted by insects to relatively large distances (Žuklys, 1957; Heimann *et al.*, 1997). Fungus is also spreading via root grafts of diseased elm trees (Heimann *et al.*, 1997) and this could contribute to formation of territorially small VCGs.

4.3. Ash dieback

4.3.1. Genetic characterization of five Lithuanian populations of Hymenoscyphus fraxineus

Lithuanian populations of *H. fraxineus* are usually called post-epidemic, as currently ash dieback disease is in its chronic phase (Burokienė *et al.*, 2015; Pliūra *et al.*, 2017). Invasive pathogens that are in post-epidemic stage can be characterized by high genetic diversity as it was demonstrated in Dutch elm disease pathogen *Ophiostoma novo-ulmi* studies (Santini *et al.*, 2005; Solla *et al.*, 2008). However, in contrary to *O. novo-ulmi*, microsatellite analysis of the herein investigated Lithuanian *H. fraxineus* populations revealed low genetic diversity but high genotypic diversity. *H. fraxineus* is a relatively "young" species (*O. novo-ulmi* is known for more than 70 years in Europe (Brasier *et al.*, 2004a)) and it may be that it was not enough time for the pathogen to generate genetic diversity. Moreover, the calculated values of Standardized Index of Association (rbarD) showed free sexual recombination with no observed clonality across all investigated populations. As a result, this generates new multilocus genotypes (new allele combinations, but not new alleles, that is necessary for genetic variation) (Sommerhalder *et al.*, 2010) and could explain high *H. fraxineus* genotypic diversity found in this work.

No genetic differentiation was detected between *H. fraxineus* isolates recovered from ash bark lesions (asexual life stage) and from fallen leaf petioles (sexual life stage). These results may indicate that pathogen has no inclination for one life stage over another. This suggestion can be supported by increased

knowledge of benefits for fungus to keep both reproductions (sexual and asexual) in fungal life cycle (Taylor *et al.*, 2015).

The present study showed comparably low genetic diversity in Lithuanian *H. fraxineus* populations. Similar results of low genetic diversity were obtained also in other genetic studies of the ash dieback pathogen (Bengtsson *et al.*, 2012; Gross *et al.*, 2014a; Hañáčková *et al.*, 2015; Cleary *et al.*, 2016; Orton *et al.*, 2018).

A total of 49 common MLGs were detected among *H. fraxineus* isolates recovered from both stages of pathogen life cycle (sexual – ascocarp formation on fallen leaf petioles and asexual – in bark lesions). This leads to an assumption that at least some *H. fraxineus* genotypes are able to live both as necrotrophs and saprotrophs, and to successfully spread asexually. This assumption contradicts suggestion by Gross *et al.* (2014a) that ash dieback pathogen is reproducing almost exclusively in a sexual way. Fones *et al.* (2016) demonstrated that asexual spores of *H. fraxineus* are capable to germinate on *F. excelsior* leaves, indicating that infection by asexually transmitted genotypes is likely. Also this could mean that some genotypes of the ash dieback pathogen start to show first signs of adaptation to their local environment (Lenormand, 2002), claiming to become dominant over other genotypes.

H. fraxineus mutation rate was low in the present study with only three new alleles described. Wierdl *et al.* (1997) demonstrated that mutation rate in SSRs is dependent on the number of repeat unit. Usually microsatellite loci with high number of repeats are more prone to mutations (Dutech *et al.*, 2007). In this study microsatellite loci with low repeat number (2-4 repeat per locus) were used and this may be the reason why low mutation rate was observed. Moreover, it was observed that rarely occurring mutations are far less beneficial to organisms (Peabody *et al.*, 2017), it may be that *H. fraxineus* also has this trait.

No genetic structure (presence of genetic clusters) was found in Lithuanian *H. fraxineus* populations by Discriminant Analysis of Principal Components (DAPC). Interestingly, genetic distance (i.e., pairwise difference of fixation indices, F_{ST}) between lesion and petiole isolates within Vilnius

population was several-fold larger than distance between any distant populations (Table 12) and this fact is hard to explain. The observed absence of genetic population structure and high genotypic diversity suggest that *H. fraxineus* population in Lithuania is extremely variable.

4.3.2. Comparison of results from two experiments of ash seedling inoculation with the same set of Hymenoscyphus fraxineus isolates

A significant positive correlation was found in proximal lesion length on seedlings between experiments Seedlings 1 and Seedlings 2 caused by the same set of the most and the least virulent *H. fraxineus* isolates, indicating that repeated inoculation experiments may truly give reproducible results. On the other hand, the correlation was only moderately strong, thus far from linear. As no similar studies are up to date available, it was not possible to compare results obtained during the present work with results obtained in other studies.

The mean length of proximal lesions in experiment Seedlings 1 was significantly higher than in experiment Seedlings 2; moreover, in experiment Seedlings 1, bark lesions of variable size developed on significantly smaller proportion of inoculated seedlings compared to experiment Seedlings 2. These differences could occur due to many reasons. First, each experiment has been initiated in different time (experiment Seedlings 1 – in September 2013 (Lygis *et al.*, 2017) and experiment Seedlings 2 – in July 2015) that could possibly have a certain impact on experimental success. Second, as suggested by Landolt *et al.* (2016), there is a possibility that weather conditions during the initial phase of experiment Seedlings 1 were more favourable for the pathogen establishment (infection phase) and further development compared to conditions during the experiment Seedlings 2. Third, the observed differences in both experiments could have occur due to genetic differences in inoculated seedlings: although all seedlings used for both experiments were obtained from the same nursery (represented the same population of *F. excelsior*), they were not genetically identical. Therefore, theoretically it is possible that seedlings used in experiment

Seedlings 2 may have been more tolerant to *H. fraxineus* than ones used in experiment Seedlings 1. Differences in resistance (tolerance) to ash dieback among *F. excelsior* individuals of Lithuanian origin have been noted by Pliūra *et al.* (2011). Differences in communities of endophytic fungi antagonistic to *H. fraxineus* in seedlings used in the two experiments could also have some impact (Haňáčková *et al.*, 2017).

In experiment Seedlings 2, no significant differences were found either between seedlings inoculated with Swiss and Lithuanian isolates, or between seedlings inoculated with lesion and petiole isolates. This result is in agreement with the results of Lygis *et al.* (2017), where no significant differences were found between *H. fraxineus* isolates originating from geographically different populations and representing two different stages of the pathogen life cycle (necrotrophic stage – bark lesions) and saprotrophic phase (fallen leaf petioles). *H. fraxineus* shows not only high morphological (Kowalski & Bartnik, 2012) and molecular diversities (Gross *et al.*, 2014b; Burokienė *et al.*, 2015; Orton *et al.*, 2018) but also by diversity in secondary metabolites secretion, as it was demonstrated by Junker *et al.* (2014) and Junker *et al.* (2017). All this may have influence in high diversity in *H. fraxineus* virulence with no obvious driving force in pathosystem.

Virulence between *H. fraxineus* isolates representing recently established (Swiss) populations and old-established (Lithuanian) populations did not differ significantly in either experiment. This confirms our assumption (Lygis *et al.*, 2017) that despite large geographical distance and different time of establishment, Swiss and Lithuanian (and, most likely, all European) populations of *H. fraxineus* are similar in many aspects (little genetic differentiation; rather short history of ash dieback in Europe for significant mutations to occur, and strong gene flow). Decline in virulence of populations of invasive organisms is generally assumed with increasing age of epidemics. This, however, does not appear to hold true for *H. fraxineus.*

The vast majority of *H. fraxineus* isolates have a strong necrotrophic potential as a bark pathogen on common ash regardless of whether they are

obtained from petioles or lesions. This result rejects hypothesis of niche differentiation among *H. fraxineus* isolates. This result again coincide with Fones *et al.* (2016) finding indicating that infection by asexually transmitted spores is possibly occuring.

All these findings encourage to continue detail *H. fraxineus* research combining different research methods.

4.3.3. Comparison of results of two inoculation experiments (Seedlings 1 & 2) and growth speed of Hymenoscyphus fraxineus in culture

No significant differences in mean radial growth speed of *H. fraxineus* cultures were found between Swiss and Lithuanian isolates, while growth speed of "lesion" and "petiole" isolates differed significantly.

Radial growth speed in culture did not correlate with length of bark lesions produced by respective *H. fraxineus* isolates in either inoculation experiment (Seedlings 1 vs. Seedlings 2). No significant difference in mean radial growth speed in culture was found between the pre-selected most virulent and least virulent *H. fraxineus* isolates as well. These results are in contrast with the results of studies performed with some other fungal tree pathogens as, for example, *O. novo-ulmi* s. l. (Brasier & Webber, 1987), *Ceratocystis polonica* (Siem.) C. Moreau (Krokene & Solheim, 2001) and *Leptographium wingfieldii* Morelet (Lieutier *et al.*, 2004), where a significant positive correlation between radial growth rate of fungal cultures and pathogen virulence was found. This contradiction is difficult to explain, although our results suggest that *H. fraxineus* pathosystem is probably independent from its mycelial growth rate and coincide with results obtained by (Junker *et al.*, 2017).

CONCLUSIONS

- In Lithuania, two species of *Phytophthora alni* species complex have been found – *P.* x *alni* and *P. uniformis*, while in Switzerland only *P.* x *alni* has been detected; the third species in complex – *P.* x *multiformis*, has not been detected;
- European populations of *P.* x *alni* showed relatively high genotypic diversity. In total, 36 MLGs have been identified, of which 24 MLGs were identified for the first time in Europe, while genotypic diversity of the investigated *P. uniformis* populations was very low (only two MLGs were identified), both *P.* x *alni* and *P. uniformis* have one dominant genotype across Europe.
- RFLP allowed identification of subspecies and intraspecific hybrids of *Ophiostoma novo-ulmi* (*O. ulmi* s. s. was not found; seemingly it has already been replaced by more pathogenic *O. novo-ulmi*): a total of 36 isolates were identified as *O. novo-ulmi* ssp. *novo-ulmi* and 13 isolates - as *O. novo-ulmi* ssp. *americana* x *O. novo-ulmi* ssp. *novo-ulmi* hybrids Lithuanian populations of *H. fraxineus* are characterized by high genotypic diversity but low genetic diversity: all identified MLGs from all sampled populations fell into one genetic cluster, i.e. no spatial structure was observed;
- Lithuanian population of *O. novo-ulmi* consists of a large number of small vegetative compatibility groups, indicating high genotypic diversity and high probability of the intraspecific recombination within populations;
- Lithuanian populations of *Hymenoscyphus fraxineus* are characterized by high genotypic diversity but low genetic diversity: all identified MLGs from all sampled populations fell into one genetic cluster, i.e. no spatial structure was observed;

- A significant correlation was observed in performance (virulence) of the same set of *H. fraxineus* isolates used in two *F. excelsior* inoculation experiments, indicating that ash seedling inoculation experiments provide reliable results of *H. fraxineus* virulence assessment (ranking), and that results of the repeated experiments are reproducible;
- Virulence of *H. fraxineus* isolates does not correlate with the mycelium growth rate of the respective isolates in culture, indicating that *H. fraxineus* pathosystem is independent from its mycelial growth rate.

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LIST OF PUBLICATIONS

Publications in journals with impact factor included into *Clarivate Analytics Web of Science* database:

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3. **Norkutė G**, Lygis V, Prospero S, 2015. *Phytophthora* diversity in declining alder stands in Lithuania and Switzerland, - Joint IUFRO

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APPENDICES

APPENDIX 1 Oomycetes and fungi isolated during the study

Species	**Sites: Lithuania – LT; Switzerland – CH**	**No. of isolates recovered in Lithuania**	**No. of isolates recovered in Switzerland**	**No. of recovered isolates – Wood**	**No of recovered isolates - Water**	**No. of recovered isolates – Soil**	**No. of recovered isolates – Total**	**Clade No. (only for *Phyto-phthora)***
1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***
Oomycota								
Phytophthora x *alni*	5LT; 26LT; 2CH; 10CH	9	8	16	-	1	17	7
Phytophthora chlamydospora	6CH	-	4	-	2	2	4	6
Phytophthora citrophthora-like	6CH	-	3	-	3	-	3	-
Phytophthora cryptogea	12CH	-	1	-	-	1	1	8
Phytophthora gallica	1LT; 14LT; 6CH; 9CH	3	5	-	4	4	8	10
Phytophthora gonapodyides	1LT; 4LT; 17LT; 1CH; 5CH; 6CH; 9CH; 11CH; 12CH	34	18	-	36	16	52	6
Phytophthora gregata	15LT; 8CH; 11CH	4	1	-	-	5	5	6
Phytophthora hydropathica	13CH	-	7	-	-	7	7	9
Phytophthora lacustris	1LT; 4LT; 14LT; 17LT; 1CH; 3CH; 4CH; 5CH; 7CH; 8CH; 9CH; 10CH; 11CH; 12CH; 13CH	62	85	4	92	51	147	6

APPENDIX 1 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Oomycota								
Phytophthora niederhauserii	2CH	-	2	-	-	2	2	7
Phytophthora plurivora	2CH; 6CH; 7CH; 9CH; 10CH; 11CH; 12CH; 13CH; 14CH	-	142	-	23	119	142	2
Phytophthora pseudosyringae	9CH	-	1	-	-	1	1	3
Phytophthora taxon Oaksoil	17LT; 6CH	6	4	-	6	4	10	6
Phytophthora uniformis	5LT; 16LT	17	-	17	-	-	17	7
Phytopythium chamaehyphon	10CH	-	3	-	1	2	3	-
Phytopythium citrinum	2CH; 9CH; 10CH; 11CH; 12CH	-	19	-	-	19	19	-
Phytopythium litorale	15LT; 2CH; 3CH; 6CH; 7CH; 9CH; 10CH; 11CH; 13CH	8	34	-	2	40	42	-
Phytopythium montanum	4LT; 10CH	1	1	-	1	1	2	-
Phytopythium vexans	2CH; 6CH; 10CH	-	6	-	-	6	6	-
Phytopythium sp.	3CH; 6CH; 9CH; 10CH; 11CH; 12CH	-	30	-	2	28	30	-
Pythium aquatile	1CH; 2CH	-	4	-	-	4	4	-

APPENDIX 1 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Oomycota								
Pythium glomeratum	2CH	-	2	-	-	2	2	-
Pythium intermedium	24LT; 25LT; 27LT;2CH	11	2	-	-	13	13	-
Pythium lutarium	2CH	-	1	-	-	1	1	-
Pythium sp.	1LT; 4LT; 17LT; 18LT; 2CH; 4CH; 5CH; 9CH; 11CH	16	10	-	18	8	26	-
Ascomycota								
Clonostachys rosea	4LT	1	-	1	-	-	1	-
Diaporte eres	26LT	1	-	1	-	-	1	-
Fusarium lateritium	5LT; 26LT	3	-	3	-	-	3	-
Paraphaeosphaeria neglecta	2CH	-	1	-	-	1	1	-
Trichoderma hamatum	26LT	2	-	1	-	1	2	-
Alternaria sp.	9CH	-	1	1	-	-	1	-
Clonostachys sp.	5LT	1	-	1	-	-	1	-
Elaphocordyceps sp.	15LT	3	-	3	-	-	3	-
Basidiomycota								
Chondrostereum purpureum	5LT	5	-	5	-	-	5	-

APPENDIX 1 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Basidiomycota								
Coprinelullus micaceus	5LT; 10CH; 12CH	2	2	4	-	-	4	-
Coprinellus xanthotrix	9CH	-	1	1	-	-	1	-
Peniophora cinerea	5LT	1	-	1	-	-	1	-
Phlebia radiata	26LT	2	-	2	-	-	2	-
Psathyrella candolleana	3CH	-	1	1	-	-	1	-
Thelephora terrestis	5LT	1	-	1	-	-	1	-
Coprinellus sp.	9CH	-	3	1	2	-	3	-
Heterobasidion sp.	12CH	-	1	1	-	-	1	-
Sistotrema sp.	15LT	3	-	3	-	-	3	-
Steccherinum sp	9CH	-	1	1	-	-	1	-
Zygomycota								
Mortierella alpina	4LT; 21LT; 24LT; 26LT	8	-	-	-	8	8	-
Mortierella elongata	23LT; 25LT; 26LT	3	-	-	-	3	3	-
Mortierella gamsii	20LT; 21LT; 22LT; 24LT	20	-	2	-	18	20	-
Mortierella horticola	25LT; 26LT	6	-	-	-	6	6	-
Mortierella minutissima	22LT; 23LT; 24LT; 25LT; 27LT	12	-	-	-	12	12	-

APPENDIX 1 (continuation)

Zygomycota								
1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***
Mortierella parvispora	1LT	3	-	3	-	-	3	-
Mortierella zonata	22LT	5	-	-	-	5	5	-
Mucor hiemalis	4LT, 10CH	1	1	-	-	2	2	-
Mortierella sp.	4LT; 22LT; 23LT; 24LT; 26LT	12	-	3	-	9	12	-
Mucor sp.	9CH	-	1	1	-	-	1	-
Rhizomucor sp	9CH	-	1	1	-	-	1	-
Umbelopsis sp.	9CH	-	1	1	-	-	1	-
All species		266	408	80	192	402	674	-

APPENDIX 2 *Phytophthora alni* species complex microsatellites scores and supplementary data

Sample	Species [A]	Country [B]	Isolation Year	Population	Supplier	MLG	M- PAU 11	M-PAU 14	M-PAU 15	M-PAU 3	M-PAU 32	M-PAU 56	M-PAU 72	M-PAU 9	PA 17	PA 23	Original code
1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***	***13***	***14***	***15***	***16***	***17***	***18***
APA2	*Pxa*	AU	2014	Austria	Corcobado, T.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/ 174/ 177	100/ 109	335	157/ 171	A2
APA3	*Pxa*	AU	2014	Austria	Corcobado, T.	Pxa-49	173/ 209	84/89/ 99	92/95	127	113	91/98	162/174	100/ 109	335	157/ 171	A20
APA5	*Pxa*	AU	2014	Austria	Corcobado, T.	Pxa-49	173/ 209	84/89/ 99	92/95	127	113	91/98	162/i74	100/ 109	335	157/ 171	A22
APA6	*Pxa*	AU	2014	Austria	Corcobado, T.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	A23
APA8	*Pxa*	AU	2014	Austria	Corcobado, T.	Pxa-50	209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174	100/ 109	335	157/ 171	A31a
APA 10	*Pxa*	AU	2014	Austria	Corcobado, T.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	A33
APA 11	*Pxa*	AU	2014	Austria	Corcobado, T.	Pxa-8	173/ 209	84/89 /99	92/95	127	107/ 113	91/98	162/174	100/ 109	335	157/ 171	A36
APA 14	*Pxa*	AU	> 2007	Austria	Corcobado, T.	Pxa-19	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174	100/ 109	335	157/ 171	A-T2
APA 15	*Pxa*	AU	> 2014	Austria	Corcobado, T.	Pxa-19	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174	100/ 109	335	157/ 171	A-T3
CZPA 2	*Pxa*	CZ	2006	DSS	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 006.06
CZPA 3	*Pxa*	CZ	2006	DSS	Černy, K. and Strnadová, V.	Pxa-19	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174	100/ 109	335	157/ 171	P 012.06

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
CZ PA4	*Pxa*	CZ	2006	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 024.06
CZPA 5	*Pxa*	CZ	2006	Vitava	Černy, K. and Strnadová, V.	Pxa-33	173/ 209	84/89/ 99	92/95	127	107	91/98	162/174	100/ 109	335	157/ 171	P 028.06
CZPA 6	*Pxa*	CZ	2007	Plouc-nice	Černy, K. and Strnadová, V.	Pxa-28	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157	P 039.07
CZPA 7	*Pxa*	CZ	2007	Ber-ounka	Vait	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 042.07
CZPA 8	*Pxa*	CZ	2007	Elbe	Černy, K. and Strnadová, V.	Pxa-34	209	84/89	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 044.07
CZPA 9	*Pxa*	CZ	2007	Sazava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 047.07
CZPA 10	*Pxa*	CZ	2007	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 050.07
CZPA 11	*Pxa*	CZ	2007	Plouc-nice	Černy, K. and Strnadová, V.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 052.07
CZPA 12	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-24	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100	335	157/ 171	P 060.07
CZPA 13	*Pxa*	CZ	2007	Mora-va	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 063.07
CZPA 14	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 131.07
CZPA 15	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 133.07
CZPA 16	*Pxa*	CZ	2007	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 135.07
CZPA 17	*Pxa*	CZ	2007	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 137.07
CZPA 18	*Pxa*	CZ	2007	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 141.07

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
CZPA 20	*Pxa*	CZ	2007	DSS	Černy, K. and Strnadová, V.	Pxa-35	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	171	P 145.07
CZPA 21	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 169.07
CZPA 22	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 171.07
CZPA 23	*Pxa*	CZ	2007	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 193.07
CZPA 24	*Pxa*	CZ	2007	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 197.07
CZPA 25	*Pxa*	CZ	2008	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 210.08
CZPA 28	*Pxa*	CZ	2008	Vitava	Černy, K. and Strnadová, V.	Pxa-36	173/ 209	84/89/ 99	92/95	127	113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 223.08
CZPA 29	*Pxa*	CZ	2008	Ohre	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 227.08
CZPA 31	*Pxa*	CZ	2008	Ohre	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 230.08
CZPA 33	*Pxa*	CZ	2009	DSS	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 298.09
CZPA 38	*Pxa*	CZ	2007	Sazava	Černy, K. and Strnadová, V.	Pxa-37	173/ 209	84/89/ 99	95	127	107	91/98	162/174	100/ 109	335	157/ 171	P061.07
CZPA 39	*Pxa*	CZ	2007	Beroun ka	Černy, K. and Strnadová, V.	Pxa-38	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162	100/ 109	335	157/ 171	P136.07
CZPA 43	*Pxa*	CZ	2007	Elbe	Černy, K. and Strnadová, V.	Pxa-39	173/ 209	84/89/ 99	95	127	107/ 113	91/98	162/174	100/ 109	335	157/ 171	P199.07
CZPA 44	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P206.08

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
CZPA 47	*Pxa*	CZ	2008	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P226.08
CZPA 49	*Pxa*	CZ	2010	Sazava	Pánek	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P377.10
CZPA 50	*Pxa*	CZ	2007	Morava	Černy, K. and Strnadová, V.	Pxa-40	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/177	100	335	157/ 171	P 080.07
CZPA 51	*Pxa*	CZ	2006	DSS	Černy, K. and Strnadová, V.	Pxa-41	173	84/89/ 99	92/95	127	107/ 113	91/94	162	100/ 109	335	157/ 171	P 011.06
CZPA 52	*Pxa*	CZ	2011	Morava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 506.11
CZPA 54	*Pxa*	CZ	2014	Elbe	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 684.14
CZPA 55	*Pxa*	CZ	2007	Elbe	Černy, K. and Strnadová, V.	Pxa-42	173/ 209	84/89/ 99	95	127	107/ 113	91/94	162/174/ 177	100/ 109	335	157/ 171	P 106.07
CZPA 56	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 051.07
CZPA 57	*Pxa*	CZ	2007	Ohre	Černy, K. and Strnadová, V.	Pxa-43	173/ 209	84/89/ 99	95	127	107/ 113	91/94/ 98	162	100/ 109	335	157/ 171	P 176.07
CZPA 58	*Pxa*	CZ	2007	Ohre	Černy, K. and Strnadová, V.	Pxa-44	173/ 209	84/89/ 99	95	127	107/ 113	91/94/ 98	162/177	100/ 109	335	157/ 171	P 129.07
CZPA 59	*Pxa*	CZ	2009	Sazava	Černy, K. and Strnadová, V.	Pxa-21	173	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 279.09
CZPA 60	*Pxa*	CZ	2007	Sazava	Černy, K. and Strnadová, V.	Pxa-19	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174	100/ 109	335	157/ 171	P 177.07
CZPA 61	*Pxa*	CZ	2007	Plouc-nice	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P 053.07
CZPA 62	*Pxa*	CZ	2007	DSS	Černy, K. and Strnadová, V.	Pxa-43	173/ 209	84/89/ 99	95	127	107/ 113	91/94/ 98	162	100/ 109	335	157/ 171	P59/07

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
LC1	*Pxa*	CZ	2007	Be-rounka	Černy, K. and Strnadová, V.	Pxa-45	173/ 209	89	95	127	113	91	162	109	335	157/ 171	239/07
LC3	*Pxa*	CZ	2006	Vitava	Černy, K. and Strnadová, V.	Pxa-46	173	84/89/ 99	92/95	127	107	91/98	162/174	100/ 109	335	157/ 171	P018/06
LC4	*Pxa*	CZ	2007	Vitava	Černy, K. and Strnadová, V.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	193/07
HPA3	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-7	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94	162/177	100/ 109	335	157/ 171	P323
HPA4	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P327
HPA5	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P333
HPA6	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P337
HPA7	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P339
HPA8	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P416
HPA9	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-27	173/ 209	84/89/ 99	95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P417
HPA 10	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P419
HPA 11	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-2	173/ 209	84/89/ 99	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P422
HPA 12	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-2	173/ 209	84/89/ 99	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P423
HPA 14	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-17	173/ 209	84/89/ 99	92/95	133	113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P430

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
HPA 15	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P431
HPA 16	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P434
HPA 17	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P437
HPA 18	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P439
HPA 19	*Pxa*	HU	2009	Rábca	Bakonyi, J.	Pxa-2	173/ 209	84/89/ 99	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P440
HPA 20	*Pxa*	HU	2004	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	H-88/04
HPA 21	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P394
HPA 22	*Pxa*	HU	2004	Zala	Bakonyi, J.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	H-89/04
HPA 23	*Pxa*	HU	2004	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	H-95/04
HPA 24	*Pxa*	HU	2004	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	H-97/04
HPA 25	*Pxa*	HU	2004	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	H-98/04
HPA 26	*Pxa*	HU	2001	Zala	Bakonyi, J.	Pxa-28	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157	Hévíz4/ 1
HPA 27	*Pxa*	HU	2001	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	Hévíz4/ 2
HPA 28	*Pxa*	HU	2001	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	Hévíz6

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
HPA 29	*Pxa*	HU	2001	Zala	Bakonyi, J.	Pxa-2	173/ 209	84/89/ 99	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	Hévíz8
HPA 30	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P409
HPA 31	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P411
HPA 32	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-27	173/ 209	84/89/ 99	95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P414
HPA 33	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P348
HPA 34	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P396
HPA 35	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P401
HPA 36	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P356
HPA 37	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P359
HPA 38	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P361
HPA 39	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P364
HPA 40	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P366
HPA 41	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P370
HPA 42	*Pxa*	HU	2009	Zala	Bakonyi, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	P375

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
LT-9	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-14.3.1
LT-10	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-14.3.2
LT-11	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-14.3.3
LT-12	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-14.3.4
LT-13	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-14.3.5
LT-24	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-43.2.1
LT-43	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-39.3.2
LT-48	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	BA-42.3.1
LT-54	*Pxa*	LT	2014	Lit-huania	Norkutė, G.	Pxa-47	173	84/89	95	127	107/113	91	162/174/177	100/109	335	157/171	BA-43.3.3
SPA5	*Pxa*	SP	NA	Spain	Solla, A.	Pxa-48	173/209	84/89/99	92/95	127	107/113	91	162/174	100/109	335	157/171	PFSPA2008
SPA6	*Pxa*	SP	NA	Spain	Solla, A.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	PFSPA2010
SPA7	*Pxa*	Spain	NA	Spain	Solla, A.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	PFSPA4017
SPA16	*Pxa*	SP	NA	Spain	Solla, A.	Pxa-6	173/209	84/89	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	PFSPA12082
SWPA1	*Pxa*	SW	2013	Mölndalsån	Redondo, M. and Oliva, J.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R2-8.1

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA 2	*Pxa*	SW	2013	Möln-dalsån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R2-8.2
SWPA 3	*Pxa*	SW	2013	Möln-dalsån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R2-10.1
SWPA 4	*Pxa*	SW	2013	Möln-dalsån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R2-10.2
SWPA 5	*Pxa*	SW	2013	Möln-dalsån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R2-11.1
SWPA 6	*Pxa*	SW	2013	Möln-dalsån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R2-11.2
SWPA 7	*Pxa*	SW	2013	Möln-dalsån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R2-11.3
SWPA 10	*Pxa*	SW	2013	Viskan	Redondo, M. and Oliva, J.	Pxa-29	173/ 209	84/89	92/95	133	107/ 113	91/98	162/174	100/ 109	335	157/ 171	R4-2.1
SWPA 12	*Pxa*	SW	2013	Viskan	Redondo, M. and Oliva, J.	Pxa-30	173/ 209	84/89/ 99	92/95	133	107/ 113	91/98	162/174	100/ 109	335	157/ 171	R4-12.2
SWPA 21	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/ 209	84/89/ 96	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R7-8.1
SWPA 22	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/ 209	84/89/ 96	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R7-9.1
SWPA 23	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/ 209	84/89/ 96	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R7-9.2
SWPA 24	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/ 209	84/89/ 96	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R7-9.3
SWPA 25	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/ 209	84/89/ 96	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R7-9.4
SWPA 26	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/ 209	84/89/ 96	92/95	133	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R7-9.5

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA27	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/209	84/89/96	92/95	133	107/113	91/94/98	162/174/177	100/109	335	157/171	R7-9.6
SWPA28	*Pxa*	SW	2013	Ronneå	Redondo, M. and Oliva, J.	Pxa-3	173/209	84/89/96	92/95	133	107/113	91/94/98	162/174/177	100/109	335	157/171	R7-9.7
SWPA30	*Pxa*	SW	2013	Käv-lingeå	Redondo, M. and Oliva, J.	Pxa-9	173/209	84/89/96	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R8-2.1
SWPA31	*Pxa*	SW	2013	Käv-lingeå	Redondo, M. and Oliva, J.	Pxa-9	173/209	84/89/96	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R8-2.2
SWPA32	*Pxa*	SW	2013	Käv-lingeå	Redondo, M. and Oliva, J.	Pxa-9	173/209	84/89/96	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R8-2.3
SWPA36	*Pxa*	SW	2013	Käv-lingeå	Redondo, M. and Oliva, J.	Pxa-2	173/209	84/89/99	92/95	133	107/113	91/94/98	162/174/177	100/109	335	157/171	R8-8B.1
SWPA37	*Pxa*	SW	2013	Käv-lingeå	Redondo, M. and Oliva, J.	Pxa-15	173/209	84/99	92/95	133	107/113	91/94/98	162/174/177	100/109	335	157/171	R8-8B.2
SWPA38	*Pxa*	SW	2013	Käv-ingeå	Redondo, M. and Oliva, J.	Pxa-2	173/209	84/89/99	92/95	133	107/113	91/94/98	162/174/177	100/109	335	157/171	R8-8B.3
SWPA39	*Pxa*	SW	2013	Käv-lingeå	Redondo, M. and Oliva, J.	Pxa-30	173/209	84/89/99	92/95	133	107/113	91/98	162/174	100/109	335	157/171	R8-8B.4
SWPA40	*Pxa*	SW	2013	Käv-lingeå	Redondo, M. and Oliva, J.	Pxa-2	173/209	84/89/99	92/95	133	107/113	91/94/98	162/174/177	100/109	335	157/171	R8-8B.5
SWPA45	*Pxa*	SW	2013	Helgeå	Redondo, M. and Oliva, J.	Pxa-9	173/209	84/89/96	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R9-8B.1
SWPA46	*Pxa*	SW	2013	Helgeå	Redondo, M. and Oliva, J.	Pxa-9	173/209	84/89/96	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R9-8B.2
SWPA47	*Pxa*	SW	2013	Helgeå	Redondo, M. and Oliva, J.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R9-12B.1
SWPA48	*Pxa*	SW	2013	Helgeå	Redondo, M. and Oliva, J.	Pxa-9	173/209	84/89/96	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	R9-12B.2

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA 49	*Pxa*	SW	2013	Helgeå	Redondo, M. and Oliva, J.	Pxa-9	173/ 209	84/89/ 96	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R9-12B.3
SWPA 50	*Pxa*	SW	2013	Helgeå	Redondo, M. and Oliva, J.	Pxa-9	173/ 209	84/89/ 96	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R9-12B.4
SWPA 52	*Pxa*	SW	2013	Ronn-ebyån	Redondo, M. and Oliva, J.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R10-8.1
SWPA 54	*Pxa*	SW	2013	Ronn-ebyån	Redondo, M. and Oliva, J.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R10-8.3
SWPA 55	*Pxa*	SW	2013	Ronn-ebyån	Redondo, M. and Oliva, J.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R10-8.4
SWPA 56	*Pxa*	SW	2013	Ronn-ebyån	Redondo, M. and Oliva, J.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R10-10.1
SWPA 57	*Pxa*	SW	2013	Ronn-ebyån	Redondo, M. and Oliva, J.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R10-10.2
SWPA 58	*Pxa*	SW	2013	Ronn-ebyån	Redondo, M. and Oliva, J.	Pxa-6	173/ 209	84/89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R10-10.3
SWPA 59	*Pxa*	SW	2013	Lyck-ebyån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R11-5.1
SWPA 70	*Pxa*	SW	2013	Lyck-ebyån	Redondo, M. and Oliva, J.	Pxa-31	173/ 209	89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R11-12.1
SWPA 71	*Pxa*	SW	2013	Lyck-ebyån	Redondo, M. and Oliva, J.	Pxa-31	173/ 209	89	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R11-12.2
SWPA 72	*Pxa*	SW	2013	Lyck-ebyån	Redondo, M. and Oliva, J.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	R12-5.2
GN76	*Pxa*	CH	2015	Switzerland	Norkutė, G.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	Rot-W8-2
GN 203	*Pxa*	CH	2015	Switzerland	Norkutė, G.	Pxa-1	173/ 209	84/89/ 99	92/95	127	107/ 113	91/94/ 98	162/174/ 177	100/ 109	335	157/ 171	Rot-W11-2.1-1

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
GN 204	*Pxa*	CH	2015	Switzerland	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	Rot-W11-2.1-4
GN 205	*Pxa*	CH	2015	Switzerland	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	Rot-W11-2.2-1
GN 207	*Pxa*	CH	2015	Switzerland	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	Rot-W11-2.2-4
GN 210	*Pxa*	CH	2015	Switzerland	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	Rot-W11-2.2-7
GN 211	*Pxa*	CH	2015	Switzerland	Norkutė, G.	Pxa-1	173/209	84/89/99	92/95	127	107/113	91/94/98	162/174/177	100/109	335	157/171	Rot-W11-2.2-8
APA7	*P. u*	AU	2014	Austria	Corcobado, T.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	A26
APA 13	*P. u*	AU	2014	Austria	Corcobado, T.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	A60
CZPA 19	*P. u*	CZ	2007	Czech Republic	Černy, K. and Strnadová, V.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	P 144.07
CZPA 34	*P. u*	CZ	2009	Czech Republic	Černy, K. and Strnadová, V.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	P 299.09
CZPA 45	*P. u*	CZ	2008	Czech Republic	Černy, K. and Strnadová, V.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	P213.08

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
CZPA 46	*P. u*	CZ	2008	Czech Repu-blic	Černy, K. and Strnadová, V.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	P220.08
LT-20	*P. u*	LT	2014	Lithu-ania	Norkutė, G.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	BA-34.1.2
LT-33	*P. u*	LT	2014	Lithu-ania	Norkutė, G.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	BA-32.1.3
SPA1	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	Zamice-1
SPA2	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	Zamice-9
SPA3	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	Zabre-1
SPA4	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	Zabre-8
SPA8	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	PFSPA4018
SPA9	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	PFSPA4020
SPA 10	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	PFSPA5024
SPA 11	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	PFSPA6034
SPA 12	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	PFSPA6035
SPA 13	*P. u*	SP	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	PFSPA7054
SPA 17	*P. u*	Spa in	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	MMSPA8059
SPA1 8	*P. u*	Spa in	NI	Spain	Solla, A.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	MMSPA8060

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA 8	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R3-8.1
SWPA 9	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R3-8.2
SWPA 11	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R4-5.1
SWPA 13	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R6-3.1
SWPA 14	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R6-9.1-II
SWPA 15	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R6-10B.1
SWPA 16	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R6-10B.2
SWPA 17	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R6-10B.3
SWPA 18	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R6-10B.4
SWPA 19	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R6-10B.6
SWPA 20	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R7-3.1
SWPA 29	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R7-9.8
SWPA 33	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R8-7.1
SWPA 34	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R8-7.2
SWPA 35	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R8-7.3
SWPA 41	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	EXTRA 1

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA 42	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	EXTRA 2
SWPA 43	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R9-6.1-I
SWPA 44	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R9-6.1.II
SWPA 51	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R10-7.1
SWPA 53	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R10-8.2
SWPA 60	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-5.2-I
SWPA 61	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-5.2-II
SWPA 62	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-5.3
SWPA 63	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-8.1
SWPA 64	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-8.2
SWPA 65	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-8.3
SWPA 66	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-9.2
SWPA 67	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-9.3
SWPA 68	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-9.4
SWPA 69	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R11-9.5

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA 73	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R12-5.3
SWPA 74	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R12-5.4
SWPA 75	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R12-6.1
SWPA 76	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R12-6.2
SWPA 77	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R12-6.3
SWPA 78	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R12-6.5
SWPA 79	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R12-6.6
SWPA 80	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R13-1.1
SWPA 81	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R13-2.1
SWPA 82	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R13-3B.1
SWPA 83	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R13-3B.2
SWPA 84	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R13-3B.3
SWPA 85	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R13-3B.4
SWPA 87	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R13-6.1
SWPA 88	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R14-3.1

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA 89	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R14-3.2
SWPA 90	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R14-3.3
SWPA 91	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R14-4.1
SWPA 92	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R14-5.2
SWPA 93	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R14-6.1
SWPA 94	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R14-7.1
SWPA 95	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R14-7.2
SWPA 96	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R14-7.3
SWPA 97	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R14-8.1
SWPA 98	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E2	173	89	95	133	113	91	162	109	335	NA	R14-9.2
SWPA 99	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R15-4.2
SWPA 100	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R15-5.1
SWPA 101	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R15-6.1
SWPA 102	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R15-6.2
SWPA 103	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R16-7.2

APPENDIX 2 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
SWPA 104	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R16-7.3
SWPA 105	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R16-7.4
SWPA 106	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R16-7.5
SWPA 107	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R16-7.6
SWPA 108	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R16-7.7
SWPA 109	*P. u*	SW	2013	Sweden	Redondo, M. and Oliva, J.	Pu-E1	173	89	95	127	113	91	162	109	335	NA	R16-10.1

[A] – *Pxa – Phytophthora* x *alni*; *P. u – Phytophthora uniformis*

[B] – Abbreviations AU – Autria, CH – Switzerland, CZ – Czech Republic, HU – Hungary, LT – Lithuania, SP – Spain, SW –Sweden

NI – No information available

NA – no alleles were scored in this locus.

APPENDIX 3 Allelic patterns of rare *Phytophthora* x *alni* multilocus genotypes (MLGs).

Name of MLG	Country	Closest MLG	N	Number of alleles observed in respective loci (in brackets indicated are the numbers of alleles found in closest MLG)										Number of lost alleles	Subgenome
				M-Pau3	M-PAU9	M-PAU11	M-PAU14	M-PAU15	PA17	PA23	M-PAU32	M-PAU56	M-PAU72		
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Pxa-27	HU	Pxa-1	2	-	-	-	-	95 (92/95)	-	-	-	-	-	1	*P.* x *m*
Pxa-28	HU, CZ	Pxa-1	2	-	-	-	-	-	-	157 (157/171)	-	-	-	1	*P.* x *m*
Pxa-29	SW	Pxa-30	1	-	-	-	84/89 (84/89/99)	-	-	-	-	91/98 (91/98)	162/174 (162/174)	1	*P.* x *m*
Pxa-30	SW	Pxa-2	2	-	-	-	-	-	-	-	-	91/98 (91/94/98)	162/174 (162/174/177)	2	*P.* x *m*
Pxa-31	SW	Pxa-6	2	-	-	-	89 (84/89)	-	-	-	-	-	-	1	*P.* x *m*
Pxa-32	CZ	Pxa-1	1	-	-	-	-	92 (92/95)	-	-	-	-	-	1	*P. u*
Pxa-33	CZ	Pxa-8	1	-	-	-	-	-	-	-	107 (107/113)	91/98 (91/98)	162/174 (162/174)	1	*P. u*
Pxa-34	CZ	Pxa-6	1	-	-	209 (173/209)	84/89 (84/89)	-	-	-	-	-	-	1	*P. u*
Pxa-35	CZ	Pxa-1	1	-	-	-	-	-	-	171 (157/171)	-	-	-	1	*P.* x *m*
Pxa-36	CZ	Pxa-1	1	-	-	-	-	-	-	-	113 (107/113)	-	-	1	*P.* x *m*
Pxa-37	CZ	Pxa-33 and Pxa-39	1	-	-	-	-	95 (92/95), (95)	-	-	107 (107), (107/113)	91/98 (91/98)	162/174 (162/174)		*P.* x *m* and *P. u*

APPENDIX 3 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Pxa-38	CZ	Pxa-19	1	-	-	-	-	-	-	-	-	-	162 (162/174)	1	*P.* x *m*
Pxa-39	CZ	Pxa-8	1	-	-	-	-	95 (92/95)	-	-	-	91/98 (91/98)	162/174 (162/174)	1	*P.* x *m*
Pxa-40	CZ	Pxa-24	1	-	100 (100/109)	-	-	-	-	-	-	-	162/177 (162/174/177)	2	*P.* x *m* and *P.u*
Pxa-41	CZ	Pxa-38	1	-	-	173 (173/209)	-	-	-	-	-	91/94 (91/94/98)	162 (162/174)	3	*P.* x *m*
Pxa-42	CZ	Pxa-27	1	-	-	-	-	95 (95)	-	-	-	91/94 (91/94/98)	-	1	*P.* x *m*
Pxa-43	CZ	Pxa-38	2	-	-	-	-	95 (92/95)	-	-	-	-	162 (162)	1	*P.* x *m*
Pxa-44	CZ	Pxa-27	1	-	-	-	-	95 (95)	-	-	-	-	162/177 (162/174/177)	1	*P.* x *m*
Pxa-45	CZ	Pxa-43	1	-	109 (100/109)	-	89 (84/89/99)	95 (95)	-	-	113 (107/113)	91 (91/94/98)	162 (162)	6	*P.* x *m*
Pxa-46	CZ	Pxa-33	1	-	-	173 (173/209)	-	-	-	-	107 (107)	91/98 (91/98)	162/174 (162/174)	1	*P.* x *m*
Pxa-47	LT	Pxa-42	1	-	-	173 (173/209)	84/89 (84/89/99)	95 (95)	-	-	-	91 (91/94)	-	3	*P.* x *m*
Pxa-48	SP	Pxa-8	1	-	-	-	-	-	-	-	-	91 (91/98)	162/174 (162/174)	1	*P.* x *m*
Pxa-49	AU	Pxa-8	2	-	-	-	-	-	-	-	113 (107/113)	91/98 (91/98)	162/174 (162/174)	1	*P.* x *m*
Pxa-50	AU	Pxa-19	1	-	-	209 (173/209)	-	-	-	-	-	-	162/174 (162/174)	1	*P. u*
Total Loss			30	0	2	5	5	4	0	2	4	7	5	-	-

Closest MLG – multilocus genotype that is closest to newly described MLG by its allele pattern; For country name abbreviations see Table 6. N – number of observed isolates; Subgenome – a subgenome from which the alleles have been lost (for information on the respective subgenomes see Aguayo et al. (2016)). *P. x m – Phytophthora* x *multiformis*; *P. u – P. uniformis*.

APPENDIX 4 *Ophiostoma novo-ulmi* isolation site

Isolate name	*O. novo-ulmi* subspecies	Host	Population	Coordinates	
				Latitude	Longitude
1	*2*	*3*	*4*	*5*	*6*
ON-3	*O. novo-ulmi* ssp. *americana* x ssp. *novo-ulmi* (EAN x NAN)	*U. glabra*	Vilnius-Verkiai	55.747940N	25.290790E
ON-5	EAN x NAN	*U. glabra*	Vilnius-Verkiai	54.749130N	25.291130E
ON-6	EAN x NAN	*U. glabra*	Vilnius-Dūkštos	54.818220N	24.925130E
ON-7	EAN x NAN	*U. glabra*	Vilnius-Dūkštos	54,818290N	24,925250E
ON-8	EAN x NAN	*U. glabra*	Vilnius-Dūkštos	54,818220N	24,925130E
ON-10	*O. novo-ulmi* ssp. *novo-ulmi* (EAN)	*U. glabra*	Pasvalys-Gulbinėnai	56,078790N	24,651960E
ON-11	EAN	*U. glabra*	Pasvalys-Paberliai	56,086730N	24,608730E
ON-12	EAN	*U. glabra*	Pasvalys-Paberliai	56,086270N	24,608350E
ON-13	EAN	*U. glabra*	Biržai-Karajimiškis	56,206260N	24,694240E
ON-14	EAN	*U. minor*	Kėdainiai-Aristava	55,301550N	24,085980E
ON15	EAN	*U. minor*	Kėdainiai-Aristava	55,302110N	24,087030E
ON-16	EAN x NAN	*U. minor*	Kėdainiai-Aristava	55,302140N	24,087060E
ON-17	EAN x NAN	*U. minor*	Kėdainiai-Aristava	55,302050N	24,086810E
ON-18	EAN	*U. minor*	Kėdainiai-Aristava	55,299747N	24,084800E
ON-19	EAN	*U. minor*	Kėdainiai-Aristava	55,301660N	24,086000E
ON-20	EAN x NAN	*U. minor*	Kėdainiai-Aristava	55,301520N	24,086070E
ON-21	EAN	*U. minor*	Kėdainiai-Aristava	55,301550N	24,086000E
ON-22	EAN	*U. minor*	Kėdainiai-Aristava	55,301890N	24,086230E
ON-23	EAN	*U. minor*	Kėdainiai-Aristava	55,301870N	24,086050E
ON-24	EAN	*U. minor*	Kėdainiai-Aristava	55,301870N	24,086120E
ON-25	EAN	*U. minor*	Kėdainiai-Aristava	55,301510N	24,086110E
ON-26	EAN	*U. minor*	Kėdainiai-Aristava	55,301620N	24,086240E
ON-27	EAN	*U. minor*	Kėdainiai-Aristava	55,301580N	24,085290E

APPENDIX 4 (continuation)

1	*2*	*3*	*4*	*5*	*6*
ON-28	EAN	*U. minor*	Kėdainiai-Aristava	55,301560N	24,085920E
ON-29	EAN	*U. minor*	Kėdainiai-Aristava	55,302030N	24,086540E
ON-30	EAN	*U. minor*	Kėdainiai-Aristava	55,301660N	24,086190E
ON-31	EAN	*U. minor*	Kėdainiai-Aristava	55,301660N	24,082512E
ON-32	EAN	*U. minor*	Kėdainiai-Aristava	55,301610N	24,085870E
ON-33	EAN	*U. minor*	Kėdainiai-Aristava	55,301680N	24,085830E
ON-34	EAN	*U. minor*	Kėdainiai-Aristava	55,301670N	24,086000E
ON-35	EAN x NAN	*U. minor*	Kėdainiai-Aristava	55,302955N	24,086890E
ON-36	EAN	*U. minor*	Kėdainiai-Aristava	55,302310N	24,086970E
ON-37	EAN	*U. minor*	Kėdainiai-Aristava	55,302720N	24,083348E
ON-38	EAN	*U. minor*	Kėdainiai-Aristava	55,301340N	24,085870E
ON-39	EAN	*U. minor*	Kėdainiai-Aristava	55,302955N	24,086890E
ON-40	EAN x NAN	*U. minor*	Kėdainiai-Aristava	55,301570N	24,086240E
ON-41	EAN x NAN	*U. minor*	Kėdainiai-Aristava	55,300235N	24,086800E
ON-42	EAN	*U. minor*	Kėdainiai-Aristava	55,300591N	24,087009E
ON-43	EAN	*U. glabra*	Kaunas-Raudondvaris	54,866863N	24,049101E
ON-44	EAN	*U. glabra*	Rokiškis-Dūsetos	55,801040N	25,790500E
ON-45	EAN	*U. glabra*	Rokiškis-Dūsetos	55,801080N	25,788420E
ON-46	EAN	*U. glabra*	Rokiškis-Dūsetos	55,796150N	25,791880E
ON-47	EAN	*U. glabra*	Rokiškis-Dūsetos	55,797030N	25,791880E
ON-48	EAN	*U. glabra*	Plungė-Kuliai	55,797100N	21,610520E
ON-49	EAN	*U. glabra*	Plungė-Kuliai	55,797030N	21,610680E
ON-50	EAN	*U. glabra*	Plungė-Kuliai	55,796990N	21,610780E
ON-51	EAN	*U. glabra*	Plungė-Kuliai	55,796750N	21,610250E
ON-52	EAN x NAN	*U. glabra*	Plungė-Kuliai	55,796590N	21,610200E
ON-53	EAN x NAN	*U. glabra*	Plungė-Kuliai	55,796600N	21,610200E

APPENDIX 5 Vegetative compatibility test results of Ophiostoma novo-ulmi. C – vegetative compatible; LG – reaction "line-gap"; L – reaction "wide gap"

	ON3	ON5	ON6	ON7	ON8	ON10	ON11	ON12	ON13	ON14	ON15	ON25	ON16	ON17	ON18	ON19	ON20	ON21	ON22	ON23	ON24	ON26	ON27	ON28	ON29	ON30	ON32	ON31
ON53	L	C	L	L	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG	C	LG	C	LG	C	C	C	LG	C	C	C	C	C
ON52	L	C	L	L	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG	C	LG	C	LG	C	C	C	LG	C	C	C	C	C
ON51	L	L	L	L	L	LG	LG	LG	LG	LG	C	C	C	C	C	C	LG	LG	LG	C	C	C	LG	C	C	C	C	C
ON50	L	L	L	L	L	LG	LG	LG	LG	LG	C	C	C	C	C	C	LG	LG	LG	C	LG	LG	LG	C	C	C	C	C
ON49	L	L	L	L	L	LG	LG	LG	LG	LG	C	C	C	C	C	C	LG	C	C	C	C	C	LG	LG	C	LG	LG	LG
ON48	L	L	L	L	L	LG	LG	LG	LG	LG	C	C	C	C	C	C	LG	C	C	C	C	C	LG	LG	C	LG	LG	LG
ON47	L	L	L	L	L	LG	LG	LG	LG	C	LG	LG	C	C	C	C	LG	C	C	LG	C	C	C	C	C	C	C	C
ON46	L	L	L	L	L	C	C	C	C	C	C	C	C	C	C	C	LG	C	LG	C	C	C	L	LG	C	C	C	C
ON45	L	L	L	L	L	C	C	C	C	L	C	C	C	C	C	C	LG	C	C	L	LG	C	LG	LG	C	C	C	C
ON44	L	L	L	L	L	C	C	C	C	L	C	C	C	C	C	LG	LG	C	C	LG	C	C	C	LG	C	C	C	C
ON43	L	L	L	L	L	C	C	C	C	C	C	C	C	C	C	C	LG	C	C	C	LG	C	C	C	C	C	C	C
ON42	L	L	L	L	L	C	C	C	LG	C	LG	LG	LG	LG	C	LG	LG	C	LG	C	LG	C	LG	LG	C	C	C	C
ON41	L	L	L	L	L	C	C	C	LG	C	LG	LG	LG	LG	C	LG	LG	LG	LG	C	LG	C	LG	LG	C	C	C	C
ON40	L	L	L	L	L	C	C	C	LG	C	LG	LG	LG	LG	C	LG	LG	C	LG	C	LG	C	LG	LG	C	C	C	C
ON39	L	L	L	L	L	C	C	C	LG	C	LG	LG	C	C	C	LG	LG	C	LG	C	LG	C	LG	LG	C	C	C	C
ON38	L	L	L	L	L	C	C	C	LG	C	LG	LG	C	C	C	LG	C	LG	LG	C	LG	LG	LG	C	LG	LG	LG	C
ON37	L	L	L	L	L	C	C	C	LG	C	LG	LG	C	C	C	LG	C	LG	LG	C	LG	LG	LG	C	LG	LG	LG	C
ON36	L	L	L	L	L	C	C	C	LG	C	LG	LG	C	C	C	LG	C	LG	LG	C	LG	LG	LG	C	LG	LG	LG	C
ON35	L	L	L	L	L	C	C	C	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG	LG
ON34	L	L	L	L	L	LG	LG	LG	C	LG	LG	LG	C	C	C	LG	C	C	LG	C	C	C	LG	C	LG	C	C	C
ON33	L	L	L	L	L	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	C	LG	C	C	C	C	C	C	C	LG	C	C	C
ON32	L	L	L	L	L	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	C	C	LG	C	C	C	C	C
ON31	L	L	L	L	L	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG	LG	C	C	C	C	C	C	C	LG	C	C	C
ON30	L	L	L	L	L	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	C	C	LG	C	C	C	C	C
ON29	L	L	L	L	L	LG	LG	LG	L	C	LG	LG	C	C	C	LG	LG	C	LG	LG	C	C	C	C	C	C	C	LG
ON28	L	L	L	L	L	C	C	LG	L	C	LG	LG	LG	LG	LG	LG	LG	C	LG	LG	C	LG	C	C	C	C	C	C
ON27	L	L	L	L	L	LG	LG	L	L	L	LG	LG	C	C	C	C	LG	C	LG	LG	C	C	C	C	C	LG	LG	C
ON26	L	L	L	L	L	C	C	LG	LG	C	C	C	C	C	C	C	LG	LG	LG	C	C	C	C	LG	C	C	C	C
ON25	L	L	L	L	L	C	C	C	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG
ON24	L	L	L	L	L	C	C	C	C	C	LG	LG	C	C	C	C	C	C	C	LG	C	C	C	C	C	C	C	C
ON23	L	L	L	L	L	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG	C	LG	C	LG	C	LG	LG	LG	C	C	C
ON22	L	L	L	L	L	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG	C	C	LG	C	LG	LG	LG	LG	LG	LG	C
ON21	L	L	L	L	L	C	C	C	LG	C	LG	LG	C	C	C	LG	LG	C	C	C	C	LG	C	C	C	LG	LG	C
ON20	L	L	L	L	L	LG	LG	LG	L	LG	LG	LG	C	C	LG	C	C	LG	LG	LG	C	LG	LG	LG	LG	LG	LG	LG
ON19	L	L	L	L	L	LG	LG	LG	C	C	LG	LG	LG	LG	LG	C	C	LG	LG	LG	C	C	C	LG	LG	LG	LG	LG
ON18	L	L	L	L	L	C	C	LG	C	C	LG	LG	C	C	C	LG	LG	C	LG	LG	C	C	C	LG	C	LG	LG	LG
ON17	L	L	L	L	L	C	C	LG	C	C	LG	LG	C	C	C	LG	C	C	LG	LG	C	C	C	LG	C	LG	LG	LG
ON16	L	L	L	L	L	C	C	LG	C	C	C	C	C	C	C	C	C	C	LG	LG	C	C	C	LG	C	LG	LG	LG
ON15	L	L	L	L	L	C	C	C	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG	LG	C	LG	LG	LG	LG	LG	LG
ON14	L	L	L	L	L	C	LG	LG	L	C	C	C	C	C	C	C	LG	C	C	C	C	C	L	C	C	C	C	C
ON13	L	L	L	L	L	LG	LG	LG	C	LG	C	C	C	C	C	C	LG	LG	C	C	C	LG	L	L	L	LG	LG	LG
ON12	L	L	L	L	L	C	C	C	LG	LG	C	C	LG	LG	LG	LG	LG	C	C	C	C	LG	L	LG	LG	LG	LG	LG
ON11	L	L	L	L	L	C	C	C	LG	LG	C	C	C	C	C	LG	LG	C	C	C	C	C	LG	C	LG	LG	LG	LG
ON10	L	L	L	L	L	C	C	C	LG	C	C	C	C	C	C	LG	LG	C	C	C	C	C	LG	C	LG	LG	LG	LG
ON8	LG	LG	LG	LG	C	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
ON7	LG	C	C	C	LG	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
ON6	LG	C	C	C	LG	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
ON5	C	C	C	C	LG	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
ON3	C	C	LG	LG	LG	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

APPENDIX 5 (continuation)

	ON33	ON34	ON35	ON36	ON38	ON37	ON39	ON40	ON41	ON42	ON43	ON44	ON45	ON46	ON47	ON48	ON49	ON50	ON51	ON52	ON53
ON53	C	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	C	LG	LG	LG	LG	LG	LG	C	C
ON52	C	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	C	LG	LG	LG	LG	LG	LG	C	C
ON51	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG
ON50	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG
ON49	LG	LG	C	L	L	L	LG	LG	LG	LG	LG	LG	C	C	C	C	C	C	C	LG	LG
ON48	LG	LG	C	L	L	L	LG	LG	LG	LG	LG	LG	C	C	C	C	C	C	C	LG	LG
ON47	C	LG	LG	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG
ON46	C	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	C	C	C	C	LG	LG
ON45	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
ON44	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	C	C	C	C
ON43	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	C	C	LG	LG
ON42	C	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	C	C	LG	LG
ON41	C	C	LG	C	C	LG	C	C	C	C	C	C	C	C	C	LG	LG	C	C	LG	LG
ON40	C	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	C	C	LG	LG
ON39	C	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG
ON38	C	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	L	L	C	C	LG	LG
ON37	C	C	LG	C	C	C	C	C	C	LG	C	C	C	C	C	L	L	C	C	LG	LG
ON36	C	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	L	L	C	C	LG	LG
ON35	LG	LG	C	LG	LG	LG	LG	LG	LG	LG	C	C	C	C	LG	C	C	C	C	LG	LG
ON34	C	C	LG	C	C	C	C	C	C	C	LG	C	C	C	LG	LG	LG	LG	LG	C	C
ON33	C	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	C	C	C	C
ON32	C	C	LG	LG	LG	LG	C	C	C	C	C	C	C	C	C	LG	LG	C	C	C	C
ON31	C	C	LG	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	C	C	C	C
ON30	C	C	LG	LG	LG	LG	C	C	C	C	C	C	C	C	C	LG	LG	C	C	C	C
ON29	LG	LG	LG	LG	LG	LG	C	C	C	C	C	LG	LG	LG	C	C	C	C	C	C	C
ON28	C	C	LG	C	C	C	LG	LG	LG	LG	C	LG	LG	L	C	LG	LG	C	C	C	C
ON27	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	C	C	C	C	LG	LG	LG	LG	LG	LG
ON26	C	C	C	LG	LG	LG	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
ON25	LG	LG	C	LG	LG	LG	LG	LG	LG	LG	C	C	C	C	C	C	C	C	C	LG	LG
ON24	C	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	LG	C	C	C	C	C	C
ON23	C	C	LG	C	C	C	C	C	C	C	C	C	C	LG	C	C	C	C	C	C	C
ON22	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	C	C	C	C	C	C	LG	LG	LG	LG
ON21	C	C	LG	LG	LG	LG	C	C	C	C	C	LG	LG	LG	LG	C	C	LG	LG	C	C
ON20	LG	C	LG	C	C	C	LG	LG	LG	LG	C	LG	LG	C	C	LG	LG	LG	LG	LG	LG
ON19	C	LG	LG	LG	LG	LG	LG	LG	LG	LG	C	C	C	C	C	C	C	C	C	C	C
ON18	LG	LG	LG	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG
ON17	LG	C	LG	C	C	C	C	LG	LG	LG	C	C	C	C	C	C	C	C	C	LG	LG
ON16	LG	C	C	C	C	C	C	LG	LG	LG	C	C	C	C	C	C	C	C	C	LG	LG
ON15	LG	LG	C	LG	LG	LG	LG	LG	LG	LG	C	LG	LG	C	LG	C	C	C	C	LG	LG
ON14	C	LG	C	C	C	C	C	C	C	C	C	L	L	C	C	LG	LG	LG	LG	LG	LG
ON13	LG	C	C	LG	LG	LG	LG	LG	LG	LG	C	C	C	C	LG	LG	LG	LG	LG	C	C
ON12	LG	LG	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG
ON11	LG	LG	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG
ON10	LG	LG	C	C	C	C	C	C	C	C	C	C	C	C	LG	LG	LG	LG	LG	LG	LG
ON8	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	LG	LG
ON7	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
ON6	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
ON5	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	C	C
ON3	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

APPENDIX 6 *Hymenoscyphus fraxineus* microsatellite scores and supplementary data of isolates

Isolate Name	Population	Abbreviation	Lesion/ Petiole	MLG	Sampling year	Locus										
						15	22	38	42	53	69	81	90	95	97	622
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
BU1L	Biržai-Joniškėlis	BIR	L	HF_LT244	2013	200	246	231	170	281	248	138	216	149	254	118
BU1P	Biržai-Joniškėlis	BIR	P	HF_LT136	2013	200	250	237	170	300	263	138	216	149	245	118
BU2L	Biržai-Joniškėlis	BIR	L	HF_LT146	2013	200	250	237	170	300	248	138	216	155	245	118
BU2P	Biržai-Joniškėlis	BIR	P	HF_LT176	2013	200	250	231	164	300	263	147	216	149	254	118
BU3L	Biržai-Joniškėlis	BIR	L	HF_LT231	2013	200	246	237	164	300	248	138	216	155	254	118
BU4L	Biržai-Joniškėlis	BIR	L	HF_LT75	2013	183	246	237	164	300	263	138	216	149	254	112
BU5P	Biržai-Joniškėlis	BIR	P	HF_LT88	2013	200	250	237	164	300	263	138	216	149	254	112
BU6L	Biržai-Joniškėlis	BIR	L	HF_LT219	2013	200	250	231	170	300	248	138	216	149	254	112
BU6P	Biržai-Joniškėlis	BIR	P	HF_LT64	2013	183	250	231	170	300	248	147	216	149	245	118
BU7L	Biržai-Joniškėlis	BIR	L	HF_LT67	2013	183	250	231	170	300	248	138	216	155	254	118
BU7P	Biržai-Joniškėlis	BIR	P	HF_LT127	2013	200	250	237	170	300	263	147	216	155	254	112
BU8L	Biržai-Joniškėlis	BIR	L	HF_LT46	2013	183	250	231	164	300	263	147	216	149	245	112
BU8P	Biržai-Joniškėlis	BIR	P	HF_LT137	2013	200	250	237	170	300	263	138	216	149	254	112
BU9L	Biržai-Joniškėlis	BIR	L	HF_LT180	2013	200	250	231	164	300	263	138	216	149	245	118
BU9P	Biržai-Joniškėlis	BIR	P	HF_LT140	2013	200	250	237	170	300	248	147	216	155	245	118
BU10L	Biržai-Joniškėlis	BIR	L	HF_LT239	2013	200	246	231	164	300	263	147	216	155	254	118
BU10P	Biržai-Joniškėlis	BIR	P	HF_LT77	2013	183	246	237	164	281	248	138	216	155	254	118
BU11P	Biržai-Joniškėlis	BIR	P	HF_LT106	2013	200	250	237	164	281	263	147	216	149	245	118
BU12L	Biržai-Joniškėlis	BIR	L	HF_LT138	2013	200	250	237	170	300	263	138	216	149	254	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
BU12P	Biržai-Joniškėlis	BIR	P	HF_LT210	2013	200	250	231	170	300	263	138	216	149	254	112
BU13L	Biržai-Joniškėlis	BIR	L	HF_LT100	2013	200	250	237	164	300	248	138	216	155	254	118
BU14L	Biržai-Joniškėlis	BIR	L	HF_LT102	2013	200	250	237	164	300	248	138	216	149	254	112
BU14P	Biržai-Joniškėlis	BIR	P	HF_LT87	2013	200	250	237	164	300	263	138	216	149	245	118
BU15L	Biržai-Joniškėlis	BIR	L	HF_LT45	2013	183	250	231	164	300	263	147	216	155	254	118
BU15P	Biržai-Joniškėlis	BIR	P	HF_LT117	2013	200	250	237	164	281	248	138	216	155	245	112
BU16P	Biržai-Joniškėlis	BIR	P	HF_LT96	2013	200	250	237	164	300	248	147	216	149	254	118
BU17L	Biržai-Joniškėlis	BIR	L	HF_LT74	2013	183	246	237	164	300	263	138	216	149	245	118
BU17P	Biržai-Joniškėlis	BIR	P	HF_LT170	2013	200	250	237	170	281	248	138	216	149	254	118
BU18P	Biržai-Joniškėlis	BIR	P	HF_LT229	2013	200	246	237	164	300	263	147	216	149	245	118
BU19P	Biržai-Joniškėlis	BIR	P	HF_LT167	2013	200	250	237	170	281	248	138	216	155	254	118
BU20P	Biržai-Joniškėlis	BIR	P	HF_LT31	2013	183	250	237	170	300	263	138	216	155	254	118
BU21L	Biržai-Joniškėlis	BIR	L	HF_LT108	2013	200	250	237	164	281	263	138	216	155	245	118
BU21P	Biržai-Joniškėlis	BIR	P	HF_LT46	2013	183	250	231	164	300	263	147	216	149	245	112
BU22L	Biržai-Joniškėlis	BIR	L	HF_LT126	2013	200	250	237	170	263	147	147	216	155	254	112
BU22P	Biržai-Joniškėlis	BIR	P	HF_LT175	2013	200	250	231	164	300	263	147	216	149	254	112
BU23L	Biržai-Joniškėlis	BIR	L	HF_LT48	2013	183	250	231	164	300	248	147	216	149	254	118
BU23P	Biržai-Joniškėlis	BIR	P	HF_LT218	2013	200	250	231	170	300	248	138	216	149	245	118
BU24L	Biržai-Joniškėlis	BIR	L	HF_LT158	2013	200	250	237	170	281	263	138	216	149	254	118
BU24P	Biržai-Joniškėlis	BIR	P	HF_LT108	2013	200	250	237	164	281	263	138	216	155	245	118
BU25L	Biržai-Joniškėlis	BIR	L	HF_LT94	2013	200	250	237	164	300	248	147	216	149	245	118
BU25P	Biržai-Joniškėlis	BIR	P	HF_LT15	2013	183	250	237	164	300	248	138	216	155	254	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
BU27L	Biržai-Joniškėlis	BIR	L	HF_LT124	2013	200	250	237	170	285	263	147	216	155	254	118
BU27P	Biržai-Joniškėlis	BIR	P	HF_LT65	2013	183	250	231	170	300	248	147	216	149	254	118
BU28L	Biržai-Joniškėlis	BIR	L	HF_LT39	2013	183	250	237	170	281	263	147	216	155	254	118
BU28P	Biržai-Joniškėlis	BIR	P	HF_LT152	2013	200	250	237	170	281	263	147	216	149	245	118
BU29P	Biržai-Joniškėlis	BIR	P	HF_LT217	2013	200	250	231	170	300	248	138	216	149	245	112
BU30L	Biržai-Joniškėlis	BIR	L	HF_LT161	2013	200	250	237	170	281	248	147	216	149	254	112
BU30P	Biržai-Joniškėlis	BIR	P	HF_LT10	2013	183	250	237	164	300	248	147	216	149	254	112
BU31L	Biržai-Joniškėlis	BIR	L	HF_LT193	2013	200	250	231	164	281	263	138	216	155	254	118
BU31P	Biržai-Joniškėlis	BIR	P	HF_LT120	2013	200	250	237	164	281	248	138	216	149	245	112
BU32P	Biržai-Joniškėlis	BIR	P	HF_LT119	2013	200	250	237	164	281	248	138	216	155	254	118
BU33L	Biržai-Joniškėlis	BIR	L	HF_LT57	2013	183	250	231	164	281	248	138	216	155	254	112
BU34L	Biržai-Joniškėlis	BIR	L	HF_LT130	2013	200	250	237	170	300	263	147	216	149	245	118
BU34P	Biržai-Joniškėlis	BIR	P	HF_LT53	2013	183	250	231	164	281	263	138	216	149	254	118
BU35L	Biržai-Joniškėlis	BIR	L	HF_LT138	2013	200	250	237	170	300	263	138	216	149	254	118
BU35P	Biržai-Joniškėlis	BIR	P	HF_LT107	2013	200	250	237	164	281	263	147	216	149	254	118
BU36P	Biržai-Joniškėlis	BIR	P	HF_LT134	2013	200	250	237	170	300	263	138	216	155	254	118
BU37P	Biržai-Joniškėlis	BIR	P	HF_LT26	2013	183	250	237	164	281	248	138	216	149	254	118
BU38L	Biržai-Joniškėlis	BIR	L	HF_LT13	2013	183	250	237	164	300	248	138	216	155	245	118
BU38P	Biržai-Joniškėlis	BIR	P	HF_LT216	2013	200	250	231	170	300	248	138	216	155	254	118
BU39L	Biržai-Joniškėlis	BIR	L	HF_LT125	2013	200	250	237	170	285	248	147	216	155	245	118
BU39P	Biržai-Joniškėlis	BIR	P	HF_LT224	2013	200	250	231	170	281	263	138	216	149	254	118
BU40P	Biržai-Joniškėlis	BIR	P	HF_LT178	2013	200	250	231	164	300	263	138	216	155	254	112

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
BU41L	Biržai-Joniškėlis	BIR	L	HF_LT131	2013	200	250	237	170	300	263	147	216	149	254	112
BU41P	Biržai-Joniškėlis	BIR	P	HF_LT158	2013	200	250	237	170	281	263	138	216	149	254	118
BU42P	Biržai-Joniškėlis	BIR	P	HF_LT56	2013	183	250	231	164	281	248	138	213	155	254	112
BU43L	Biržai-Joniškėlis	BIR	L	HF_LT201	2013	200	250	231	164	281	248	138	216	155	245	118
BU43P	Biržai-Joniškėlis	BIR	P	HF_LT230	2013	200	246	237	164	300	248	147	216	149	254	118
BU44L	Biržai-Joniškėlis	BIR	L	HF_LT66	2013	183	250	231	170	300	248	138	216	155	254	112
BU44P	Biržai-Joniškėlis	BIR	P	HF_LT9	2013	183	250	237	164	300	248	147	216	149	245	118
BU45L	Biržai-Joniškėlis	BIR	L	HF_LT155	2013	200	250	237	170	281	263	138	216	155	254	118
BU45P	Biržai-Joniškėlis	BIR	P	HF_LT181	2013	200	250	231	164	300	263	138	216	149	254	112
BU46L	Biržai-Joniškėlis	BIR	L	HF_LT241	2013	200	246	231	170	300	248	138	216	155	254	118
BU46P	Biržai-Joniškėlis	BIR	P	HF_LT147	2013	200	250	237	170	300	248	138	216	155	254	112
BU47L	Biržai-Joniškėlis	BIR	L	HF_LT148	2013	200	250	237	170	300	248	138	216	155	254	118
BU47P	Biržai-Joniškėlis	BIR	P	HF_LT121	2013	200	250	237	164	281	248	138	216	149	254	112
BU48L	Biržai-Joniškėlis	BIR	L	HF_LT138	2013	200	250	237	170	300	263	138	216	149	254	118
BU48P	Biržai-Joniškėlis	BIR	P	HF_LT123	2013	200	250	237	170	296	248	147	216	149	254	118
BU49L	Biržai-Joniškėlis	BIR	L	HF_LT149	2013	200	250	237	170	300	248	138	216	149	245	112
BU50L	Biržai-Joniškėlis	BIR	L	HF_LT197	2013	200	250	231	164	281	248	147	216	155	254	118
BU53L	Biržai-Joniškėlis	BIR	L	HF_LT227	2013	200	250	231	170	281	248	138	216	149	254	112
BU54L	Biržai-Joniškėlis	BIR	L	HF_LT148	2013	200	250	237	170	300	248	138	216	155	254	118
BU56L	Biržai-Joniškėlis	BIR	L	HF_LT135	2013	200	250	237	170	300	263	138	216	149	245	112
BU57L	Biržai-Joniškėlis	BIR	L	HF_LT168	2013	200	250	237	170	281	248	138	216	149	245	118
BU58L	Biržai-Joniškėlis	BIR	L	HF_LT215	2013	200	250	231	170	300	248	138	216	155	254	112

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
BU59L	Biržai-Joniškėlis	BIR	L	HF_LT221	2013	200	250	231	170	281	263	147	216	149	254	118
BU60L	Biržai-Joniškėlis	BIR	L	HF_LT83	2013	200	250	234	164	281	248	138	216	149	254	112
KR1L	Kretinga-Vėžaičiai	KRE	L	HF_LT67	2013	183	250	231	170	300	248	138	216	155	254	118
KR1P	Kretinga-Vėžaičiai	KRE	P	HF_LT21	2013	183	250	237	164	281	248	147	216	155	254	118
KR2L	Kretinga-Vėžaičiai	KRE	L	HF_LT162	2013	200	250	237	170	281	248	147	216	149	254	118
KR2P	Kretinga-Vėžaičiai	KRE	P	HF_LT44	2013	183	250	237	170	281	248	147	216	149	254	118
KR3L	Kretinga-Vėžaičiai	KRE	L	HF_LT132	2013	200	250	237	170	300	263	147	216	149	254	118
KR3P	Kretinga-Vėžaičiai	KRE	P	HF_LT59	2013	183	250	231	164	281	248	138	216	149	245	112
KR4L	Kretinga-Vėžaičiai	KRE	L	HF_LT218	2013	200	250	231	170	300	248	138	216	149	245	118
KR4P	Kretinga-Vėžaičiai	KRE	P	HF_LT159	2013	200	250	237	170	281	263	138	216		245	118
KR5P	Kretinga-Vėžaičiai	KRE	P	HF_LT29	2013	183	250	237	170	300	263	147	216	155	254	118
KR6L	Kretinga-Vėžaičiai	KRE	L	HF_LT1	2013	197	250	237	170	300	248	147	216	149	254	118
KR6P	Kretinga-Vėžaičiai	KRE	P	HF_LT170	2013	200	250	237	170	281	248	138	216	149	254	118
KR7P	Kretinga-Vėžaičiai	KRE	P	HF_LT90	2013	200	250	237	164	300	248	147	213	149	254	112
KR8P	Kretinga-Vėžaičiai	KRE	P	HF_LT105	2013	200	250	237	164	281	263	147	216	155	254	118
KR9L	Kretinga-Vėžaičiai	KRE	L	HF_LT153	2013	200	250	237	170	281	263	147	216	149	254	118
KR9P	Kretinga-Vėžaičiai	KRE	P	HF_LT188	2013	200	250	231	164	300	248	138	216	149	245	112
KR10L	Kretinga-Vėžaičiai	KRE	L	HF_LT119	2013	200	250	237	164	281	248	138	216	155	254	118
KR10P	Kretinga-Vėžaičiai	KRE	P	HF_LT193	2013	200	250	231	164	281	263	138	216	155	254	118
KR11P	Kretinga-Vėžaičiai	KRE	P	HF_LT61	2013	183	250	231	170	300	263	138	216	149	245	118
KR12P	Kretinga-Vėžaičiai	KRE	P	HF_LT196	2013	200	250	231	164	281	263	138	216	149	254	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
KR13L	Kretinga-Vėžaičiai	KR	L	HF_LT184	2013	200	250	231	164	300	248	147	216	149	254	118
KR13P	Kretinga-Vėžaičiai	KR	P	HF_LT97	2013	200	250	237	164	300	248	138	216	155	245	112
KR14L	Kretinga-Vėžaičiai	KR	L	HF_LT182	2013	200	250	231	164	300	248	147	216	155	245	118
KR14P	Kretinga-Vėžaičiai	KR	P	HF_LT134	2013	200	250	237	170	300	263	138	216	155	254	118
KR15L	Kretinga-Vėžaičiai	KR	L	HF_LT186	2013	200	250	231	164	300	248	138	216	155	254	112
KR15P	Kretinga-Vėžaičiai	KR	P	HF_LT8	2013	183	250	237	164	300	248	147	216	155	254	118
KR16L	Kretinga-Vėžaičiai	KR	L	HF_LT76	2013	183	246	237	164	300	248	138	216	155	245	118
KR16P	Kretinga-Vėžaičiai	KR	P	HF_LT102	2013	200	250	237	164	300	248	138	216	149	254	112
KR17L	Kretinga-Vėžaičiai	KR	L	HF_LT136	2013	200	250	237	170	300	263	138	216	149	245	118
KR17P	Kretinga-Vėžaičiai	KR	P	HF_LT232	2013	200	246	237	164	281	248	138	216	149	254	118
KR18L	Kretinga-Vėžaičiai	KR	L	HF_LT24	2013	183	250	237	164	281	248	138	216	155	254	118
KR19L	Kretinga-Vėžaičiai	KR	L	HF_LT17	2013	183	250	237	164	300	248	138	216	149	254	118
KR19P	Kretinga-Vėžaičiai	KR	P	HF_LT120	2013	200	250	237	164	281	248	138	216	149	245	112
KR20P	Kretinga-Vėžaičiai	KR	P	HF_LT147	2013	200	250	237	170	300	248	138	216	155	254	112
KR21L	Kretinga-Vėžaičiai	KR	L	HF_LT183	2013	200	250	231	164	300	248	147	216	155	254	118
KR21P	Kretinga-Vėžaičiai	KR	P	HF_LT118	2013	200	250	237	164	281	248	138	216	155	254	112
KR22L	Kretinga-Vėžaičiai	KR	L	HF_LT26	2013	183	250	237	164	281	248	138	216	149	254	118
KR22P	Kretinga-Vėžaičiai	KR	P	HF_LT104	2013	200	250	237	164	281	263	147	216	155	245	118
KR23L	Kretinga-Vėžaičiai	KR	L	HF_LT28	2013	183	250	237	170	300	263	147	216	155	245	112
KR23P	Kretinga-Vėžaičiai	KR	P	HF_LT145	2013	200	250	237	170	300	248	147	216	149	254	118
KR24P	Kretinga-Vėžaičiai	KR	P	HF_LT190	2013	200	250	231	164	300	248	138	216	149	254	118
KR25L	Kretinga-Vėžaičiai	KR	L	HF_LT96	2013	200	250	237	164	300	248	147	216	149	254	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
KR25P	Kretinga-Vėžaičiai	KRE	P	HF_LT49	2013	183	250	231	164	300	248	138	216	155	254	118
KR26L	Kretinga-Vėžaičiai	KRE	L	HF_LT79	2013	183	246	237	170	281	263	138	213	149	254	112
KR26P	Kretinga-Vėžaičiai	KRE	P	HF_LT208	2013	200	250	231	170	300	263	138	216	155	245	112
KR27L	Kretinga-Vėžaičiai	KRE	L	HF_LT119	2013	200	250	237	164	281	248	138	216	155	254	118
KR27P	Kretinga-Vėžaičiai	KRE	P	HF_LT163	2013	200	250	237	170	281	248	138	213	149	254	118
KR28L	Kretinga-Vėžaičiai	KRE	L	HF_LT147	2013	200	250	237	170	300	248	138	216	155	254	112
KR28P	Kretinga-Vėžaičiai	KRE	P	HF_LT139	2013	200	250	237	170	300	248	147	216	155	245	112
KR29L	Kretinga-Vėžaičiai	KRE	L	HF_LT186	2013	200	250	231	164	300	248	138	216	155	254	112
KR29P	Kretinga-Vėžaičiai	KRE	P	HF_LT103	2013	200	250	237	164	300	248	138	216	149	254	118
KR30L	Kretinga-Vėžaičiai	KRE	L	HF_LT32	2013	183	250	237	170	300	263	138	216	149	245	118
KR30P	Kretinga-Vėžaičiai	KRE	P	HF_LT148	2013	200	250	237	170	300	248	138	216	155	254	118
KR31L	Kretinga-Vėžaičiai	KRE	L	HF_LT207	2013	200	250	231	170	300	263	147	216	149	254	118
KR32P	Kretinga-Vėžaičiai	KRE	P	HF_LT165	2013	200	250	237	170	281	248	138	216	155	245	118
KR33P	Kretinga-Vėžaičiai	KRE	P	HF_LT237	2013	200	246	237	170	281	248	138	216	155	254	118
KR34P	Kretinga-Vėžaičiai	KRE	P	HF_LT189	2013	200	250	231	164	300	248	138	216	149	254	112
KR35L	Kretinga-Vėžaičiai	KRE	L	HF_LT154	2013	200	250	237	170	281	263	138	216	155	254	112
KR35P	Kretinga-Vėžaičiai	KRE	P	HF_LT128	2013	200	250	237	170	300	263	147	216	155	254	118
KR36L	Kretinga-Vėžaičiai	KRE	L	HF_LT141	2013	200	250	237	170	300	248	147	216	155	254	112
KR36P	Kretinga-Vėžaičiai	KRE	P	HF_LT158	2013	200	250	237	170	281	263	138	216	149	254	118
KR37L	Kretinga-Vėžaičiai	KRE	L	HF_LT169	2013	200	250	237	170	281	248	138	216	149	254	112
KR37P	Kretinga-Vėžaičiai	KRE	P	HF_LT169	2013	200	250	237	170	281	248	138	216	149	254	112
KR38P	Kretinga-Vėžaičiai	KRE	P	HF_LT25	2013	183	250	237	164	281	248	138	216	149	245	112

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
KR39L	Kretinga-Vėžaičiai	KRE	L	HF_LT144	2013	200	250	237	170	300	248	147	216	149	254	112
KR39P	Kretinga-Vėžaičiai	KRE	P	HF_LT119	2013	200	250	237	164	281	248	138	216	155	254	118
KR40P	Kretinga-Vėžaičiai	KRE	P	HF_LT102	2013	200	250	237	164	300	248	138	216	149	254	112
KR42L	Kretinga-Vėžaičiai	KRE	L	HF_LT92	2013	200	250	237	164	300	248	147	216	155	245	118
KR43L	Kretinga-Vėžaičiai	KRE	L	HF_LT110	2013	200	250	237	164	281	263	138	216	149	254	118
KR46L	Kretinga-Vėžaičiai	KRE	L	HF_LT62	2013	183	250	231	170	300	263	138	216	149	254	112
KR47L	Kretinga-Vėžaičiai	KRE	L	HF_LT213	2013	200	250	231	170	300	248	147	216	149	245	112
KR49L	Kretinga-Vėžaičiai	KRE	L	HF_LT110	2013	200	250	237	164	281	263	138	216	149	254	118
KR50L	Kretinga-Vėžaičiai	KRE	L	HF_LT182	2013	200	250	231	164	300	248	147	216	155	245	118
KR51L	Kretinga-Vėžaičiai	KRE	L	HF_LT204	2013	200	250	231	164	281	248	138	216	149	254	118
KR52L	Kretinga-Vėžaičiai	KRE	L	HF_LT19	2013	183	250	237	164	281	263	138	216	155	254	112
KR53L	Kretinga-Vėžaičiai	KRE	L	HF_LT136	2013	200	250	237	170	300	263	138	216	149	245	118
KR54L	Kretinga-Vėžaičiai	KRE	L	HF_LT99	2013	200	250	237	164	300	248	138	216	155	254	112
KR55L	Kretinga-Vėžaičiai	KRE	L	HF_LT38	2013	183	250	237	170	300	248	138	216	149	254	118
KR56L	Kretinga-Vėžaičiai	KRE	L	HF_LT211	2013	200	250	231	170	300	263	138	216	149	254	118
KR57L	Kretinga-Vėžaičiai	KRE	L	HF_LT189	2013	200	250	231	164	300	248	138	216	149	254	112
KR58L	Kretinga-Vėžaičiai	KRE	L	HF_LT88	2013	200	250	237	164	300	263	138	216	149	254	112
KU1P	Kėdainiai-Skaistgirys	KED	P	HF_LT151	2012	200	250	237	170	300	248	138	216	149	254	118
KU2L	Kėdainiai-Skaistgirys	KED	L	HF_LT41	2012	183	250	237	170	281	248	147	216	155	254	112
KU2P	Kėdainiai-Skaistgirys	KED	P	HF_LT150	2012	200	250	237	170	300	248	138	216	149	254	112
KU3L	Kėdainiai-Skaistgirys	KED	L	HF_LT89	2012	200	250	237	164	300	263	138	216	149	254	118
KU3P	Kėdainiai-Skaistgirys	KED	P	HF_LT110	2012	200	250	237	164	281	263	138	216	149	254	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
KU4P	Kėdainiai-Skaistgirys	KED	P	HF_LT7	2012	183	250	237	164	300	248	147	216	155	254	112
KU5P	Kėdainiai-Skaistgirys	KED	P	HF_LT202	2012	200	250	231	164	281	248	138	216	149	245	112
KU6P	Kėdainiai-Skaistgirys	KED	P	HF_LT174	2012	200	250	231	164	300	263	147	216	149	245	118
KU7L	Kėdainiai-Skaistgirys	KED	L	HF_LT35	2012	183	250	237	170	300	248	138	216	155	245	118
KU7P	Kėdainiai-Skaistgirys	KED	P	HF_LT20	2012	183	250	237	164	281	263	138	216	149	245	112
KU8P	Kėdainiai-Skaistgirys	KED	P	HF_LT197	2012	200	250	231	164	281	248	147	216	155	254	118
KU9P	Kėdainiai-Skaistgirys	KED	P	HF_LT198	2012	200	250	231	164	281	248	147	216	149	254	112
KU10L	Kėdainiai-Skaistgirys	KED	L	HF_LT205	2012	200	250	231	170	300	263	147	216	155	245	112
KU10P	Kėdainiai-Skaistgirys	KED	P	HF_LT154	2012	200	250	237	170	281	263	138	216	155	254	112
KU13L	Kėdainiai-Skaistgirys	KED	L	HF_LT34	2012	183	250	237	170	300	248	147	216	155	254	118
KU14P	Kėdainiai-Skaistgirys	KED	P	HF_LT189	2012	200	250	231	164	300	248	138	216	149	254	112
KU15L	Kėdainiai-Skaistgirys	KED	L	HF_LT38	2012	183	250	237	170	300	248	138	216	149	254	118
KU15P	Kėdainiai-Skaistgirys	KED	P	HF_LT114	2012	200	250	237	164	281	248	147	216	155	254	118
KU16P	Kėdainiai-Skaistgirys	KED	P	HF_LT209	2012	200	250	231	170	300	263	138	216	155	254	112
KU17P	Kėdainiai-Skaistgirys	KED	P	HF_LT38	2012	183	250	237	170	300	248	138	216	149	254	118
KU18P	Kėdainiai-Skaistgirys	KED	P	HF_LT172	2012	200	250	231	164	300	263	147	216	155	245	118
KU19L	Kėdainiai-Skaistgirys	KED	L	HF_LT144	2012	200	250	237	170	300	248	147	216	149	254	112
KU19P	Kėdainiai-Skaistgirys	KED	P	HF_LT190	2012	200	250	231	164	300	248	138	216	149	254	118
KU20L	Kėdainiai-Skaistgirys	KED	L	HF_LT50	2012	183	250	231	164	300	248	138	216	149	254	118
KU20P	Kėdainiai-Skaistgirys	KED	P	HF_LT93	2012	200	250	237	164	300	248	147	216	155	254	118
KU21L	Kėdainiai-Skaistgirys	KED	L	HF_LT149	2012	200	250	237	170	300	248	138	216	149	245	112
KU22P	Kėdainiai-Skaistgirys	KED	P	HF_LT73	2012	183	250	231	170	281	248	138	216	149	254	112

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
KU23L	Kėdainiai-Skaistgirys	KED	L	HF_LT129	2013	200	250	237	170	300	263	147	216	149	245	112
KU23P	Kėdainiai-Skaistgirys	KED	P	HF_LT114	2013	200	250	237	164	281	248	147	216	155	254	118
KU24P	Kėdainiai-Skaistgirys	KED	P	HF_LT228	2013	200	250	231	170	281	248	138	216	149	254	118
KU25L	Kėdainiai-Skaistgirys	KED	L	HF_LT197	2013	200	250	231	164	281	248	147	216	155	254	118
KU27L	Kėdainiai-Skaistgirys	KED	L	HF_LT148	2013	200	250	237	170	300	248	138	216	155	254	118
KU27P	Kėdainiai-Skaistgirys	KED	P	HF_LT101	2013	200	250	237	164	300	248	138	216	149	245	118
KU28P	Kėdainiai-Skaistgirys	KED	P	HF_LT192	2013	200	250	231	164	281	263	147	216	149	245	118
KU30P	Kėdainiai-Skaistgirys	KED	P	HF_LT226	2013	200	250	231	170	281	248	138	216	149	245	118
KU31L	Kėdainiai-Skaistgirys	KED	L	HF_LT89	2013	200	250	237	164	300	263	138	216	149	254	118
KU31P	Kėdainiai-Skaistgirys	KED	P	HF_LT194	2013	200	250	231	164	281	263	138	216	149	245	112
KU32P	Kėdainiai-Skaistgirys	KED	P	HF_LT101	2013	200	250	237	164	300	248	138	216	149	245	118
KU34L	Kėdainiai-Skaistgirys	KED	L	HF_LT187	2013	200	250	231	164	300	248	138	216	155	254	118
KU34P	Kėdainiai-Skaistgirys	KED	P	HF_LT5	2013	183	250	237	164	300	263	138	216	155	254	112
KU35L	Kėdainiai-Skaistgirys	KED	L	HF_LT187	2013	200	250	231	164	300	248	138	216	155	254	118
KU36L	Kėdainiai-Skaistgirys	KED	L	HF_LT113	2013	200	250	237	164	281	248	147	216	155	254	112
KU37P	Kėdainiai-Skaistgirys	KED	P	HF_LT7	2013	183	250	237	164	300	248	147	216	155	254	112
KU38L	Kėdainiai-Skaistgirys	KED	L	HF_LT43	2013	183	250	237	170	281	248	147	216	149	254	112
KU38P	Kėdainiai-Skaistgirys	KED	P	HF_LT189	2013	200	250	231	164	300	248	138	216	149	254	112
KU39L	Kėdainiai-Skaistgirys	KED	L	HF_LT243	2013	200	246	231	170	281	248	138	216	149	245	112
KU40L	Kėdainiai-Skaistgirys	KED	L	HF_LT143	2013	200	250	237	170	300	248	147	216	149	245	118
KU40P	Kėdainiai-Skaistgirys	KED	P	HF_LT10	2013	183	250	237	164	300	248	147	216	149	254	112
KU41L	Kėdainiai-Skaistgirys	KED	L	HF_LT151	2013	200	250	237	170	300	248	138	216	149	254	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
KU41P	Kėdainiai-Skaistgirys	KED	P	HF_LT12	2013	183	250	237	164	300	248	138	216	155	245	112
KU42L	Kėdainiai-Skaistgirys	KED	L	HF_LT204	2013	200	250	231	164	281	248	138	216	149	254	118
KU42P	Kėdainiai-Skaistgirys	KED	P	HF_LT22	2013	183	250	237	164	281	248	147	216	149	245	112
KU43L	Kėdainiai-Skaistgirys	KED	L	HF_LT109	2013	200	250	237	164	281	263	138	216	155	254	118
KU43P	Kėdainiai-Skaistgirys	KED	P	HF_LT219	2013	200	250	231	170	300	248	138	216	149	254	112
KU47L	Kėdainiai-Skaistgirys	KED	L	HF_LT206	2013	200	250	231	170	300	263	147	216	149	254	112
KU48L	Kėdainiai-Skaistgirys	KED	L	HF_LT92	2013	200	250	237	164	300	248	147	216	155	245	118
KU51L	Kėdainiai-Skaistgirys	KED	L	HF_LT235	2013	200	246	237	170	281	248	147	216	149	245	112
KU55L	Kėdainiai-Skaistgirys	KED	L	HF_LT166	2013	200	250	237	170	281	248	138	216	155	254	112
KU57L	Kėdainiai-Skaistgirys	KED	L	HF_LT227	2013	200	250	231	170	281	248	138	216	149	254	112
KU58L	Kėdainiai-Skaistgirys	KED	L	HF_LT151	2013	200	250	237	170	300	248	138	216	149	254	118
UKM1P	Ukmergė-Deltuva	UKM	P	HF_LT242	2013	200	246	231	170	300	248	138	216	149	254	112
UKM2P	Ukmergė-Deltuva	UKM	P	HF_LT60	2013	183	250	231	170	300	263	147	216	155	254	112
UKM3L	Ukmergė-Deltuva	UKM	L	HF_LT116	2013	200	250	237	164	281	248	147	216	149	254	118
UKM4P	Ukmergė-Deltuva	UKM	P	HF_LT167	2013	200	250	237	170	281	248	138	216	155	254	118
UKM5L	Ukmergė-Deltuva	UKM	L	HF_LT51	2013	183	250	231	164	281	263	147	216	149	245	112
UKM5P	Ukmergė-Deltuva	UKM	P	HF_LT161	2013	200	250	237	170	281	248	147	216	149	254	112
UKM6P	Ukmergė-Deltuva	UKM	P	HF_LT65	2013	183	250	231	170	300	248	147	216	149	254	118
UKM7L	Ukmergė-Deltuva	UKM	L	HF_LT138	2013	200	250	237	170	300	263	138	216	149	254	118
UKM7P	Ukmergė-Deltuva	UKM	P	HF_LT145	2013	200	250	237	170	300	248	147	216	149	254	118
UKM8P	Ukmergė-Deltuva	UKM	P	HF_LT156	2013	200	250	237	170	281	263	138	216	149	245	118
UKM9L	Ukmergė-Deltuva	UKM	L	HF_LT225	2013	200	250	231	170	281	248	147	216	149	254	112

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
UKM9P	Ukmergė-Deltuva	UKM	P	HF_LT222	2013	200	250	231	170	281	263	138	216	155	254	118
UKM10P	Ukmergė-Deltuva	UKM	P	HF_LT206	2013	200	250	231	170	300	263	147	216	149	254	112
UKM11L	Ukmergė-Deltuva	UKM	L	HF_LT100	2013	200	250	237	164	300	248	138	216	155	254	118
UKM11P	Ukmergė-Deltuva	UKM	P	HF_LT185	2013	200	250	231	164	300	248	138	216	155	245	118
UKM12L	Ukmergė-Deltuva	UKM	L	HF_LT170	2013	200	250	237	170	281	248	138	216	149	254	118
UKM12P	Ukmergė-Deltuva	UKM	P	HF_LT72	2013	183	250	231	170	281	248	138	216	149	245	112
UKM13L	Ukmergė-Deltuva	UKM	L	HF_LT93	2013	200	250	237	164	300	248	147	216	155	254	118
UKM13P	Ukmergė-Deltuva	UKM	P	HF_LT86	2013	200	250	237	164	300	263	147	216	155	245	118
UKM14L	Ukmergė-Deltuva	UKM	L	HF_LT114	2013	200	250	237	164	281	248	147	216	155	254	118
UKM14P	Ukmergė-Deltuva	UKM	P	HF_LT69	2013	183	250	231	170	281	263	138	216	155	254	112
UKM15L	Ukmergė-Deltuva	UKM	L	HF_LT164	2013	200	250	237	170	281	248	138	216	155	245	112
UKM15P	Ukmergė-Deltuva	UKM	P	HF_LT182	2013	200	250	231	164	300	248	147	216	155	245	118
UKM16L	Ukmergė-Deltuva	UKM	L	HF_LT89	2013	200	250	237	164	300	263	138	216	149	254	118
UKM16P	Ukmergė-Deltuva	UKM	P	HF_LT99	2013	200	250	237	164	300	248	138	216	155	254	112
UKM17L	Ukmergė-Deltuva	UKM	L	HF_LT98	2013	200	250	237	164	300	248	138	216	155	245	118
UKM17P	Ukmergė-Deltuva	UKM	P	HF_LT6	2013	183	250	237	164	300	263	138	216	149	254	118
UKM18L	Ukmergė-Deltuva	UKM	L	HF_LT102	2013	200	250	237	164	300	248	138	216	149	254	112
UKM18P	Ukmergė-Deltuva	UKM	P	HF_LT95	2013	200	250	237	164	300	248	147	216	149	254	112
UKM19L	Ukmergė-Deltuva	UKM	L	HF_LT110	2013	200	250	237	164	281	263	138	216	149	254	118
UKM20L	Ukmergė-Deltuva	UKM	L	HF_LT91	2013	200	250	237	164	300	248	147	216	155	245	112
UKM20P	Ukmergė-Deltuva	UKM	P	HF_LT173	2013	200	250	231	164	300	263	147	216	155	254	118
UKM21L	Ukmergė-Deltuva	UKM	L	HF_LT50	2013	183	250	231	164	300	248	138	216	149	254	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
UKM21P	Ukmergė-Deltuva	UKM	P	HF_LT198	2013	200	250	231	164	281	248	147	216	149	254	112
UKM22L	Ukmergė-Deltuva	UKM	L	HF_LT9	2013	183	250	237	164	300	248	147	216	149	245	118
UKM22P	Ukmergė-Deltuva	UKM	P	HF_LT36	2013	183	250	237	170	300	248	138	216	155	254	118
UKM23L	Ukmergė-Deltuva	UKM	L	HF_LT157	2013	200	250	237	170	281	263	138	216	149	254	112
UKM23P	Ukmergė-Deltuva	UKM	P	HF_LT68	2013	183	250	231	170	281	263	138	216	155	245	112
UKM24L	Ukmergė-Deltuva	UKM	L	HF_LT78	2013	183	246	237	170	300	248	147	216	149	254	118
UKM24P	Ukmergė-Deltuva	UKM	P	HF_LT154	2013	200	250	237	170	281	263	138	216	155	254	112
UKM25L	Ukmergė-Deltuva	UKM	L	HF_LT4	2013	183	250	237	164	300	263	147	216	149	254	112
UKM25P	Ukmergė-Deltuva	UKM	P	HF_LT99	2013	200	250	237	164	300	248	138	216	155	254	112
UKM26L	Ukmergė-Deltuva	UKM	L	HF_LT47	2013	183	250	231	164	300	263	138	216	149	254	118
UKM26P	Ukmergė-Deltuva	UKM	P	HF_LT122	2013	200	250	237	164	281	248	138	216	149	254	118
UKM27L	Ukmergė-Deltuva	UKM	L	HF_LT169	2013	200	250	237	170	281	248	138	216	149	254	112
UKM27P	Ukmergė-Deltuva	UKM	P	HF_LT200	2013	200	250	231	164	281	248	138	216	155	245	112
UKM28L	Ukmergė-Deltuva	UKM	L	HF_LT70	2013	183	250	231	170	281	263	138	216	149	245	112
UKM28P	Ukmergė-Deltuva	UKM	P	HF_LT63	2013	183	250	231	170	300	248	147	216	155	254	112
UKM29P	Ukmergė-Deltuva	UKM	P	HF_LT148	2013	200	250	237	170	300	248	138	216	155	254	118
UKM30L	Ukmergė-Deltuva	UKM	L	HF_LT171	2013	200	250	231	164	285	248	138	216	149	254	112
UKM31L	Ukmergė-Deltuva	UKM	L	HF_LT220	2013	200	250	231	170	300	248	138	216	149	254	118
UKM31P	Ukmergė-Deltuva	UKM	P	HF_LT96	2013	200	250	237	164	300	248	147	216	149	254	118
UKM32L	Ukmergė-Deltuva	UKM	L	HF_LT84	2013	200	250	237	164	296	263	138	216	149	254	112
UKM32P	Ukmergė-Deltuva	UKM	P	HF_LT55	2013	183	250	231	164	281	248	147	216	149	254	112
UKM33L	Ukmergė-Deltuva	UKM	L	HF_LT165	2013	200	250	237	170	281	248	138	216	155	245	118

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
UKM33P	Ukmergė-Deltuva	UKM	P	HF_LT26	2013	183	250	237	164	281	248	138	216	149	254	118
UKM34L	Ukmergė-Deltuva	UKM	L	HF_LT82	2013	183	246	231	170	281	248	138	216	155	254	118
UKM34P	Ukmergė-Deltuva	UKM	P	HF_LT195	2013	200	250	231	164	281	263	138	216	149	254	112
UKM35L	Ukmergė-Deltuva	UKM	L	HF_LT112	2013	200	250	237	164	281	248	147	216	155	245	118
UKM35P	Ukmergė-Deltuva	UKM	P	HF_LT177	2013	200	250	231	164	300	263	138	216	155	245	118
UKM36L	Ukmergė-Deltuva	UKM	L	HF_LT37	2013	183	250	237	170	300	248	138	216	149	254	112
UKM36P	Ukmergė-Deltuva	UKM	P	HF_LT47	2013	183	250	231	164	300	263	138	216	149	254	118
UKM37L	Ukmergė-Deltuva	UKM	L	HF_LT199	2013	200	250	231	164	281	248	147	216	149	254	118
UKM39L	Ukmergė-Deltuva	UKM	L	HF_LT234	2013	200	246	237	170	281	263	147	216	155	245	118
UKM39P	Ukmergė-Deltuva	UKM	P	HF_LT3	2013	183	250	237	164	300	268	147	216	149	254	112
UKM40L	Ukmergė-Deltuva	UKM	L	HF_LT236	2013	200	246	237	170	281	248	147	216	149	254	118
UKM40P	Ukmergė-Deltuva	UKM	P	HF_LT227	2013	200	250	231	170	281	248	138	216	149	254	112
UKM41L	Ukmergė-Deltuva	UKM	L	HF_LT115	2013	200	250	237	164	281	248	147	216	149	245	118
UKM41P	Ukmergė-Deltuva	UKM	P	HF_LT154	2013	200	250	237	170	281	263	138	216	155	254	112
UKM42L	Ukmergė-Deltuva	UKM	L	HF_LT81	2013	183	246	231	170	300	263	138	216	155	254	118
UKM42P	Ukmergė-Deltuva	UKM	P	HF_LT50	2013	183	250	231	164	300	248	138	216	149	254	118
UKM43L	Ukmergė-Deltuva	UKM	L	HF_LT40	2013	183	250	237	170	281	263	147	216	149	254	118
UKM44L	Ukmergė-Deltuva	UKM	L	HF_LT165	2013	200	250	237	170	281	248	138	216	155	245	118
UKM45L	Ukmergė-Deltuva	UKM	L	HF_LT133	2013	200	250	237	170	300	263	138	216	155	245	112
UKM46L	Ukmergė-Deltuva	UKM	L	HF_LT139	2013	200	250	237	170	300	248	147	216	155	245	112
UKM49L	Ukmergė-Deltuva	UKM	L	HF_LT89	2013	200	250	237	164	300	263	138	216	149	254	118
UKM50L	Ukmergė-Deltuva	UKM	L	HF_LT85	2013	200	250	237	164	300	263	147	216	155	245	112

APPENDIX 6 (continuation)

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
UKM51L	Ukmergė-Deltuva	UKM	L	HF_LT161	2013	200	250	237	170	281	248	147	216	149	254	112
UKM52L	Ukmergė-Deltuva	UKM	L	HF_LT11	2013	183	250	237	164	300	248	147	216	149	254	118
UKM55L	Ukmergė-Deltuva	UKM	L	HF_LT220	2013	200	250	231	170	300	248	138	216	149	254	118
UKM57L	Ukmergė-Deltuva	UKM	L	HF_LT58	2013	183	250	231	164	281	248	138	216	155	254	118
UKM59L	Ukmergė-Deltuva	UKM	L	HF_LT27	2013	183	250	237	170	285	248	138	216	155	245	118
UKM60L	Ukmergė-Deltuva	UKM	L	HF_LT17	2013	183	250	237	164	300	248	138	216	149	254	118
VI1L	Vilnius-Verkiai	VIL	L	HF_LT16	2012	183	250	237	164	300	248	138	216	149	254	112
VI2P	Vilnius-Verkiai	VIL	P	HF_LT18	2012	183	250	237	164	281	263	138	216	155	245	118
VI4P	Vilnius-Verkiai	VIL	P	HF_LT52	2012	183	250	231	164	281	263	147	216	149	254	118
VI5L	Vilnius-Verkiai	VIL	L	HF_LT2	2012	183	250	237	164	296	248	138	213	155	254	118
VI6L	Vilnius-Verkiai	VIL	L	HF_LT16	2012	183	250	237	164	300	248	138	216	149	254	112
VI6P	Vilnius-Verkiai	VIL	P	HF_LT148	2012	200	250	237	170	300	248	138	216	155	254	118
VI7P	Vilnius-Verkiai	VIL	P	HF_LT101	2012	200	250	237	164	300	248	138	216	149	245	118
VI8P	Vilnius-Verkiai	VIL	P	HF_LT184	2012	200	250	231	164	300	248	147	216	149	254	118
VI9P	Vilnius-Verkiai	VIL	P	HF_LT94	2012	200	250	237	164	300	248	147	216	149	245	118
VI10P	Vilnius-Verkiai	VIL	P	HF_LT43	2012	183	250	237	170	281	248	147	216	149	254	112
VI11P	Vilnius-Verkiai	VIL	P	HF_LT203	2012	200	250	231	164	281	248	138	216	149	254	112
VI13P	Vilnius-Verkiai	VIL	P	HF_LT98	2012	200	250	237	164	300	248	138	216	155	245	118
VI14L	Vilnius-Verkiai	VIL	L	HF_LT179	2012	200	250	231	164	300	263	138	216	155	254	118
VI14P	Vilnius-Verkiai	VIL	P	HF_LT102	2012	200	250	237	164	300	248	138	216	149	254	112
VI15P	Vilnius-Verkiai	VIL	P	HF_LT142	2012	200	250	237	170	300	248	147	216	149	245	112
VI16P	Vilnius-Verkiai	VIL	P	HF_LT71	2012	183	250	231	170	281	248	138	216	155	245	118

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
VI17L	Vilnius-Verkiai	VIL	L	HF_LT99	2013	200	250	237	164	300	248	138	216	155	254	112
VI17P	Vilnius-Verkiai	VIL	P	HF_LT42	2013	183	250	237	170	281	248	147	216	149	245	118
VI18P	Vilnius-Verkiai	VIL	P	HF_LT80	2013	183	246	231	164	300	248	147	216	149	245	118
VI19P	Vilnius-Verkiai	VIL	P	HF_LT120	2013	200	250	237	164	281	248	138	216	149	245	112
VI20P	Vilnius-Verkiai	VIL	P	HF_LT220	2013	200	250	231	170	300	248	138	216	149	254	118
VI21P	Vilnius-Verkiai	VIL	P	HF_LT43	2013	183	250	237	170	281	248	147	216	149	254	112
VI22L	Vilnius-Verkiai	VIL	L	HF_LT197	2013	200	250	231	164	281	248	147	216	155	254	118
VI24L	Vilnius-Verkiai	VIL	L	HF_LT166	2013	200	250	237	170	281	248	138	216	155	254	112
VI24P	Vilnius-Verkiai	VIL	P	HF_LT160	2013	200	250	237	170	281	248	147	216	155	254	118
VI25P	Vilnius-Verkiai	VIL	P	HF_LT199	2013	200	250	231	164	281	248	147	216	149	254	118
VI26P	Vilnius-Verkiai	VIL	P	HF_LT103	2013	200	250	237	164	300	248	138	216	149	254	118
VI27L	Vilnius-Verkiai	VIL	L	HF_LT14	2013	183	250	237	164	300	248	138	216	155	254	112
VI28L	Vilnius-Verkiai	VIL	L	HF_LT216	2013	200	250	231	170	300	248	138	216	155	254	118
VI28P	Vilnius-Verkiai	VIL	P	HF_LT116	2013	200	250	237	164	281	248	147	216	149	254	118
VI29L	Vilnius-Verkiai	VIL	L	HF_LT102	2013	200	250	237	164	300	248	138	216	149	254	112
VI29P	Vilnius-Verkiai	VIL	P	HF_LT204	2013	200	250	231	164	281	248	138	216	149	254	118
VI31L	Vilnius-Verkiai	VIL	L	HF_LT240	2013	200	246	231	170	300	248	147	216	149	254	118
VI31P	Vilnius-Verkiai	VIL	P	HF_LT212	2013	200	250	231	170	300	248	147	216	155	254	118
VI32L	Vilnius-Verkiai	VIL	L	HF_LT238	2013	200	246	237	170	281	248	138	216	149	254	118
VI32P	Vilnius-Verkiai	VIL	P	HF_LT100	2013	200	250	237	164	300	248	138	216	155	254	118
VI33P	Vilnius-Verkiai	VIL	P	HF_LT44	2013	183	250	237	170	281	248	147	216	149	254	118
VI35P	Vilnius-Verkiai	VIL	P	HF_LT155	2013	200	250	237	170	281	263	138	216	155	254	118

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1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
VI36L	Vilnius-Verkiai	VIL	L	HF_LT111	2013	200	250	237	164	281	248	147	216	155	245	112
VI37P	Vilnius-Verkiai	VIL	P	HF_LT144	2013	200	250	237	170	300	248	147	216	149	254	112
VI38L	Vilnius-Verkiai	VIL	L	HF_LT54	2013	183	250	231	164	281	248	147	216	155	254	118
VI38P	Vilnius-Verkiai	VIL	P	HF_LT102	2013	200	250	237	164	300	248	138	216	149	254	112
VI39P	Vilnius-Verkiai	VIL	P	HF_LT191	2013	200	250	231	164	281	263	147	213	155	245	118
VI40L	Vilnius-Verkiai	VIL	L	HF_LT214	2013	200	250	231	170	300	248	138	213	155	254	112
VI40P	Vilnius-Verkiai	VIL	P	HF_LT30	2013	183	250	237	170	300	263	138	216	155	245	112
VI41L	Vilnius-Verkiai	VIL	L	HF_LT109	2013	200	250	237	164	281	263	138	216	155	254	118
VI41P	Vilnius-Verkiai	VIL	P	HF_LT94	2013	200	250	237	164	300	248	147	216	149	245	118
VI44L	Vilnius-Verkiai	VIL	L	HF_LT148	2013	200	250	237	170	300	248	138	216	155	254	118
VI45L	Vilnius-Verkiai	VIL	L	HF_LT233	2013	200	246	237	170	300	248	138	216	155	254	118
VI46L	Vilnius-Verkiai	VIL	L	HF_LT103	2013	200	250	237	164	300	248	138	216	149	254	118
VI47L	Vilnius-Verkiai	VIL	L	HF_LT23	2013	183	250	237	164	281	248	147	216	149	254	118
VI48L	Vilnius-Verkiai	VIL	L	HF_LT223	2013	200	250	231	170	281	263	138	216	149	245	112
VI49L	Vilnius-Verkiai	VIL	L	HF_LT25	2013	183	250	237	164	281	248	138	216	149	245	112
VI51L	Vilnius-Verkiai	VIL	L	HF_LT215	2013	200	250	231	170	300	248	138	216	155	254	112
VI52L	Vilnius-Verkiai	VIL	L	HF_LT33	2013	183	250	237	170	300	248	147	216	155	254	112
VI56L	Vilnius-Verkiai	VIL	L	HF_LT158	2013	200	250	237	170	281	263	138	216	149	254	118