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Health of elms and Dutch elm disease in Estonia

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Abstract During three years, 2014–2016, Dutch elm disease (further DED) was investigated on 1225 elm trees at 4 different sampling sites and 2 sub-sites in Estonia. For the first time, both subspecies of the invasive pathogen *Ophiostoma novo-ulmi*: *O. novo-ulmi* subsp. *novo-ulmi*, and *O. novo-ulmi* subsp. *americana*, were detected by *col1* and *cu* genes in Estonia and north-eastern Europe. *Ophiostoma novo-ulmi* subsp. *americana* was identified only at one site in northern Estonia, in Tallinn. In addition, during our assessments, the health of elms there appeared worse than at other sampling sites: *O. novo-ulmi* subsp. *americana* demonstrated higher aggressiveness. Simultaneous occurrence of both subspecies and their hybrids was not detected. A repeat survey of 109 elms in 2014 and 2016 demonstrated ca. 22% probability of mortality within 24 months, irrespective of urban vs. rural habitat. In sub-site A1 in Tallinn, *O. novo-ulmi* subsp. *americana* has been found since 2013. DED signs were noted on 39% of all 1225 surveyed trees. Among the assessed elm species, *Ulmus laevis* showed higher resistance than *U. glabra*: 82% and 66% of trees, respectively, showed high vitality. In addition, no *U. laevis* trees were found dead, compared to 18% of the *U. glabra*.

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Introduction

Changing climatic conditions (incl. warmer winters) and global trade have aggravated the invasion of plant pathogens (Brasier 2008; Dehnen-Schmutz et al. 2010; Rytkönen et al. 2008, 2011; Müller et al. 2016; Liebhold et al. 2017; Ghelardini et al. 2017). Several invasive pathogens, e.g., *Hymenoscyphus fraxineus, Dothistroma septosporum, Lecanosticta acicola, Diplodia sapinea,* have invaded Estonia, as expected, the result of climate change and international trade (Hanso and Drenkhan 2009, 2013; Drenkhan et al. 2014, 2015, 2016; Adamson et al. 2015a, b, 2018a, b).

The health status of elms in Tallinn, northern Estonia, worsened substantially in 2013. DED was confirmed as the cause (R. Drenkhan, pers. comm.). It is possible that the threat to *Ulmus* spp. has risen in Estonia due to the trade of infected elm plants or wood as shown by La Porta et al. (2008) and Solheim et al. (2011) in other countries, similar to what has happened in Sweden, Norway, the UK and USA (Brasier and Kirk 2010; Solheim et al. 2011; Menkis et al. 2016). Mortality due to DED has decimated elm populations, like in Sweden, where all the native elm species (*U. glabra*, *U. laevis*, *U. minor*) are on the Red List as critically endangered (Barstow and Rivers 2017; Barstow and Harvey-Brown 2017; Barstow

et al. 2017). The DED agent is known to kill trees rapidly (Phillips and Burdekin 1992; Schmidt 2006).

In the forests of Estonia, two native elm species, *U. glabra* and *U. laevis*, occur rarely – forming only 0.1% of the total volume of forest trees (Raudsaar et al. 2016). *Ulmus glabra* can be found throughout Estonia, while *U. laevis* is much rarer and is growing mainly along riversides (Kukk and Kull 2005). *Ulmus laevis* is listed as "near threatened" in Estonia according to the IUCN Red List of Threatened Species (Lilleleht 2008; IUCN 2018; Leht 2018); thus, the value of the tree species is even higher because of ecological and cultural reasons (Martín et al. 2018). Elms are common and valuable amenity trees in urban spaces (Aaspõllu 1999; Kaar 2011) and in rural areas (incl. historical parks and forests) all over Estonia (Abner et al. 2007, 2012). Some of these historical parks had been formed from boreo-nemoral forests (Kalda 1995; Tamm 2007), which are considered good environments for broad-leaved trees such as elms in Estonia (Paal 1998). Groups and even monocultures of elm trees can be found in these parks (Kristian 1939).

Generally, elm is an ecologically important tree genus in the dendroflora of the northern Baltic region. Loss of elms causes a loss of many associated organisms (Thor et al. 2010). For example, 39 different epiphytic lichens, obligate on broadleaved trees, live on *U. glabra* in Estonia, incl. two near-threatened species and one endangered (Jüriado et al. 2009). Two fungi, *Rhodotus palmatus* and *Hymenochaete ulmicola*, obligately associated to elms, are endangered (Corfixen and Parmasto 2005; Kalamees 2011).

Taxonomically and pathologically related species, *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, had infected *Ulmus* species in many parts of Europe (Clinton and McCormick 1936; Schmidt 2006). In Estonia, according to the European and Mediterranean Plant Protection Organization (EPPO) global database, *O. ulmi* has been reported since 1979 (EPPO 2017). Actually, *O. ulmi* has been reported in Estonia since the 1930s (Lepik 1940). In 2006, *O. novo-ulmi* was detected for the first time in Estonia (Hanso and Drenkhan 2007), but at that time it was distinguished neither as a subspecies nor as a hybrid. In Europe, DED is caused by two different subspecies of the pathogen – *O. novo-ulmi* subsp. *novo-ulmi* and *O. novo-ulmi* subsp. *americana* (Brasier and Kirk 2001; Brasier et al. 2004; Martín et al. 2010). Information about the occurrence of the subspecies and their hybrids in regions closest to Estonia comes from southern Norway, Sweden (Brasier and Kirk 2010) and Lithuania (Motiejūnaitė et al. 2016).

Mortality by DED varies also depending on the host (*Ulmus*) species, levels of susceptibility and genetic variation (Martín et al. 2018), as well as stand density and possible rootgrafts (Santini and Faccoli 2013) of trees in natural stands or urban areas, but is also influenced by pathogen spore concentration (Flower et al. 2017). In its natural habitats in Poland, for example, *U. minor* suffered more and *U. laevis* less than *U. glabra* (Łakomy et al. 2016). *Ulmus laevis* is considered less susceptible to DED because it is less attractive to the beetles (Collin 2002).

Insect vectors (e.g. *Scolytus* spp.) are essential agents in spreading *O. novo-ulmi*. It is thought that northern Europe is protected from DED because of a lack of these insect vectors (Caulton et al. 1998; La Porta et al. 2008). *Scolytus* beetles of elms have not been found in Finland (Voolma et al. 2004; Hannunen and Marinova-Todorova 2016), but several of them (e.g., *S. scolytus*, *S. laevis*, *S. multistriatus*, *S. triarmatus*) inhabit Estonia, with some of them discovered as early as the first half of the last century (Voolma et al. 2000, 2004).

So far, DED has not been documented in areas closer to Estonia – Finland and north-west Russia, where the natural species composition is the same (Hannunen and Marinova-Todorova 2016; EPPO 2017); therefore, our investigation should represent the northeastern-most survey of DED in Europe.

The aim of the research is to assess the health conditions of elms affected by DED agents to protect local elm populations over the long term.

The specific objectives of this work were: (1) to isolate and identify the causative agents of DED in Estonia, (2) to monitor the health status of elms in different sampling sites and habitats over three consecutive years, and (3) to assess and compare the vitality of elms affected by the two subspecies of *Ophiostoma novo-ulmi* in two non-consecutive years, 2014 and 2016.

Material and methods

Study sites and health survey of elms

The health status of elms was surveyed over three years (2014–2016) at four different sampling sites (incl. two sub-sites – A1 and B1) in Estonia. One sample collected

by R. Drenkhan in 2013 from Tallinn was also included, as it was the key for this research as DED started to spread in the town quite intensively. The sites were selected based on published information regarding the occurrence of elms in Estonia (Kukk and Kull 2005; Saarse and Veski 2001) and dendrofloristic inventories of parks (Laas and Treumuth 2006; Abner et al. 2007, 2012; Rist 2015). Samples were collected in various urban and rural areas. In total, 1225 mature elm trees were assessed. Of them, 109 (*U. glabra*) were assessed twice in summer of 2014 and summer of 2016 in sub-sites A1 and B1. The time between the assessments was 24 months and both sub-sites have an area of ca. 10 ha (see Fig. 1). The sub-sites A1 and B1 were chosen by pathogen sub-species to analyse the effect of the pathogens on hosts under natural conditions. In this study, urban space includes streets, city parks and urban forests. Selected rural habitats were close to roads (avenues) or situated in rural parks (historical manor parks) and forests. More than half (57%) of the surveyed trees (694) were in urban space, while 531 (43%) were at rural sites. A total of 238 symptomatic samples were collected for laboratory analyses during the three years of this survey.

During the survey, eight different elm taxa and at least two cultivars were assessed. 1016 (82.9%) of the investigated trees were *Ulmus glabra*, 181 (14.8%) were *U. laevis* and 28 trees (2.3%) were hybrids or non-native *Ulmus* species (Table 1).

All the mature elm trees growing at a site were mapped, and identified by species and varieties, according to Hillier Nurseries (1991). The variety (planted along the Euroroute R1 in 2015) 'New Horizon' (Johannes Grothaus, pers. comm.) is a hybrid *Ulmus davidiana* var. *japonica* × *U. pumila*. The presence of bark beetles was assessed according

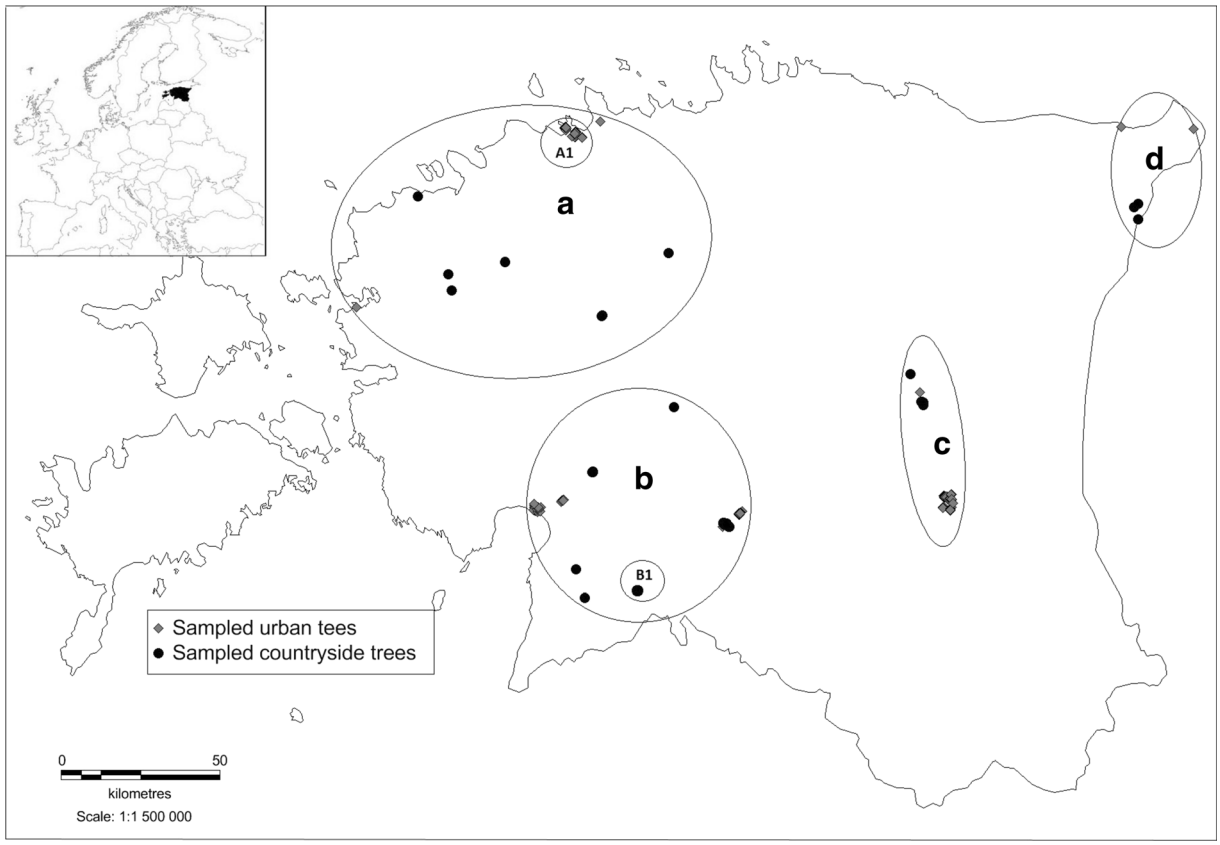


Fig. 1 Locations of the four sampling sites (**a**, **b**, **c**, **d**) in Estonia. The same sample trees (N = 109) were estimated in two non-consecutive years (2014 and 2016) in smaller sub-sites A1, Tallinn and B1, Tihemetsa. At sampling site **a** there are 15 different habitats and 8 of them in Tallinn (in A1–5). At sampling site **b** there are 12 different habitats (incl. B1–1 habitat), at sample site **c** – 10 and at sample site D – 3. DED was isolated from samples collected from sampling sites **a**, **b** and **c**, but not from site **d**

Table 1 Amount of *Ulmus* spp. trees analysed in four sampling sites (see Fig. 1) and habitats

Location	Habitat	Total	Surveyed species			
			Ulmus glabra[a]	*U. laevis*	*U.* hybrid[b]	*U.* spp.[c]
Urban space		694	614	53	20	7
	Park	325	302	21		2
	Street	255	200	30	20	5
Rural		531	402	128		1
	Park	271	225	45		1
	Road avenue	21	5	16		
	Forest	239	172	67		
Number of trees		1225	1016	181	20	8
Total assessed trees (%)		100	82.9	14.8	1.6	0.7

[a] Number of surveyed trees contains different *Ulmus glabra* cultivars: 'Camperdownii' (11),'Exoniensis' (25)

[b] *Ulmus davidiana* var. *japonica* × *U. pumila* 'New Horizon' (20)

[c] Other non-native species: *U. minor* (5), *U. procera* (1), *U. minor* f. *suberosa* (1), *U. pumila* (1)

to Atkinson (2017) and Süda (2006). Symptomatic foliage conditions, incl. wilting, yellowing and browning of leaves, were considered to be caused by DED agent (Solheim et al. 2011).

The survey was carried out from June to October in three consecutive years (2014–2016). Five general crown vitality classes were determined by visual assessment according to Rosenvald et al. (2015) with some original genus-specific modifications and corrections for *Ulmus* spp. (see Fig. 2).

Crown conditions were estimated as: healthy (no visible wilting of leaves in the crown); branch loss (several branches dead and/or up to one-quarter of the crown consisting of wilting of leaves and dry branches); damaged (many dead branches and/or up to half of the crown consisting of wilting of leaves and dead branches); dying (less than a half of live branches remaining) and dead trees (no live branches remaining).

If external disease symptoms were obvious and typical for DED (Santini and Faccoli 2013) and dark brown dots or rings in xylem of symptomatic twigs were confirmed (Łakomy et al. 2016), the samples were taken for laboratory analyses. Twigs or shoots were cut with telescopic secateurs that were sterilized after each cut. Each sample was separately packed into a labelled sterile plastic bag, transported to the lab and stored for a maximum of 1 week at +4 °C until the fungal isolations.

The presence of elm bark beetles was determined on the trunk of every assessed tree at up to 2 m height, noting the occurrence of entrance holes and larval galleries (Santini and Faccoli 2013).

The maps were compiled using MapInfo Professional version 15 (Pitney Bowes Software 2015).

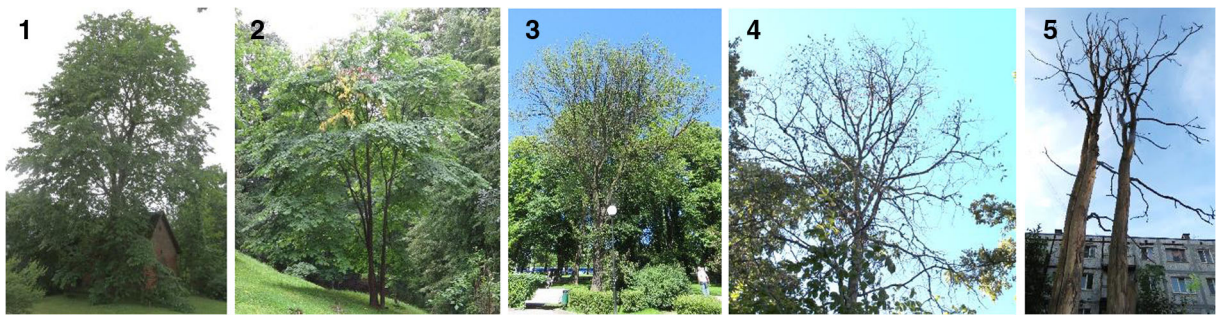


Fig. 2 Crown vitality classes of surveyed *Ulmus* spp. trees (here illustrated by *U. glabra*): 1, healthy; 2, branch loss; 3, damaged; 4, dying; 5, dead

Climate conditions in the years of assessment

Climate data from meteorological stations near the sites (Keskkonnaagentuur 2015, 2016, 2017) during the survey years (2014–2016), along with average values since 1981, are shown in Table 2.

Fungal isolation and DNA extraction

Pathogens and other fungi (Appendix Table 6) were isolated from the symptomatic shoots similar to Drenkhan et al. (2017) with some modifications:

1. Bark of the symptomatic shoots was peeled off with a sterile scalpel and a thin layer of wood was removed up to dark brown rings in xylem. After that, small pieces of the infected wood tissue were placed on sterile MEA (Malt Extract Agar) and incubated at room temperature for 7–14 days.
2. Subcultures were made by transferring small amounts of mycelium from colonies into new plates and incubated for ca. 14 days.
3. Ca. 0.04 g of mycelium taken from the culture was transferred into 2.0 ml micro centrifuge tubes for DNA extraction using a Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, EU).

DNA was stored at −20 °C until further analyses.

PCR and sequencing

Species-specific PCR primers mtsr1 (5′-AGTG GTGTACAGGTGAG-′3) and mtsr2 (5′-CGAG TGGTTAGTACAATCC-′3) (Gibb and Hausner 2005) were used for quick detection of *O. ulmi* and *O. novo-ulmi* from mycelial DNA. Then subspecies of *Ophiostoma novo-ulmi* were detected from mycelial DNA by the gene *col1* species-specific primed PCR (SSPP) that was performed using the primer pair F-primer (5′-GCAGTTGTTGACATGTATG-′3) and R-primer (5′-TGCTTGACGTAGATCTCG-3′) described by Konrad et al. (2002). The *cu* gene region was amplified with the primers CU1 (5′-GGGCAGCTTACCAG AGTGAAC-3′) and CU2 (5′-GCGTTATGATGTAG CGGTGGC-3′) (Pipe et al. 1997) and then digested by restriction enzyme *Hph* I (New England Biolabs, USA) to also identify subspecies of *Ophiostoma novo-ulmi* (see Konrad et al. 2002; Dvořák et al. 2007, and the manufacturer's instructions). The purpose of analysing the two genes (*col1* and *cu*) of *Ophiostoma novo-ulmi* was to detect hybridization of the pathogen (Dvořák et al. 2007; Tziros et al. 2017).

Detection of *Ophiostoma* sp. and other fungi was performed from DNA extracted from pure cultures using the fungal-specific ITS PCR primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA -3′; Gardes and Bruns 1993) and ITS4 (5′- CCTCCGCTTATTGA TATGC -3′; White et al. 1990) and carried out as described by Drenkhan et al. (2017).

PCR products of randomly chosen samples of all symptomatic hosts from the above-mentioned sampling

Table 2 Minimum, maximum, and mean temperatures and precipitation for the years 2014, 2015, and 2016 and for 1981–2016, including vegetation periods, from two meteorological stations

Sampling sites and meteorological stations	Calendar year	Air temperature (°C)				Precipitation sum (mm)	
		Annual min	Annual max	Annual mean	Vegetation period mean	Vegetation period	Annual
A and Tallinn-Harku	2014	–18.7	31.6	6.8	13.0	328.6	575.9
	2015	–12.3	28.4	7.5	12.3	328.6	590.0
	2016	–20.9	27.7	6.6	13.2	456.2	773.7
	1981–2016			7.5	12.8	393.6	622.8
B and Pärnu-Sauga	2014	–22.7	31.8	7.0	13.5	427.9	740.5
	2015	–14.5	29.3	7.5	12.6	372.5	729.9
	2016	–25.1	29.8	6.7	13.7	425.5	745.7
	1981–2016			7.0	13.6	402.8	773.5

sites were sequenced in order to find the subspecies and spread of their hybrids.

The PCR products were visualized on 1% agarose (SeaKem® LE Agarose, Lonza) gels under UV light using the Quantum ST4-system (VilberLourmat SAS, Marne-la-allée, France). All amplifications were performed at least twice to ensure consistent banding patterns.

Randomly chosen samples from different sites and hosts were sequenced at the Estonian Biocentre in Tartu, using the primers ITS5 (5′-GGAAGTAAAAGTCG TAACAAGG-3′; White et al. 1990), and primers F and R for sequencing of *col1* gene (Konrad et al. 2002). The sequences were edited using the BioEdit program, Version 7.2.5 (Hall 2013) and deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) (see Table 3). BLAST searches for the fungal taxa confirmation were performed using the GenBank database (https://www.ncbi.nlm.nih.gov/). ITS sequence similarity threshold was ≥99% for *Ophiostoma* spp. and ≥ 97% for other fungal species detection. The *col1* gene sequence similarity threshold was ≥99% for *Ophiostoma novo-ulmi* subspecies detection.

Statistical analyses

Statistical analyses were carried out to evaluate for changes in host vitality, for probable change in tree condition, and for analyses of the impact of habitat (rural versus urban area) on the health of elms.

Two sets of analyses were carried out for estimation of health condition change of elms between 2014 and 2016 in sub-sites A1 and B1 (Fig. 5). In this case, the regression formula (1) was used:

$$\mathrm{Vc}_{2016} = a_0 + a_1 \cdot \mathrm{Vc}_{2014}, \quad (1)$$

where Vc_{2016} represents the vitality class in 2016; Vc_{2014} is the vitality class in 2014, and a_0, a_1 are regression coefficients.

For prognosis of changing tree conditions over 2 years, regression formula (2) was used to guarantee regression pervasion through point (5; 5) (see Fig. 6):

$$\mathrm{Vc}_{2016} - 5 = a \cdot (\mathrm{Vc}_{2014} - 5), \quad (2)$$

where Vc_{2016} is the vitality class in 2016; Vc_{2014} is the vitality class in 2014, and a represents the regression coefficient.

Regression analyses were implemented for sub-sites A1 and B1 together (see Fig. 6) and separately to compare the health conditions (formula 4) of elm trees on these sites.

Location identifiers (sub-sites A1 and B1) were nominal characteristics. The nominal features were changed to numerical. It was assigned one for sub-site A1 (C_A = 1) to calculate this characteristic for sub-site B1 with the next formula (3):

$$C_B = \frac{a_B}{a_A}, \quad (3)$$

where C_B is the numeric value for nominal characteristic "Sub-site B1", and a_B and a_A are constants for regression formula 2 according to the data (sub-site B1 and A1). Values depending on location (C_B and C_A) were added to the data. For the following regression analyses (see Fig. 6) the numeric characteristic of location was added, and the formula was used as follows (4):

$$\mathrm{Vc}_{2016} - 5 = a \cdot (\mathrm{Vc}_{2014} - 5) \cdot C, \quad (4)$$

where Vc_{2016} represents the vitality class in 2016; Vc_{2014} is the vitality class in 2014, C is the characteristic of sub-site A1 or B1, and a is a coefficient.

Regression analyses were used for calculating health conditions in different habitats of native elm species. Exotic species and cultivars were excluded from these calculations.

Results

Dutch elm disease agents at different sampling sites and on different hosts

In total 238 samples were collected, from which 76 pure cultures of *Ophiostoma novo-ulmi* were successfully isolated (see Fungal isolation and DNA extraction in Material and methods). The species-specific PCR primers were used to check all the strains, which were *O. novo-ulmi* (Gibb and Hausner 2005). *Col1* gene sequencing confirmed the first occurrence of both known *O. novo-ulmi* subspecies in Estonia (see Table 3), and *O. ulmi* was not found. *Ophiostoma novo-ulmi* subsp. *novo-ulmi* was found in sampling sites B and C situated in southwest and central Estonia (Fig. 1). *Ophiostoma novo-ulmi* subsp. *americana* was found

Table 3 Origin of isolations from symptomatic samples of different *Ulmus* species and molecularly detected DED agents by gene *col1* and digested gene *cu* with enzyme *Hph* I, accession No in GenBank (NCBI) by *col1* gene and ITS

No	Identification No	Sampling				Host species	Tested isolations of SSPP *Ophiostoma novo-ulmi*	Molecular identification *Ophiostoma novo-ulmi* subsp.		Accession no. GenBank	
		site or sub-site	date	location	habitat			by *col1*	by *cu*+ *Hph* I	by *col1* gene	by ITS
1	1050	A	18.06.2013	urban space	park	*Ulmus glabra*	+	*americana*	*americana*	MF784565	MF754038
2	4651	A	02.07.2015	urban space	park	*U. glabra*	+	*americana*	*americana*		
3	4652	A	02.07.2015	urban space	street	*U. glabra*	+	*americana*	*americana*		
4	6839	A1	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*	MF784568	MF766443
5	6833	A1	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*		
6	6835	A1	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*		
7	6836	A1	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*		
8	6842	A1	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*		
9	6845	A	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*		
10	6880; 6840	A1	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*		
11	8326	A1	14.07.2016	urban space	street	*U. glabra*	+	*americana*	*americana*		
12	4533	B	01.07.2015	urban space	forest	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*	MF784566	MF754039
13	4534	B	01.07.2015	urban space	forest	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*		
14	4618	B	01.07.2015	urban space	forest	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*		
15	4638	B	19.07.2015	rural	park	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*	MF784567	MF766442
16	4751	B	24.08.2015	rural	avenue	*U. laevis*	+	*novo-ulmi*	*novo-ulmi*		
17	6851	B1	11.05.2016	rural	park	*U. glabra* 'Camperdownii'	+	*novo-ulmi*	*novo-ulmi*	MF784570	MF766445
18	6856	B	09.07.2016	urban	park	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*	MF784571	MF766446
19	6963	B	09.07.2016	rural	park	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*		
20	6843	B1	10.07.2016	rural	park	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*		
21	6855	B1	10.07.2016	rural	park	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*		
22	6975	B	20.07.2016	rural	forest	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*		
23	6948	B	20.07.2016	rural	forest	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*	MF784572	MF766447
24	6952	B	20.07.2016	rural	forest	*U. laevis*	+	*novo-ulmi*	*novo-ulmi*	MF784573	MF766448
25	6844	C	03.07.2016	urban	park	*U. glabra*	+	*novo-ulmi*	*novo-ulmi*	MF784569	MF766444

Identification No – in collection of laboratory of forest pathology of Estonian University of Life Sciences

SSPP – species-specific PCR primers (Gibb & Hausner, 2005) were used to detect *Ophiostoma novo-ulmi* or *O. ulmi* for all the isolations

col1 – the colony type gene (Konrad et al. 2002); the sequence similarity threshold ≥ 99% for *Ophiostoma novo-ulmi* subspecies detection

cu+*Hph* I – ceratoulmin gene - RFLP banding pattern of *cu* from *O. novo-ulmi* digested with enzyme *Hph* I (Konrad et al. 2002)

ITS – internal transcribed spacer; the sequence similarity threshold is ≥ 99% for *Ophiostoma* spp. detection

GenBank (NCBI- https://www.ncbi.nlm.nih.gov/)

only in Tallinn, at a location in sub-site A1 (see Fig. 1). *O. novo-ulmi* subsp. *novo-ulmi* was detected on *U. glabra* and its variety 'Camperdownii', and on *U. laevis*. *O. novo-ulmi* subsp. *americana* was found on *U. glabra* in Tallinn (see Table 3). No *O. novo-ulmi* was found in northeast Estonia (sampling site D) – neither in urban spaces nor at rural sites. *Ophiostoma novo-ulmi* hybrids by colony type (*col1*) gene and ceratoulmin gene (*cu*) were not detected at any sampling sites in Estonia (see Table 3).

Insects on assessed trees

All the assessed trees (N = 1225) were examined for signs of elm bark beetles, e.g., entrance holes, larval galleries etc. Thirty randomly selected *U. glabra* sample trees had entrance holes of bark beetles. Larval galleries were found only on dead trees at sample sites A and B, but not at site C. Those galleries belonged to *Scolytus multistriatus*, *S. scolytus* or *S. triarmatus* (K. Voolma, entomologist, pers. comm.). Signs of the latter two species were considered so similar that it was impossible to identify the species without seeing the adult beetles (see Süda 2006).

Health condition of different elm species in Estonia

At the sampling sites, the dieback or death of elm trees was considered to be caused by DED. Among all assessed trees (N = 1225) *U. laevis* showed significantly ($p < 0.001$) higher vitality than *U. glabra*, with 82% of *U. laevis* and only 66% of *U. glabra* trees rated as vitality class 1 and 2. 18% of *U. glabra* trees, but no *U. laevis* trees were found dead (Fig. 3).

Correlation between DED symptoms and vitality class of native elm species (*U. glabra* and *U. laevis*) showed that 29% or 30% of DED-symptomatic trees were in vitality classes 2 and 3, but 80% in class 4 (Fig. 4). This data shows that health of elm species in Estonia (indicated by vitality class) correlates significantly ($p < 0.001$) with the DED symptoms.

Vitality changes of elms in sub-sites A1 and B1 from 2014 to 2016

In this health analysis, we considered trees that were assessed in 2014 and 2016 (N = 109). All these trees were *U. glabra,* 72 at sub-site A1 and 37 at B1. Over this period, the proportion of healthy *Ulmus* spp. fell significantly ($p < 0.001$). In 2014 about 50% of assessed trees were healthy, but in 2016 only five (5%) of the 109 trees investigated were still healthy. Ten sampled trees (9%) were found dead in 2014. In 2016 a total of 32 trees (29%) were dead (Fig. 5).

From 2014 to 2016, the vitality of elms clearly diminished according to the repeated vitality estimation of the same 109 trees in sub-sites A1 and B1. The calculated regression line (formula 2, see the "Statistical analyses" section) shows that during these 24 months the health condition of each sampled tree decreased by ca. 0.77 vitality class (Fig. 5). In 2014 we classified 22 trees (20%) as vitality class 1 to 4, but after 24 months all these trees were dead.

One purpose of this study was to describe the vitality change of elms in this period at two sub-sites (A1 and B1) as *O. novo-ulmi* subsp. *americana* was causing damage in one location (Tallinn) at the sub-site A1, but *O. novo-ulmi* subsp. *novo-ulmi* was causing damage at sub-site B1. The vitality changes between 2014 and 2016 (24 months) of the surveyed trees was calculated by formula 2 (see Fig. 6, for details see Table 4). The effect of the two sub-sites (separately A1 and B1) on the vitality of elms was calculated by regression formulas 3 and 4 (see "Statistical analyses" section). This analysis showed that the vitality of elms was significantly lower ($p < 0.00001$) at sub-site A1 than at sub-site B1 (see Fig. 6). 18 elms (25%) at A1 and 4 elms (12%) at B1 were living in 2014, but dead by 2016.

Taking into account that the assessed DED-infected elms (N = 109) at sub-sites A1 and B1, the probability of dying after 24 months for elms in vitality classes 1–4 was 22%, and in class 4 (already dying trees) 78% (see Table 5). The data did not indicate that the health condition of elm trees improved during the monitoring years (see Figs. 5 and 6).

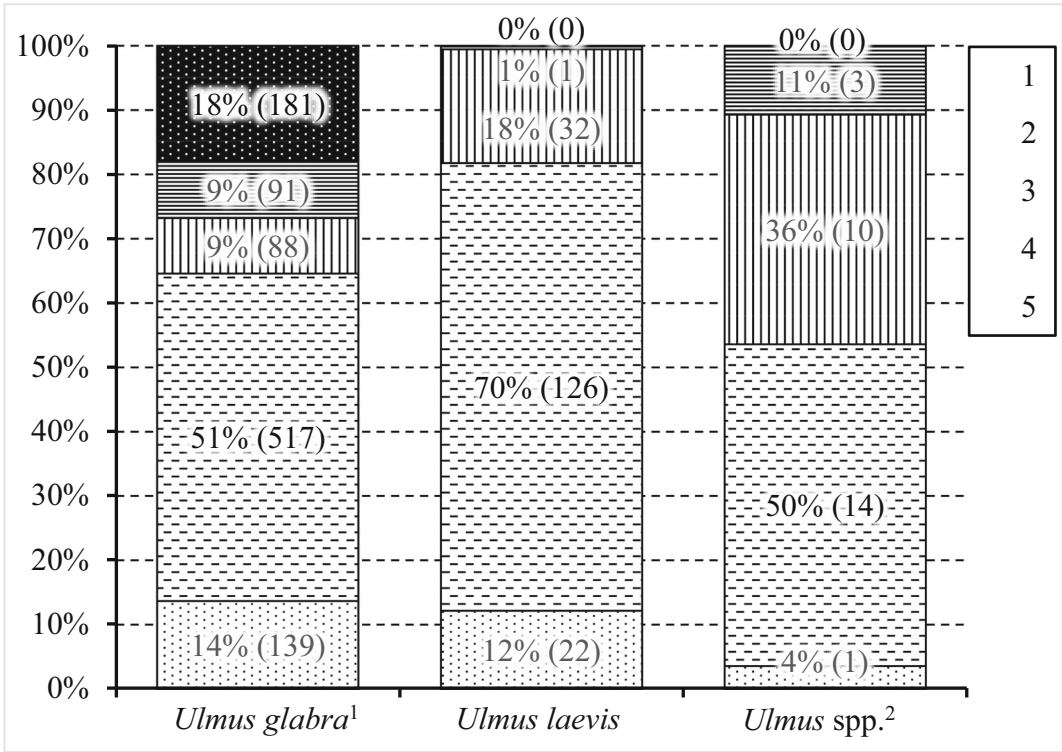
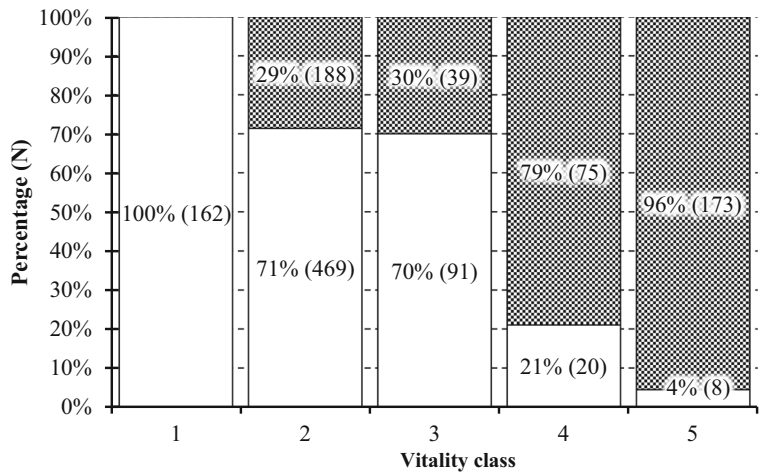


Fig. 3 Health conditions of *U. glabra, U. laevis* and other *Ulmus* spp. based on their representation in vitality classes 1–5, see Fig. 2. The percentage of sampled trees and the number of trees (N) are listed per vitality class. [1]The number of surveyed trees consists mainly of *Ulmus glabra* and its cultivars: 'Camperdownii' (11 trees), 'Exoniensis' (25). [2]Identified exotic *Ulmus* species: *Ulmus davidiana* var. *japonica* × *U. pumila* 'New Horizon' (20), *U. procera* (1), *U. minor* (5), *U. minor* f. *suberosa* (1), *U. pumila* (1)

Fig. 4 The relative incidence of symptoms of DED (white area without symptoms, squared area – with symptoms) on *U. glabra* and *U. laevis* depending on vitality class (see Fig. 2), the percentage of representatives and number of assessed trees (N) in a definite vitality class



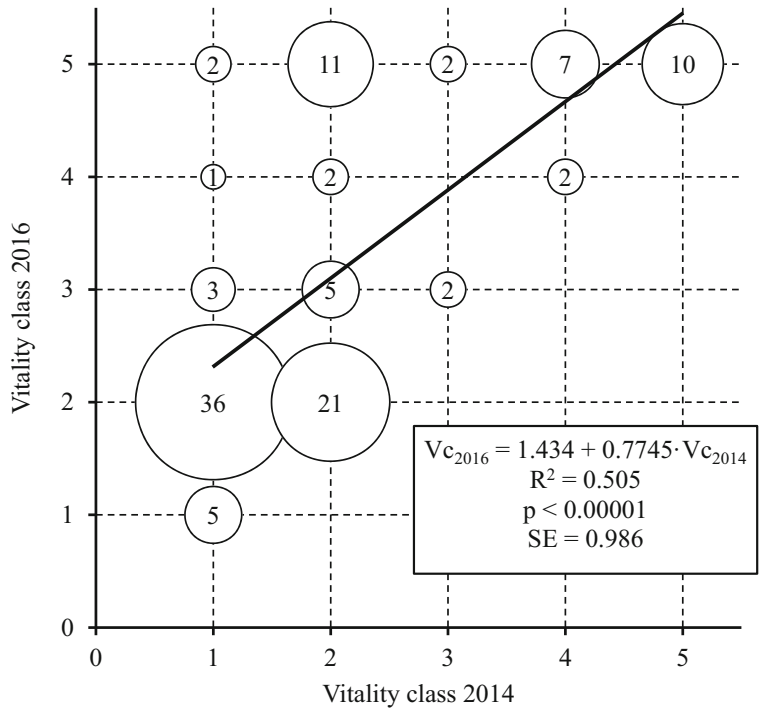


Fig. 5 Change in the vitality of surveyed elms (N = 109) during 24 months (from 2014 summer to 2016 summer). The numbers (in circles) indicate how many trees have kept or lost their vitality. The bigger circles show the higher number of trees in a definite vitality class in 2016. The rising line shows the growing decline of vitality for 109 surveyed elms at two sub-sites (A1 and B1)

Health conditions of elms at two different locations

Of the assessed elm trees, 36% urban sites and 43% of rural sites had symptoms of DED, a statistically insignificant difference ($p > 0.05$). This did not show differences in health conditions of elms at any sampling site. The differences between the meteorological characteristics (mean temperatures and precipitation sums) of the two meteorological stations compared over the surveyed years (2014–2016) were statistically insignificant ($p > 0.05$), and other abiotic reasons were not analysed in this work. Not all isolated fungi (except *Ophiostoma novo-ulmi* subsp.) from elm shoots are known to be pathogens to elms (see Appendix Table 6).

Discussion

Although DED is one of the most investigated diseases from many perspectives, there are still various questions about plant-pathogen interactions (Bernier et al. 2014). In this study, two subspecies of the invasive pathogen – *Ophiostoma novo-ulmi* subsp. *novo-ulmi* and *O. novo-ulmi* subsp. *americana* – were documented for the first time in Estonia and north-eastern Europe. The occurrence of *O. ulmi,* the

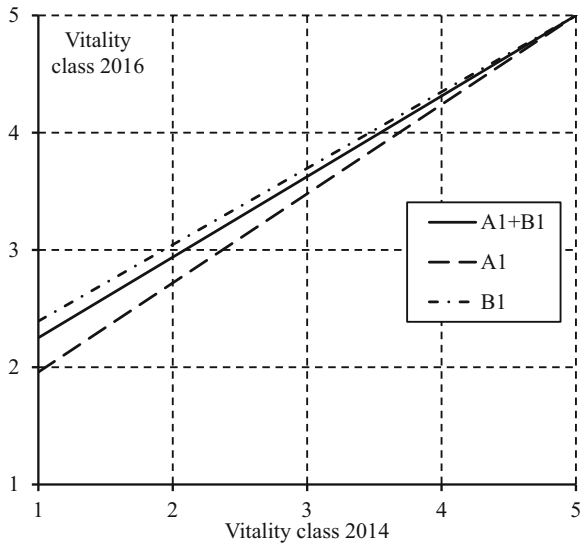


Fig. 6 Probability of changing health of elms at sub-sites A1 + B1 (solid line) together and two sub-sites A1 and B1, separately calculated. The solid line here is from the same data seen in Fig. 5

Table 4 Description of vitality changes of DED-infected elms during 24 months for the trees assessed in 2014 and 2016 at sub-sites A1 and B1

Sub-site	Number of observed trees	a (a·C)	SE	R^2	*p* value
A1 and B1	109	0.70955	0.99	0.505	< 0.0001
A1	72	0.67704	1.03	0.490	< 0.0001
B1	37	0.76344	0.84	0.583	< 0.0001

Parameters of regression analyses (number of observed trees, *a* – regression coefficient) for formula 2 (see Statistical analyses) with standard errors (SE), coefficients of determination (R^2) and significances (*p* value)

previous agent of DED, was not detected during this study. In northern Estonia, only *O. novo-ulmi* subsp. *americana* (11 isolates from randomly chosen symptomatic trees) was found in 5 different habitats in Tallinn, but not at the other locations. It proved to be more aggressive than *O. novo-ulmi* subsp. *novo-ulmi*, as 28% of surveyed elms (18) after 24 months were found dead at sub-site A1. At sub-site B1, where *O. novo-ulmi* subsp. *novo-ulmi* was causing damage, only 4 (12%) were dead. Regression analyses indicated that after being infected by DED (caused by both subspecies), the mean probability of elm trees to die within 2 years was ca. 22% among the 109 elm trees surveyed. Concluding all 1225 elms then *U. laevis* survived better (none of the trees was found dead) than the other naturally occurring *U. glabra*.

If both subspecies occur in the same region, there is a possibility for hybridization between them. No hybrids between *O. novo-ulmi* subspecies were detected in Estonia.

DED in Estonia

DED was diagnosed in Estonia by the 1930's (Lepik 1940). The pathogen was expected to spread across most of the country (Lepik 1940; Kaar 2011). At that time, the pathogen in Europe was known as *O. ulmi* (Brasier and Buck 2001). This agent may leave many infected elm trees alive because it was not so devastating (Kirisits 2013). In the beginning of the twenty-first century, *O. ulmi* has still been found in some parts of Europe (Solla et al. 2008). In neighbour countries

Table 5 The probability of decline over two non-consecutive years 2014 and 2016 (total 24 months) for DED in each health vitality class of elms at sub-sites A1 and B1

	Vitality in year x				
Vitality in year x + 2	Healthy	Branch loss	Damaged	Dying	Dead
Healthy	0.106				
Branch loss	0.767	0.538			
Damaged	0.064	0.128	0.500		
Dying	0.021	0.054	0.000	0.222	
Dead	0.042	0.283	0.500	0.778	1.000
N	47	39	4	9	10

N number of observed trees

DED was still present as *O. ulmi*, e.g. in Latvia (since 2012), Lithuania (since 1979) and in the European part of Russia (since 1979) (EPPO 2017). However, *O. novo-ulmi* is considered to be widespread in southern Russia (Gibbs 1978) and later on *O. novo-ulmi* subsp. *novo-ulmi* was detected there (Brasier and Kirk 2001). There exists a restricted distribution of *O. ulmi* in southern parts of Sweden and Norway (EPPO 2017), whereby *O. novo-ulmi* and the both subspecies having been found as well (Brasier and Kirk 2001). DED (but without molecular identification of its agent) had been found in Finland in 1988 (EPPO 2017), but it was not found there later (Hannunen and Marinova-Todorova 2016).

Ophiostoma ulmi was not identified in Estonia during this investigation, similar to what has been observed in many regions of Europe, where the more aggressive *O. novo-ulmi* displaced the earlier naturally occurring species *O. ulmi* (Brasier and Buck 2001; Brasier and Kirk 2001). Similarly, the native *Hymenoscyphus albidus* populations were displaced by an aggressive ash dieback agent (*H. fraxineus*) in Europe (e.g. Drenkhan et al. 2016).

A new wave of DED has occurred in Estonia since the 1990's, particularly in the areas grouped in this investigation as sample sites A and B. The losses of elms were suspected to be higher than during the previous era, i.e., the first half of the twentieth century (M. Hanso, pers. comm.). Likewise, in many other parts of Europe, and in some parts of Asia (Kirisits 2013) and North America (Dobbs et al. 2017) DED killed the majority of mature elm trees. The scarcity of elms (Kukk and Kull 2005) may be one reason for the slower spread of the pathogen, and we have only a few pure stands in limited or concentrated areas, e.g. in central Estonia (Kaar 2011). Another reason why some of the regions are unaffected by DED, or the degradation is not severe, can be explained by the scarcity of DED insect vectors (Voolma et al. 2000; Süda 2006) and also unattractiveness of *U. laevis* to them (Sacchetti et al. 1990; Santini and Faccoli 2013; Martín et al. 2018). This was observed also in this work at sites A and B, where elm bark beetles were found only on sampled trees of *U. glabra* and not on *U. laevis*. However, it is probable that invasive pathogen *O. novo-ulmi* subsp. *americana* may have been imported to the port city of Tallinn by infected plants, because there is no evidence that the pathogen was detected at any other location in Estonia (Table 3).

DED and health condition of elms

Both subspecies that cause DED are equally dangerous to elms regardless of the location, e.g., at urban sites or at rural locations; the difference was statistically insignificant ($p > 0.05$).

The assessment of elms during two non-consecutive years, 2014 and 2016, demonstrates the health status of trees at sub-sites A1 and B1, but the health conditions of elms cannot automatically be extrapolated to all of Estonia. This study demonstrates a disastrous decline of elm trees vitality, at particular sampling sites in Estonia, where the trees were affected by *O. novo-ulmi* subspecies, since about 22% of trees were dead after 2 years (see Fig. 5). It shows the same tendency as seen in the history of severe attacks of DED in other countries in Europe and North America (Phillips and Burdekin 1992; Schmidt 2006).

From 2014 to 2016, in Tallinn (sub-site A1) the elms died much quicker than in sub-site B1, located in the south-west of Estonia; ca. 25% of assessed trees were already dead after 24 months. A statistically significant ($p < 0.00001$) difference of elm health conditions between sub-sites A1 and B1 was demonstrated, apparently due to the different subspecies of the pathogen. Samples from sub-site A1 showed the presence of non-hybrid *O. novo-ulmi* subsp. *americana* by genes *col1* and *cu* (see Table 3), which is recognized as more aggressive to elms (Brasier and Kirk 2001) than non-hybrid *O. novo-ulmi* subsp. *novo-ulmi,* found at sub-site B1. Since another inventory at site B (Tihemetsa park in south-west Estonia) showed that 52% of assessed *U. glabra* trees died in 10 years (Laas and Treumuth 2006; Rist 2015), it may indicate that the *O. novo-ulmi* subsp. *novo-ulmi* that exists in site B is a less aggressive

pathogen (see Table 3). Nevertheless, population genetics at the genome level may give more information origin of the pathogens and pathogenicity.

In those regions where the occurrence of both subspecies of the pathogen overlap, their hybrids have also been found (Konrad et al. 2002; Dvořák et al. 2007) and even complex hybrids can occur (Brasier et al. 2004). Closest to Estonia, records of both subspecies come from the southern part of Norway and Sweden (Brasier and Kirk 2010). No hybrids were detected during this research. This can be explained by quite long distances between the sites (over 100 km) where different subspecies were found.

No other serious pathogens were isolated from symptomatic shoots from the elms at different sampling sites in Estonia (see Appendix Table 6); most of them are known endophytes (Blumenstein 2015; Martín et al. 2013b). It indicates that the most important pathogens on elms are subspecies of *O. novo-ulmi*. It is also important that in the northern Baltics the elms grow near the northern limit of their natural distribution area (Laasimer 1965), which increases their sensitivity to climate change and susceptibility to pathogens (Hanso and Drenkhan 2007, 2013). However, elms are native to Estonia and at the sampling sites only mature trees of Estonian origin were assessed (S. Järve, dendrologist, pers. comm.). Thus, we consider that hosts are similarly diverse as in native populations, but the elm populations are not genetically analysed in Estonia. The worst health situation for elms was in the Tallinn area (sub-site A1), not in other similar conditions in the northern and western parts of Estonia, and also at the sampling site D in eastern Estonia. The elms had been monitored by pathologists, arborists and other specialists for a longer period across Estonia. Furthermore, the differences in weather characteristics between sub-sites A1 and B1 were statistically insignificant (see Table 2). Additionally, differences in DED symptoms on elms were statistically insignificant ($p > 0.05$) in the urban space sites versus rural sites, incl. forests. It demonstrates that environmental characteristics (weather, site type, etc.) are not the causes of the devastating health conditions of elms in sub-site A1, particularly in Tallinn (see Fig. 1).

Only climate data (temperature and precipitation) were analysed to compare different sites. Other abiotic characteristics were not analysed in this work as those are unlikely diminish vitality of elms, because they are extremely hardy against abiotic stresses (Townsend and Douglass 2004; Scheffer et al. 2008; Büchel et al. 2016), adapt to city conditions (Santini et al. 2010) and lack specific soil type requirements (Buiteveld et al. 2014). The soils of comparable sites are sandy type (Umbri-Densic and Haplic Podzol to Albeluvisols) according to Astover et al. (2012) and the precise Estonian Soil Map (2018). However, with this kind of field experiment, the geographical difference of locations in sub-sites A1 and B1 was not ideal compared to a classical field trial, but it is still necessary to assess and analyse pathogen impact on natural populations, because it is not always possible to conduct classical field trials.

DED affects host species differently

Ulmus spp. have inherent differences in tolerance to DED (Guries and Smalley 2000; Townsend 2000; Venturas et al. 2014; Solla et al. 2014). The variation in susceptibility may depend on their anatomy and physiology, such as differences in vessels in the early wood, pit openings, other xylem features and branch sizes (Solla and Gil 2002; Martín et al. 2009, 2013a). *Ulmus glabra* was more affected by DED pathogens at the assessed sampling sites in Estonia (Fig. 3). *Ulmus laevis* was less affected ($p < 0.001$), similar to its characteristic in Poland (Łakomy et al. 2016) as well as results of inoculation tests in France (Pinon et al. 2005).

Some studies demonstrate that *U. laevis* is less attractive to the *Scolytus* beetles (Sacchetti et al. 1990; Santini and Faccoli 2013). This study confirms that finding (sites A and B), where elm bark beetles were found only on sampled trees of *U. glabra* and not on *U. laevis*. However, inoculation tests with *U. laevis* had also demonstrated

some susceptibility of this elm species (Pinon et al. 2005; Solla et al. 2005). Some elm cultivars usually stayed uninfected by DED pathogens, as was shown in a previous research work in Estonia (Aaspõllu 1999), but mature *U. glabra* 'Camperdownii' died due to DED in Tihemetsa park (sub-site B1). Still, some native mature elm trees seemed less susceptible to DED pathogens in heavily diseased areas in Estonia, suggesting that native elms species do not die out. These are viewed to be potential future trees for the environmental conditions of Estonia.

Alternatively, it has been suggested that resistant elm cultivars or hybrids (e.g., *Ulmus davidiana* var. *japonica* × *U. pumila* 'New Horizon') could be used in urban spaces (Brunet et al. 2013; Buiteveld et al. 2014). Nevertheless, since most of these elm hybrids have *U. pumila* as one of the parental species (Brunet et al. 2013), these hybrids do not grow well in northern climates, because *U. pumila* shoots are sensitive to late frosts in early spring, e.g., in Tallinn Botanical Garden, northern Estonia (A. Kaur, dendrologist, pers. comm.). In this study, all the assessed hybrid elms (20 trees) demonstrated some frost dieback symptoms on previous-year shoots. It clearly means that non-native species should be tested before large-scale planting in new conditions, such as north-eastern Europe.

DED pathogen identification

The pathogen cultures isolated from 76 different elms shoot samples showing typical symptoms of DED infection. Species detection of the causative agents of DED, *Ophiostoma novo-ulmi* and its subspecies, was carried out from isolated cultures by species-specific PCR primers (e.g. Gibb and Hausner 2005; Konrad et al. 2002). The primers were found to be effective only for pure cultures and were not species-specific when testing biological samples, e.g. symptomatic host tissues. Species-specific PCR primers are needed to quickly and reliably detect *Ophiostoma* species and subspecies from symptomatic host tissues. These primers will be useful for imported and exported planting material and for the general molecular monitoring of DED agents.

Conclusions

This study provides new information on Dutch elm disease in the north-eastern part of Europe, where the invasive pathogen *Ophiostoma novo-ulmi,* with its two subspecies (subsp. *novo-ulmi* and subsp. *americana*), was identified for the first time in Estonia. All 25 isolates, tested by *col1* and *cu* genes, were not hybrids. In the natural conditions of two non-consecutive calendar years at different sampling sites in the northern Baltics, the mean probability of native elm trees to die was ca. 22% by DED agents within 24 months. *Ophiostoma novo-ulmi* subsp. *americana* demonstrated higher aggressiveness toward elm trees in spite of the limitations inherent to the study; its victims died more than two times faster than elms infected by *O. novo-ulmi* subsp. *novo-ulmi*.

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Compliance with ethical standards The authors declare that ethical standards have been followed and that no human participants or animals were involved in this research.

Conflict of interest The authors declare that they have no competing interests.

Appendix

Table 6 List of other isolated and determined fungi from different elm trees' shoots in all sampling sites

No	Sampling				Host species	Molecular identification
	site	date	location	habitat		
1	A	02.07.2015	urban	park	*Ulmus glabra*	*Sphaeropsis ulmicola*
2	A1	14.07.2016	urban	street	*U. minor*	*Aureobasidium* sp.
3	A	31.07.2016	rural	road	*U. glabra*	*Phomopsis* sp.
4	A	31.07.2016	urban	park	*U. glabra*	*Dothiorella sarmentorum*
5	B	01.07.2015	urban	forest park	*U. glabra*	*Phomopsis* sp.
6	B	01.07.2015	urban	forest park	*U. glabra*	*Fusarium lateritum*
7	B	04.07.2015	urban	street	*U. glabra*	*Phomopsis* sp.
8	B	17.07.2015	urban	street	*U. glabra*	*Aureobasidium pullulans*
9	B	19.07.2015	rural	park	*U. glabra*	*Aureobasidium pullulans*
10	B	21.08.2015	rural	park	*U. glabra 'Camperdownii'*	*Cladosporium allicinum*
11	B	01.10.2015	rural	park	*U. glabra*	*Undosporium allicinium*
12	B	09.07.2016	urban	park	*U. glabra*	*Pleospolares* sp.
13	B1	10.07.2016	rural	park	*U. glabra*	*Phaeobotryon* sp.
14	B1	10.07.2016	rural	park	*U. glabra*	*Fusarium* sp.
15	B1	10.07.2016	rural	park	*U. glabra*	*Cladosporium* sp.
16	B1	10.07.2016	rural	park	*U. glabra*	*Phomopsis* sp.
17	B1	10.07.2016	rural	park	*U. glabra*	*Lophiostoma* sp.
18	B	20.07.2016	rural	forest	*U. glabra*	*Diaporthe* sp.
19	B	31.07.2016	rural	park	*U. glabra*	*Phomopsis* sp.
20	C	17.06.2016	urban	park	*U. glabra*	*Dothiorella ulmicola*
21	C	17.06.2016	urban	park	*U. glabra*	*Phoma* sp.
22	C	17.06.2016	urban	park	*U. glabra*	*Etypa crustata*
23	C	17.06.2016	urban	park	*U. glabra*	*Lophiostoma* sp.
24	C	17.06.2016	urban	park	*U. glabra*	*Boeremia exiqua* var. *exiqua*
25	C	17.06.2016	urban	park	*U. glabra*	*Cladosporium* sp.
26	C	30.06.2015	urban	street	*U. glabra*	*Sphaeropsis ulmicola*
27	C	30.06.2015	urban	street	*U. glabra*	*Mucor hiemalis f*
28	C	30.06.2015	urban	street	*U. glabra*	*Sphaeropsis ulmicola*
29	C	13.07.2016	urban	street	*U. hybrid*[a]	*Cladosporium* sp.
30	C	13.07.2016	urban	street	*U. hybrid*[a]	*Alternaria* sp.
31	C	13.07.2016	urban	street	*U. hybrid*[a]	*Leptosphaeria rubefaciens*
32	D	18.07.2016	rural	forest	*U. glabra*	*Mortierella hyalina*

[a] *Ulmus davidiana* var. *japonica* × *U. pumila* 'New Horizon'

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