Molecular relationship among isolates of *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* in the Baltic Sea area with a special reference to the island of Gotland

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**Abstract**

Two-hundred-two fungal isolates of *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* originating primarily from the Gotland island of Sweden and from mainland Sweden, Latvia, Lithuania, Ukraine and western Russia were compared using genealogical concordance of six genetic markers. The genetic markers included flanking region of the MAT-2 locus (SC1), cerato-ulmin gene (CU), internal transcribed spacer of rRNA (ITS), small subunit of nuclear rRNA (nSSU), large subunit of nuclear rRNA (nLSU) and elongation factor 1 alfa (EF1a). The complete alignment of combined dataset was 2,901 bp long. Cluster analysis of sequence information sub-divided isolates of each *Ophiostoma* species into distinct clades. Results showed that for each *Ophiostoma* species the lowest genetic distance was among isolates from Gotland. The latter demonstrated that introduction of *Ophiostoma* species to the island of Gotland occurred on a very few occasions. Analysis has also showed that a number of *O. novo-ulmi* genotypes were shared between populations from Gotland and mainland Sweden, suggesting that introduction of this species to Gotland has occurred from mainland Sweden.

**Keywords:** invasive pathogens, genotype diversity, Baltic Sea, Dutch elm disease, *Ulmus*

**Introduction**

Dutch elm disease (DED) is a lethal vascular wilt disease, which since the beginning of 20th century has led to a massive destruction of elm trees (*Ulmus* spp.) in Eurasia and North America, threatening the existence of susceptible elm species over large geographical areas (Phillips & Burdekin 1982). The first epidemics of the disease started at the beginning of the 20th century, and within a short period DED spread over Europe and North America causing severe losses. Then, there was a general decline in the level of disease in Europe during the 1940s and 1950s, but in the late 1960s there was renewed concern about DED, and by 1970 it was clear that new and more destructive epidemics had already started (Brasier 1990). As a result, by the late 1970s over 75% of the original elm population of 23 million in southern England was dead or dying, and about 70% of the elms in Netherlands were killed (Phillips & Burdekin 1982).

Brasier (1979) provided the evidence that the two discussed-above waves of DED pandemics were caused by two biologically different groups of fungi. The causal agent of the first wave belongs to the species *Ophiostoma ulmi*. Causal agent of the second, more aggressive wave, exhibited morphological and molecular differences from *O. ulmi*, and consequently was described as a new species, *Ophiostoma novo-ulmi* (Brasier 1991). Subsequent research has indicated that during the second DED wave there might have been two simultaneous epidemics present in Europe, one developing in Western Europe from North American sources and the other spreading into Europe from Russia or western Asia. As a result, *O. novo-ulmi* was separated into two subspecies, *O. novo-ulmi* ssp. *americana* and *O. novo-ulmi*...
ssp. *novo-ulmi* (Brasier 2001). In Europe, these two entities of DED fungi have been widely hybridizing, and they are one of the foremost examples of a plant pathogenic fungus for which this previously little known phenomenon has been documented (Brasier 2001; Konrad et al. 2002).

In Sweden, the disease is known since early 1950s, and then it was caused by the less aggressive *O. ulmi*, which by then was introduced. Subsequently, the incidence and spread of DED gradually increased, in particular during the 1980s, when the more aggressive *O. novo-ulmi* was introduced (Östbrant et al. 2009). The first clear evidence of the aggressive DED was the total destruction of Örups elm forest. Since 1986 DED is regarded as a serious threat to Swedish elm population. It was therefore decided that the disease must be controlled in order to sustain for as long as possible elm component, highly valuable for cultural landscapes and urban environment (Bergquist & Karltorp 1986). Despite that, the situation seemingly is getting worse. For example, mapping of elms in Stockholm City in 2008 has revealed that 40% of them had been already killed by the disease (Östbrant et al. 2009). However, as compared with other areas of Sweden and Europe, the disease arrived just recently to Gotland Island, and for the first time it was observed there only in year 2005 (Menkis et al. 2016b). However, even in case of presence in Gotland just of one *Ophiostoma* species, the research on genetic diversity in its population is very relevant. First, it would allow estimating number of entries of the pathogen into the island (diversity low, – entries few, and *vice versa*). Consequently, this would indicate the “vulnerability” of Gotland to repeated DED invasions in the future, what, for example, could suggest phytosanitary regulations. Detected high diversity in *Ophiostoma* population(s) would imply possibility for genetic recombination and hybridization, thus increasing threats for elm trees. By contrast, in case of low diversity (few entries, largely clonal population, transmitted by asexual spores and/or vegetative mycelium), this would increase chances for success of DED control, both by active DED eradication and by selection of disease resistant elm clones. As DED reached Gotland only after 50 years of its presence in the mainland Sweden, we hypothesize that the entries were few and the genetic diversity of DED pathogens in the island is low. However, in order to support or reject this hypothesis, structure and genetics of Gotland *Ophiostoma* spp. populations must be investigated. Insights into such questions can be gained by using molecular fingerprinting, which allow to assess genetic diversity and relatedness between different individuals of the species. Although a number of such methods have been developed, in fungi, no single method has established as a dominant and each method has its own advantages and limitations (Soll 2000). In this study, we compared two different PCR based molecular fingerprinting methods including the arbitrary primed PCR and genealogical concordance of several genetic markers.

**Materials and Methods**

*Isolation of fungal cultures*

Plant materials for fungal culturing were sampled during 2009-2018 from the island of Gotland, mainland Sweden, Latvia, Lithuania, western Russia and Ukraine. Fungal culturing was carried out from elm branches with characteristic DED symptoms. Bark was removed and three pieces of infected xylem tissues were placed in each Petri dish with Hagem agar medium (Stenlid 1985) containing 0.5% chloramphenicol and 0.1% cycloheximide antibiotics. The *Ophiostoma* fungi are relatively easy to isolate into agar culture as they are more tolerant to high concentrations of these antibiotics than many other fungi. Petri dishes were incubated at 20°C in darkness and checked daily for mycelial outgrowth. Outgrowing mycelia were transferred to new Petri dishes without antibiotics. For each fungal strain, one colony was maintained at room temperature at ca. 21°C and another was stored frozen at -
20°C. All cultures isolated in the present study were deposited in the culture collection of the department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

DNA work
Genomic DNA from fungal cultures was isolated using CTAB method. Prior to isolation of DNA, fungal mycelia was placed in 2ml centrifugation tubes together with glass beads and homogenized using a Fast prep shaker (Precellys 24, Bertin Technologies, Rockville, MD). 800 µL of extraction CTAB buffer (3% cetyltrimethylammonium bromide, 2 mM EDTA, 150 mM Tris–HCl, 2.6 M NaCl, pH 8) was added to each tube and incubated at 65°C for 1 h. After centrifugation, the supernatant was transferred to new 1.5 mL centrifugation tubes and mixed with one volume of chloroform by gentle vortexing. After centrifugation for 8 min at 10,000 rpm, the supernatant was precipitated with 2 volumes of cold isopropanol, washed with 70% ethanol and dissolved in 50 µL TE buffer. In each sample, concentration of genomic DNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Diluted (1–10 ng/µL) genomic DNA samples were used for amplification by PCR.

Firstly, genetic diversity of the isolates was studied using the arbitrary primed PCR (AP-PCR) and M13 as a primer (Vasiliauskas & Stenlid 1998). All AP-PCR procedures were carried out as described by Franzén et al. (2007). Resulting banding patterns of AP-PCR were separated by electrophoresis on 1% agarose gels (Agarose D1, Conda, Spain) in 1x SB buffer (Brody & Kern 2004) for 6h at 150V. The gels were stained with ethidium bromide and obtained images were analysed in Quantity One version 4.6.3 (Bio-Rad laboratories, CA, USA) software. Secondly, the intraspecific genetic diversity of Ophiostoma isolates was studied using genealogical concordance of six genetic markers including flanking region of the MAT-2 locus (SC1), cerato-ulmin gene (CU), internal transcribed spacer of rRNA (ITS), small subunit of nuclear rRNA (nSSU), large subunit of nuclear rRNA (nLSU) and elongation factor 1 alpha (EF1a) using their specific primers and PCR conditions (White et al. 1990; Paoletti et al. 2005; Menkis & Vasaitis 2011).

Cluster analyses
Cluster analysis was used in order to study intraspecific genetic diversity of Ophiostoma isolates by a combination of six genetic marker dataset. Sequence alignment for each genetic marker was constructed using the Clustal W algorithm of BioEdit (Hall 1999) and adjusted manually. Phylogeny of the combined six genetic marker dataset was generated using UPGMA algorithm at https://www.ebi.ac.uk/Tools/phylogeny/. To determine if the genetic markers were in significant conflict, the partition homogeneity test option in PAUP was used between the loci in all possible pair-wise combinations, using 100 replicates and the heuristic general search option. The null hypothesis of congruence was rejected if p < 0.001. The generated phylogeny was analysed using iTOL at https://itol.embl.de/.

Results and Discussion
A total of 202 Ophiostoma isolates including 170 from the island of Gotland, 25 from mainland Sweden, three from Latvia, two from Lithuania, one from Ukraine and one from western Russia were used in the present study. Firstly, electrophoresis analysis of the SC1 locus showed the presence of both O. ulmi and O. novo-ulmi fungi on Gotland (Fig. 1). Results showed that the ratio of O. ulmi to O. novo-ulmi isolates was 50:50 in 2009, but 30:70 in 2012. Ophiostoma ulmi was only very occasionally isolated after 2012. This rapid change in species abundance was likely caused by intraspecific competition among Ophiostoma
species in the same geographical area, when an aggressive DED pathogen *O. novo-ulmi* replaced less aggressive *O. ulmi* (Brasier et al. 2004). If not yet, it can be expected that *O. ulmi* disappears from Gotland in the nearest future due to this replacement. Furthermore, the molecular size of SC1 fragment of *O. novo-ulmi* from Gotland was similar to *O. novo-ulmi* isolates from mainland Sweden, resembling *O. novo-ulmi ssp. americana*. In comparison, this fragment in isolates from Latvia was of molecular size corresponding to *O. novo-ulmi ssp. novo-ulmi* (Fig. 1). This may suggest that *Ophiostoma novo-ulmi* isolates from Gotland and mainland Sweden have similar genetics and origin.

Figure 1. Part of the flanking region of the MAT-2 locus (SC1), based on Paoletti et al. (2005), showing the presence of *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* on Gotland. The molecular size of the fragment shows that isolates of *Ophiostoma novo-ulmi* from Gotland are more similar to the isolates from the mainland Sweden than from Latvia.

Secondly, polymorphic fingerprint patterns of AP-PCR have also showed the presence of two distinct *Ophiostoma* species on Gotland, but with low genetic diversity within each of them (Fig. 2). However, reproducibility and completion of AP-PCR for all isolates was problematic as the method appears to be very sensitive to different factors e.g. DNA concentration, PCR instrument or PCR chemistry (Perez et al. 1998).

Figure 2. Electrophoresis diagram based on AP-PCR showing low intraspecific genetic diversity among *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* isolates from the island of Gotland.
This showed the need of other fingerprinting method. Koufopanou et al. (1997) have shown that compatibility of different gene genealogies can be used to study population genetics. Genealogies constructed from different parts of clonal genomes will be identical, while those from parts of organisms which undergone recombination will be different. In the present study, sequencing and alignment of six genetic markers resulted in combined dataset that was 2,901 bp long. In agreement with partial AP-PCR data, phylogenetic analysis clearly separated O. ulmi and O. novo-ulmi, and showed certain genetic variation among O. novo-ulmi isolates originating from the Baltic Sea area. Consequently, in the given dataset all O. novo-ulmi isolates from Gotland were identical to each other, thereby resembling a clonal population structure, and clustered together (Fig. 3). Moreover, O. novo-ulmi isolates from Gotland and 17 O. novo-ulmi isolates from mainland Sweden were on the same cluster, showing similar genetics and origin. However, when all isolates of O. novo-ulmi from mainland Sweden were taken together, this showed higher genetic variability and heterogeneous population structure (Fig. 3). Even higher genetic distance was among O. novo-ulmi isolates from Gotland and isolates from Latvia, Lithuania, Ukraine and western Russia (Fig 3), thereby suggesting different genetics and origin of the latter isolates.

Figure 3. A circular phylogeny showing molecular relationship among isolates of Ophiostoma ulmi and Ophiostoma novo-ulmi from the island of Gotland, mainland Sweden, Latvia, Lithuania, Ukraine and western Russia. The phylogeny is based on sequence information of combined six genetic marker dataset. Geographical distribution and identities of fungal species are indicated by different colours and labelled. Abbreviations: LV-Latvia, LT-Lithuania, UA-Ukraine and RU-Russia.

The results of the present study have supported the hypothesis. The detection of low genetic diversity of Ophiostoma fungi on the island of Gotland is interesting and may have implications for DED control. Indeed, this would allow deployment of viruses (also known as d-factor) into local Ophiostoma isolates in order to reduce their virulence. The best approach is to release viruses in places where DED exhibit low genetic diversity, as it is expected that a
A genetically uniform pathogen population will be more susceptible to the viruses than a diverse population (Dunn 2000). Fungal viruses can be used to suppress DED in local high value elm populations, such as in Gotland. They may be deployed alone or as a component in an integrated control strategy. If the fungal viruses are established in the pathogen’s population it can be expected that the low spore germination rate and growth would provide the tree with more time to fight the disease, thus reducing the overall mortality rate. The reduction in spore viability would also reduce viable spore loads, preventing infection of elms (Dunn 2000). Furthermore, *Scolytus multistriatus*, the only insect vector of DED on Gotland (Menkis et al. 2016a), is considered as a poor to moderate vector because it carries relatively few spores compared with other *Scolytus* vectors (Webber 1990). Thus, a small reduction in spore viability could be enough to significantly reduce pathogen transmission. Although the likelihood that fungal viruses can help control DED in Gotland is high, the proof-of-concept experiments should be performed prior to field release.

**Conclusions**

At the time of this study, the less aggressive DED pathogen *Ophiostoma ulmi* and the highly aggressive -*Ophiostoma novo-ulmi* were present on Gotland, each exhibiting limited genetic diversity likely due very few occasions of introduction. A number of *O. novo-ulmi* genotypes were shared between populations from Gotland and mainland Sweden, suggesting that introduction of this species to Gotland has occurred from mainland Sweden. Low genetic diversity of *Ophiostoma* species on Gotland provides the possibility to use fungal viruses for biocontrol of DED.

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**References**


