Virulence and population structure in *Neonectria ditissima*, causative agent of European canker in apple

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Abstract

This study investigated the virulence, pathogenicity, and population structure of *Neonectria ditissima*, the causative agent of European canker (EC) in apple. Twelve isolates were phenotyped for virulence and pathogenicity in a glasshouse assay using one-year-old 'Katja' apple trees. Significant differences in virulence were observed among isolates, with area under disease progression curve (AUDPC) values ranging from 58.66 to 4347.79. Disease incidence varied considerably among isolates and was strongly correlated with virulence ($r = 0.85$, $p < 0.001$). The estimated broad-sense heritability for virulence was remarkably high ($H^2 = 0.98$), indicating that variation in AUDPC was predominantly attributable to genetic differences between isolates. Population structure was analyzed using simple sequence repeat (SSR) markers for 25 isolates and whole genome sequencing (WGS) data for 38 isolates. Principal coordinate analysis of SSR data suggested potential clustering patterns, though not clearly correlated with geographic origin or virulence. Principal component analysis of WGS data revealed clustering patterns related to both host of origin and geographic distribution. Isolates from *Fagus* hosts grouped closely despite diverse geographic origins, while isolates from different European regions showed distinct clustering patterns. Additionally, ITS sequencing of 46 isolates, including related *Neonectria* species, confirmed the species identity of the studied isolates and demonstrated the ability of ITS sequences to differentiate most *Neonectria* species except *N. ditissima* and *N. major*. This research provides valuable insights into the genetic diversity and virulence characteristics of *N. ditissima*, which will inform resistance breeding strategies for apple and improve understanding of host-pathogen interactions in European canker disease.

Keywords: European canker, *Neonectria ditissima*, virulence, apple, population structure.

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Abbreviations

ANOVA	Analysis of variance
AUDPC	Area under disease progression curve
BLUE	Best linear unbiased estimate
DNA	Deoxy ribonucleic acid
gDNA	Genomic DNA
GLM	Generalised linear model
EC	European canker
IBD	Identity by descent
ITS	Internal transcribed spacer
LD	Linkage disequilibrium
LG	Linkage group
LGR	Linear growth rate
LMM	Linear mixed model
PCA	Principle component analysis
PCoA	Principle coordinate analysis
PCR	Polymerase chain reaction
PVE	Percent of variance explained
qPCR	Quantitative PCR
QTL	Quantitative trait locus
RT-qPCR	Reverse transcription qPCR
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats a.k.a microsatellites
PAMP	Pathogen associated molecular pattern
PTI	PAMP triggered immunity
WGS	Whole genome sequencing

1.1 Introduction

Apple (*Malus x domestica* Borkh.) was, as of 2017, the fruit crop yielding the highest economic value on the global market (O'Rourke 2021). The 2017 global apple industry produced over 80 million tonnes at a value of around 37.8 billion USD. In Sweden, apple was the leading fruit crop with a total production volume of 29.4 tonnes at an estimated value of 348.9 million SEK in 2020 (*Trädgårdsundersökningen 2020. Kvantiteter och värden avseende 2020 års produktion* 2021).

Swedish apple production is almost exclusively sold for domestic consumption, but Swedish apples only account for around 20 % of the fresh fruit market (*Kraftsamling för svenska äpplen* 2023). Unfair competition on the European open market due to lack of permitted countermeasures for common crop diseases is generally perceived by Swedish producers to be a major obstacle for increasing market shares. Environmental policy prevents the use of many crop protection chemicals, and producers are relying on a decreasing number of approved substances (Nybom et al. 2016; *Bekämpningsmedelsregistret - Sök UPMA* 2021). A wide array of pests and diseases cause significant losses in apple production and European canker (EC) is regarded as the most serious threat to Swedish orchards (Garkava Gustavsson & Skytte af Sätra 2023). Due to the lack of other effective countermeasures, resistance breeding is necessary to achieve more sustainable apple production in Nordic climate in the future (Nybom et al. 2016). Understanding of pathogen biology and host resistance pave the way for efficient breeding of new resistant cultivars.

1.1.1 European canker

EC is caused by the fungus *Neonectria ditissima* [Tul. & C. Tul.] Samuels & Rossman, anamorph *Cylindrocarpon heteronema* (Castlebury et al. 2006), formerly *Neonectria galligena* Bres. The genus *Neonectria* belongs to the class *Sordariomycetes* in the phylum *Ascomycota*. The disease affects apple, pear (*Pyrus communis* L.), and a number of other deciduous trees, e.g. mountain ash (*Sorbus aucuparia* L.), birch (*Betula* spp. L.), beech (*Fagus* spp L.), and hawthorn (*Crataegus* spp. Tourn.), (Weber 2014). Disease outbreaks are especially severe in apple, regulary causing significant economic losses in commercial orchards. Maritime climates with mild and rainy weather e.g. in northern Europe favor the pathogen (Beresford & Kim 2011). EC constitutes only a minor problem in most other species, including pear, although disease in some forest species e.g. *Fagus grandifolia* and *Betula alleghanensis* is also economically significant due to the reduced value of timber from affected trees (Flack & Swinburne 1977). *N. ditissima* can also infect and damage the fruit (Weber 2014), and in some areas fruit infection causes major economic losses (Gelain et al. 2020). The disease

damages fruit also in Swedish storage facilities (Skytte af Sätra et al. 2024). Symptoms on infected trees manifest as canker lesions i.e., patches of bark undergoing necrosis which spreads slowly outwards in mostly circular – elliptic shapes until eventually girdling the shoot (Weber 2014). Distal portions of the plant die upon girdling and the disease is therefore most severe when young trees are affected on the trunk. Growth of tissues bordering the canker forms ridges which are later recolonised by the fungus, creating a characteristic multi layered shape of older cankers. During infection, mycelium colonises the cortex, killing the tissue as it spreads and after some time, usually a few weeks or more from the time of infection, sporodochia are formed and conidia are spread by rain splash, insects or gardening tools (Weber 2014). Conidia are released during the entire growing season except during hot and dry conditions in summer and freezing conditions in winter. After around one year, the sexual fruiting bodies, perithecia, may appear on the cankered tissue, releasing wind borne ascospores. The ascospore discharge is greatest during spring and autumn, especially after rain, when the drying of previously wet perithecia triggeres spore release.
Infection occurs mainly through wounds caused by e.g. fruit picking, pruning, leaf shed, bark cracking or hail damage (Amponsah et al. 2015). A period of wetness after spore adhesion greatly increases the ability of *N. ditissima* to infect its host (Beresford & Kim 2011).

Figure 1. Canker on apple tree in a nursery. Photography by the author, Lund, Sweden (Bengtsson 2022).

1.1.2 Genetic diversity

N. ditissima is hypothesized to have a centre of origin in North America (Plante et al. 2002). Thus far, only low diversity and weak population structure have been observed in other parts of the world (Ghasemkhani et al. 2016a; Gómez-Cortecero et al. 2016), but more thorough studies including systematic sample collection in different regions and hosts are needed to draw certain conclusions. Plante et al. (2002) found that < 1 % of the genetic variation was attributable to host of origin in a set of isolates collected on several deciduous tree hosts including several species of *Fagus, Betula,* and *Acer*. Beech bark disease on *Fagus* is caused by *N. ditissima, N. coccinea* and *N. faginata* (Castlebury et al. 2006). EC on apple seems, on the contrary, to be exclusively caused by *N. ditissima.* Cases of both heterothallic
(non selfing) and homothallic (selfing) reproduction systems have been reported within *Neonectria* (Debuchy & Turgeon 2006). *N. ditissima* however, seems to be mainly heterothallic, and Von Krüger (1974) showed obligate heterothallism between two mating groups in a mating study performed on isolates from apple trees in Germany. El-Gholl et al. (1986) on the other hand reported perithecium formation in single spore cultures derived from abscised germ tube cells from isolates collected on American mahogany (*Swietenia mahogoni* (L.) Jacq.). Stauder et al. (2020) studied *N. ditissima* isolates collected on *Fagus grandifolia, Betula alleghanensis, Betula lenta, Sorbus americana, Acer pennsylvanicum, Acer spicatum,* and *Sassafras albidum* and showed exclusively heterothallic mating controlled by the *MAT1* locus, with all samples carrying one of two idiomorphs. Heterothallic mating has also been suggested by Ghasemkhani et al. (2016a) based on genetic diversity among single spore isolates collected from the same perithecium. The study also demonstrated greater genetic variation within orchards (89%) than between orchards (11%) which is comparable to other studies of pathogens of cultivated plants (Gargouri et al. 2011; Nath et al. 2013; Sun et al. 2013). Ghasemkhani et al. (2016a) suggest that gene flow from a common source, e.g. a European fruit tree nursery, combined with heterothallic mating in orchards could explain the observed pattern.

1.1.3 Virulence

As of yet, little is known of the molecular interactions between *N. ditissima* and its hosts. Florez Palacios (2019) studied expression profiles of four candidate virulence genes (*g8150*, *g4542*, *g5809* and *g7123*) and four apple pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) genes (*PGIP*, *CERK1*, *LYK4* and *CYP1*) during EC infection of apple. All four genes were significantly up-regulated with expression peaks five to six weeks post infection. In addition, a knock-out mutation of *g8150*, the most up-regulated of the four genes, predicted to encode a protein kinase, resulted in a low virulence phenotype.

In a follow up study, better suited reference genes (*actin* and *mips*) were developed and applied to expression profiling of *g4542*, *g5809* and *g7123* (Florez et al. 2020). Up-regulation in planta was detected during three to five weeks post infection.
Plante & Bernier (1997) found virulence to be under genetic control based on an examination of trait segregation in eight full sib families. The study found no evidence for host specific virulence. Loss of virulence during *in vitro* culture is a commonly observed phenomenon in pathogenic fungi (Naiki 1983; Chang et al. 2020; Breen et al. 2022). Mutations in genomic DNA as well as epigenetic modifications can cause such changes in behaviour. The mechanism of lost virulence in isolates of *N. ditissima* is not well understood (Scheper et al. 2019), but virulence in such an isolate can, at least in some cases, be restored after passaging through a host. Thus, one of six isolates restored virulence after passaging through an apple host and an additional passage increased virulence further in the restored isolate, perhaps pointing towards epigenetic effects in that case. Deng et al. (2015) compared draft genome sequences of two isolates with differing virulence. They found genetic differences between the isolates at virulence associated loci, but no causative relationship was shown experimentally.

1.1.4 Resistance breeding

Successful breeding for resistance to EC in Sweden depends on the accurate screening of disease resistance in the Nordic breeding germplasm (Garkava-Gustavsson et al. 2013, 2016). Taking into account that the virulence of *N. ditissima* on apple differs between pathogen isolates, and can decrease over time as a result of *in vitro* culture (Gómez-Cortecero et al. 2016; Scheper et al. 2019), specific attention should be given to variability of the pathogen. Such variability makes comparisons between resistance screening studies in apple problematic. Thus, more knowledge of the fungus and of *N. ditissima – M. domestica* interactions is needed to develop robust phenotyping protocols for future resistance breeding.
Partial resistance towards EC in commercial apple cultivars varies from high susceptibility to moderate levels of resistance (Garkava-Gustavsson et al. 2013, 2016; Nybom et al. 2016; Skytte af Sätra et al. 2023). Higher resistance levels are displayed by some root stocks, e.g. *M. domestica* ‘G. 969’ (Garkava-Gustavsson et al. 2024) and ornamental *Malus* spp. e.g. *M. robusta* ‘Robusta 5’ (Bus et al. 2019). The resistance of ‘Robusta 5’ has been shown to be controlled by a major quantitative trait locus (QTL) on linkage group (LG) 14 (Bus et al. 2019). QTL mapping of EC resistance in commercial dessert apple cultivars have thus far yielded weak to moderate QTLs on LG 2, 5, 6, 8, 10, 15 and 16, with highest percentage of variance explained (PVE) of 19 % (LG 15) (Karlström et al. 2022), and on LG 1, 8, 15, and 16, with the highest PVE of 16.1%, on a QTL in LG 16,

(Skytte af Sätra et al. 2023). Bus et al. (2021) mapped EC resistance in two full sib families and found two QTLs. One on LG 8 with a PVE 12.3 %, and one on LG 16 with a PVE 17.6 %.

Disease phenotyping

The outcome of a host – pathogen interaction depends on the properties of both species, and the environment in which the interaction takes place (Agrios 2008). Host resistance can be divided into two main categories: quantitative- and qualitative resistance. Quantitative resistance refers to the ability of a host to resist disease development to a certain degree. Qualitative resistance refers to the ability of a host to completely resist disease development when exposed to a pathogen. Because the outcome of the interaction also depends on properties of the pathogen, variation of such traits within a pathogen species can also be phenotyped to gain a better understanding of the dynamics governing the interaction (Naiki 1983; Plante & Bernier 1997; Morel et al. 2018). Assuming host exposure of the pathogen, i.e. the epidemiological properties determining the ability of the pathogen to survive outside the host and spread over distance etc. are neglected, phenotyping of pathogen traits related to disease progression can be performed using two parameters: pathogenicity and virulence (Andrivon 1993). Definition of these terms has varied historically over time and across fields of research (Thomas & Elkinton 2004; Sacristán & García-Arenal 2008). According to the definitions adapted by the American phytopathological society (Andrivon 1993; Sacristán & García-Arenal 2008), pathogenicity can be defined as the qualitative ability of a pathogen to cause disease, regardless of the severity of the symptoms. It is measured as disease incidence which is the proportion of exposures resulting in disease symptoms. Virulence is defined as the quantitative ability of the pathogen to cause disease symptoms and reduce the fitness of the host, given that infection has occurred. The goal being to measure the severity of the symptoms and the impact on the host. Virulence can be measured in several ways depending on the specific host - pathogen combination studied, the host production goals, and experimental practical limitations etc. Protocols for virulence phenotyping are often focused on measuring the colonisation and damage to host tissue or organs.

Area under disease progression curve (AUDPC) (Shaner & Finney 1977) as used for EC resistance phenotyping by Skytte af Sätra et al. (2023) is a well established method for quantification of disease symptoms in interactions between various fungal pathogens and their plant hosts (Jeger & Viljanen-Rollinson 2001). Because the method is established for phenotyping of EC resistance in apple, it should in this case also be well suited for phenotyping of virulence in the context of this specific interaction. The method entails repeated measurements of lesion length with intervals adapted to the speed of the symptom development.

Some researchers, however, suggest that under circumstances where disease progress follows a typical sigmoid progress curve the protocol might be simplified to include as few as two measurements without loss of useful information (Jeger & Viljanen-Rollinson 2001; Haynes & Weingartner 2004). The suggested simplification can be used under the following circumstances: 1. The effect of resistance should be expressed as disease progress rate rather than asymptotic level. 2. Disease should be introduced simultaneously across the trial. 3. Infection should be continuous and not dependent upon discrete environmental events. An alternative approach compatible with protocol for lesion measurements for AUDPC was proposed by Wenneker et al. (2017). There, lesion measurements between a common start date and individual lesion girdling are used to approximate lesion growth rate (LGR), defined as the slope of the regression of lesion size versus time. The proposed advantage being that artifacts related to halted lesion growth after girdling are avoided.

1.1.5 Aim of the study

A key aspect of successful resistance breeding is to validate resistance phenotyping methods. To ensure the relevance of reference isolates and experimental procedures knowledge of pathogen genetic diversity and trait variability is crucial. The aim of this study is to support ongoing resistance breeding efforts by providing knowledge of key properties of the pathogen that may affect experimental design and enterpretation of results in resistance phenotyping experiments.

1.2 Materials and methods

1.2.1 *In vitro* culture and sample preparation

To produce genetic material for SSR- and ITS genotyping, mycelium samples of 17 isolates (Appendix 1) from previous culture (Ghasemkhani et al. 2016a; Bourras et al. 2025), or recently collected from orchards in Norway and Estonia, stored in 50 % glycerol kept at – 80 °C, were transferred to fresh potato dextrose agar (PDA) under sterile conditions and cultured under ambient temperature and light conditions until sufficient amounts of mycelium was produced. Genomic deoxyribonucleic acid (gDNA) was extracted using Genomic DNA Purification Kit (Thermo Fisher Scientific) following the manufacturer’s protocol.

1.2.2 Verification of isolate species identity

The 17 cultured isolates (Appendix 1) were examined using a quantitative polymererase chain reaction (qPCR) protocol described by Ghasemkhani et al. (2016b), targeting a β-tubuline coding gene specific to- and conserved within *N. ditissima*.

1.2.3 ITS sequencing and phylogenetic analysis

Sanger sequencing of Internal transcribed spacer- (ITS) regions of ribosome encoding genes (Gardes & Bruns 1993) was performed on PCR products of 17 isolates (Appendix 1). The PCR reactions were performed with a 2720 Thermal Cycler (Applied Biosystems) with 5 minutes of denaturation at 95°C followed by 35 cycles of 30 seconds denaturation at 95°C, 30 seconds of annealing at 58°C and 30 seconds of elongation at 72°C, followed by a final period of elongation at 72°C using primers ITS1F and ITS4 (Appendix 2). The PCR products were purified using a GeneJET PCR purification Kit (Thermo Fisher Scientific) according to the manufacturers protocol. DNA concentration of the purified products was measured by spectrophotometry using DS-11 FX (DeNovix) and the products were diluted to 5 ngµL^{-1} in milli-Q H_2O. 15 µL of diluted product was premixed with 2 µL of 10 µM ITS1F primer and shipped for externally performed sequencing (Eurofin Genomics). A nucleotide-BLAST search of the most common ITS-genotype within the data set was performed on the NCBI data base. 14 sequences of related *Neonectria* spp. were added to the data set to be included in the analysis. Raw ITS sequencing data was curated manually and, along with downloaded sequences, trimmed to the length of ITS sequencing data of 20 isolates (Appendix 1) available from a previous study conducted at SLU (Ghasemkhani et al. 2016b). Sequence alignment of all 51 sequences was performed using the ClustalW alignment algorithm (Li 2003) as implemented in MEGA version 12 (Kumar et al. 2024). A phylogenetic tree was generated using the Neighbor joining method (Saitou & Nei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Phylogenetic analysis and generation of the dendrogram was performed in MEGA version 12 (Kumar et al. 2024).

1.2.4 Virulence and pathogenicity phenotyping

Virulence

A glasshouse assay was conducted to evaluate the virulence and pathogenicity of 12 *N. ditissima* isolates (Appendix 1). The assay was set up before the start of this MSc project and without involvement of the author. One year old trees of *Malus domestica* cv. 'Katja' on 'M.9' rootstocks planted in 3.5 L plastic pots were inoculated on 2024-12-12 (block 1), and 2024-12-18 (block 2) by applying a spore suspension to wounds made by removing lateral buds using a scalpel; a horizontal section below, and a vertical section behind the buds created ~1mm deep and ~5 mm long wounds placed at the 11th, 14th and 17th lateral buds. Vaseline was applied after inoculation and thereafter removed five days later. The experiment consisted of two blocks each containing six replicates, with three inoculation sites per plant, resulting in a total of 432 observations. Virulence was measured as AUDPC based on six measurements of lesion length performed every second week in each block between 2025-01-16 and 2025-03-27 (Block 1), and between 2025-01-23 and 2025-04-03 (Block 2), using a digital caliper. The AUDPC values were calculated as:

$$AUDPC = \sum_{i=1}^{n} \left[\frac{Y_{i+1} + Y_i}{2}\right] [X_{i+1} - X_i]$$

where Y_i i is the lesion length at the ith assessment. X_i is number of days at the ith observation, and n is the total number of observations. Inoculation sites lacking infection were excluded from the analysis.

Lesion tissue was defined as all bark adjacent to the inoculation site undergoing any characteristic visible change of colour and texture. Lesions which could not be accurately measured due to the complete death and drying out of distal shoot portions as a result of girdling were assigned the length measured at the last previous assessment. Fused lesions were measured as one, and individual values were approximated by assigning the individual lesion a fraction of the total (fused) length based on the proportions at the last assessment before fusion.

For statistical analysis of virulence, both ANOVA and linear mixed models (LMM) were employed. ANOVA was used to test for overall differences between isolates, while the mixed model approach incorporated block and entry nested within block as random effects to account for the experimental design. Best Linear Unbiased Estimates (BLUEs) for each isolate AUDPC were calculated using the emmeans package in R. *Post-hoc* comparisons between isolates were performed using Duncan's multiple range test. Significance groupings were

determined with α = 0.05. Isolates sharing the same letter were considered not significantly different from each other.
Broad-sense heritability (H^2) for isolate virulence was calculated as r^2.

Figure 2. Lesion measured during the fourth assessment in the glasshouse assay. Photography by the author, Lomma Sweden (Bengtsson 2025).

Pathogenicity

Disease incidence was recorded as a binary outcome based on the presence of symptoms at the sixth assessment, 105 (block 1), and 106 (block 2) days post inoculation. Statistical analysis of pathogenicity was performed using R version 4.5 (R core team 2025). Due to the binary nature of disease incidence data and its non-normal distribution, a generalized linear model (GLM) with binomial distribution and logit link function was used. The model included pathogen isolate as the fixed effect of interest and block as a fixed effect to account for the structured temporal differences between blocks. Individual lesions were treated as pseudoreplicates and averaged to one measurement per tree. Isolate disease incidence was calculated as the proportion of infected inoculation sites for each isolate. The statistical model was specified as:

$$logit(\pi ij) = \mu + \alpha i + \beta j$$

where πij represents the probability of disease incidence for the i^{th} isolate in the j^{th} block, μ is the overall mean, αi is the effect of the i^{th} isolate, and βj is the effect of the j^{th} block.

Wald Chi-square tests were conducted to test for overall differences among isolates using analysis of deviance via the Anova function in car (Fox & Weisberg 2019). To quantify the uncertainty of individual isolate effects, exact binomial confidence intervals were calculated using the Clopper-Pearson method (Clopper & Pearson 1934). Isolates with non-overlapping confidence intervals were considered to have differing disease incidence. Results were visualized using the ggplot2 (Wickham 2016), displaying disease incidence proportions for each isolate along with exact binomial 95% confidence intervals. Correlation between virulence and pathogenicity was assessed using Spearman's rank correlation coefficient.

1.2.5 SSR genotyping

SSR genotyping was performed on 25 isolates (Appendix 1). In addition to the 17 isolates cultured during this study, Extracted DNA of 8 isolates obtained from a previous study (Ghasemkhani et al. 2016a) was included. SSR genotyping was performed using 7 previously described loci (Marra & Corwin 2009; Ghasemkhani et al. 2016a). PCR reactions were performed in a mixture containing 7.5 µL DreamTaq Green PCR Mix (2X), 0.5 µL forward primer (Appendix 2), 0.5 µL reverse primer, 4.5 µL nuclease free H_2O and 2 µL DNA diluted to ~30 ngμL^{-1} with a total volume of 15 µL. The PCR products were diluted 60-fold in milli-Q H_2O and mixed with a GeneScan™ - 600 LIZ™ size standard (Thermo Fisher Scientific). Capillary electrophoresis was conducted on an ABI 3130xl DNA analyzer (Applied Biosystems) using a 36 cm capillary array and POP-7™ polymer. Electrophoresis data was analyzed with GeneMarker® software Version 1.85, (SoftGenetics LLC).

Principal coordinate analysis (PCoA) of SSR genotypes

PCoA of SSR-data from 25 isolates (Appendix 1) was performed using GenAlex 6.5 (Peakall & Smouse 2006, 2012). To identify patterns indicating correlations between PCoA grouping and various parameters such as virulence , disease incidence, and isolate geographic origin, coloured labels showing parameter values were added to plots using ggplot2 (Wickham 2016) package in R (R core team 2025).

1.2.6 Whole genome sequence analysis

Whole genome sequencing data of 39 *N. ditissima* isolates pruned for local linkage disequilibrium (LD) were generated in a previous project and obtained from Bourras et al. (L. Gustavsson, pers. comm.). Excluding SNPs with minor allele frequencies ≤ 0.01 left a set of 35,635 SNPs. The data was used to evaluate genetic relatedness among the isolates. Genome-wide identity-by-descent (IBD) for all pairwise combinations of isolates was estimated using the PI_HAT parameter in PLINK v1.9 (Purcell et al. 2007). Following exclusion of isolates with IBD > 0.2, population structure was assessed by principal component analysis (PCA). PCA was performed using PLINK v1.9. Visualisation of the PCA results was performed in R (R core team 2025) using ggplot2 (Wickham 2016). The PCA results were integrated with isolate metadata (Appendix 1), allowing visualisation of population structure in relation to isolate geographic origin and isolate host of origin.

1.3 Results

1.3.1 Isolate identity

The qPCR assay showed similar peak flourescence for all studied samples with peak fluorescence at 83°C (Fig. 3), indicating that all isolates are likely *N. ditissima*

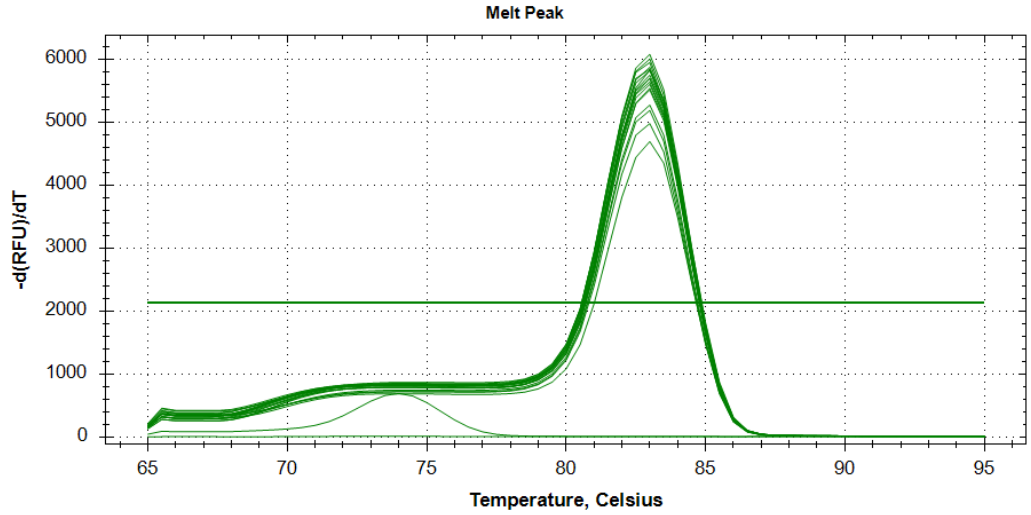

Figure 3. Melt curve analysis of qPCR showing similar peak fluorescense temperature for all 17 isolates. The two samples that did not amplify are negative controls.

1.3.2 Phylogenetic analysis

Phylogenetic analysis of ITS sequences (Fig. 9) showed expected grouping of isolates according to proposed species identity for *N. neomacrospora* and *N. bordenii.* The proposed *N. ditissima* (syn. *N. galligena*) isolates grouped together except for E_B1 and E_B2 which grouped apart from the others in direction towards *N. neomacrospora. N. major* isolates could not be distinguished from *N. ditissima*. *N. ramulariae* grouped together with *N. lugdunensis.*

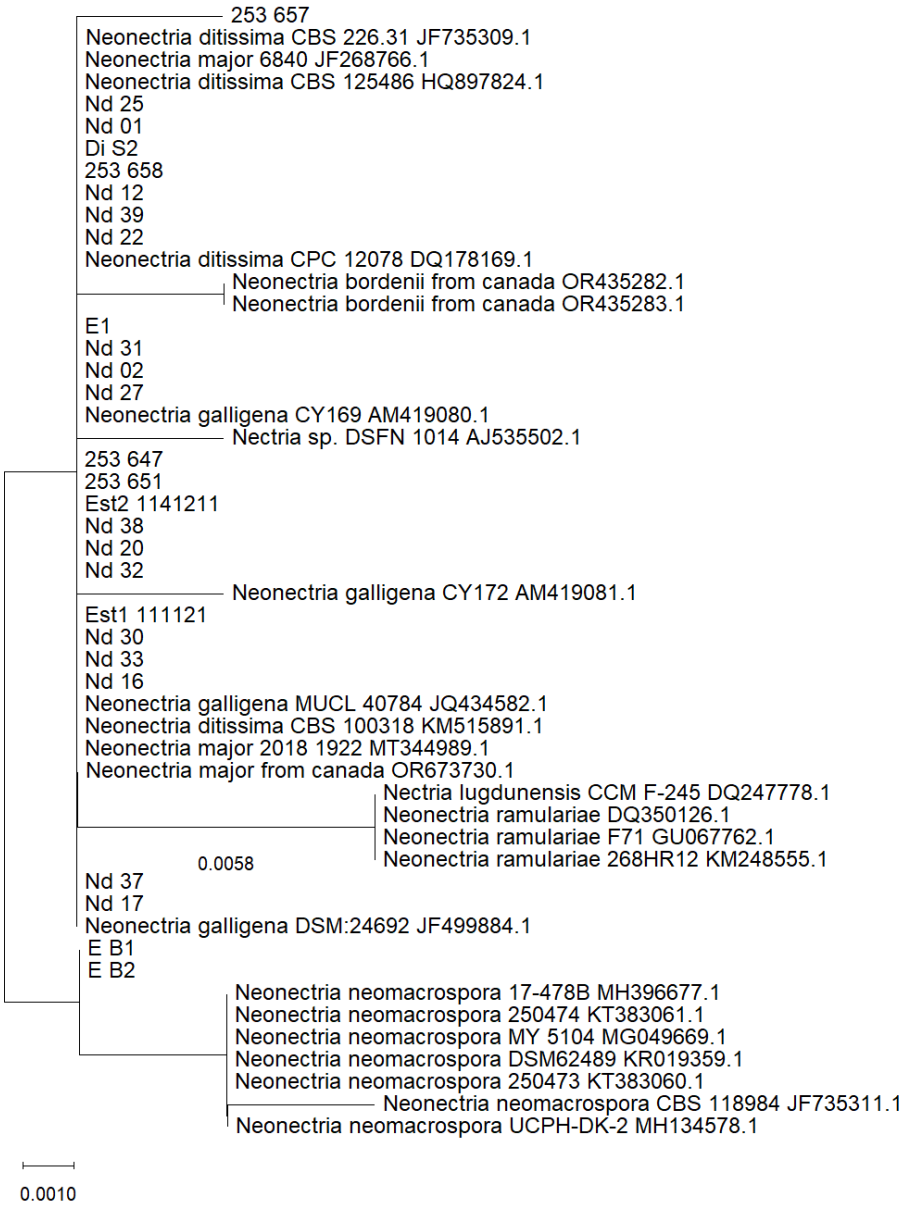

Figure 4. Dendrogram showing phylogenetic grouping of 51 fungal isolates based on ITS sequences

1.3.3 Virulence and pathogenicity

The AUDPC of the studied isolates varied between 58.66 for isolate E1 and 4347.79 for isolate 253_657 (Tab. 1), with significant differences between BLUEs for isolate AUDPC ($p < 0.001$). Duncan's multiple range test identified seven statistically distinct groups at the 95% confidence level (Fig. 4). There was a strong positive correlation between blocks (r = 0.99 $p < 0.001$), (Fig. 5), and accordingly broad sense heritability (H^2) was estimated to 0.98. Disease incidence varied between 0.14 and 1.0 (Fig. 6), with two isolates; E1, and E_B1 showing much lower values (0.14 and 0.19 respectively) than the other isolates. AUDPC was significantly correlated with disease incidence (r = 0.85, $p < 0.001$). AUDPC was not correlated to AUDPC in two cut-shoot experiments conducted at SLU (Garkava-Gustavsson pers. com.) in 2022, (r = 0.28 $p = 0.60$), (Fig. 7), and 2024 (r = 0.40 $p = 0.33$), (Fig. 8). E1 and E_B1 stand out in these comparisons having displayed high virulence and disease incidence in both previous experiments.

Table 1. BLUE of Isolate AUDPC with Duncan groups showing significant differences among 12 N. ditissima isolates

Isolate	AUDPC	Duncan group
253_657	4347.79	a
253_656	4080.72	ab
Nd_12	3943.06	ab
EST1	3386.28	bc
Nd_35	2935.1	cd
Nd_37	2385.98	d
Nd_18	1564.62	e
Nd_9	1168.37	ef
Nd_39	816.86	efg
EST2	605.08	fg
E_B1	203.49	g
E1	58.66	g

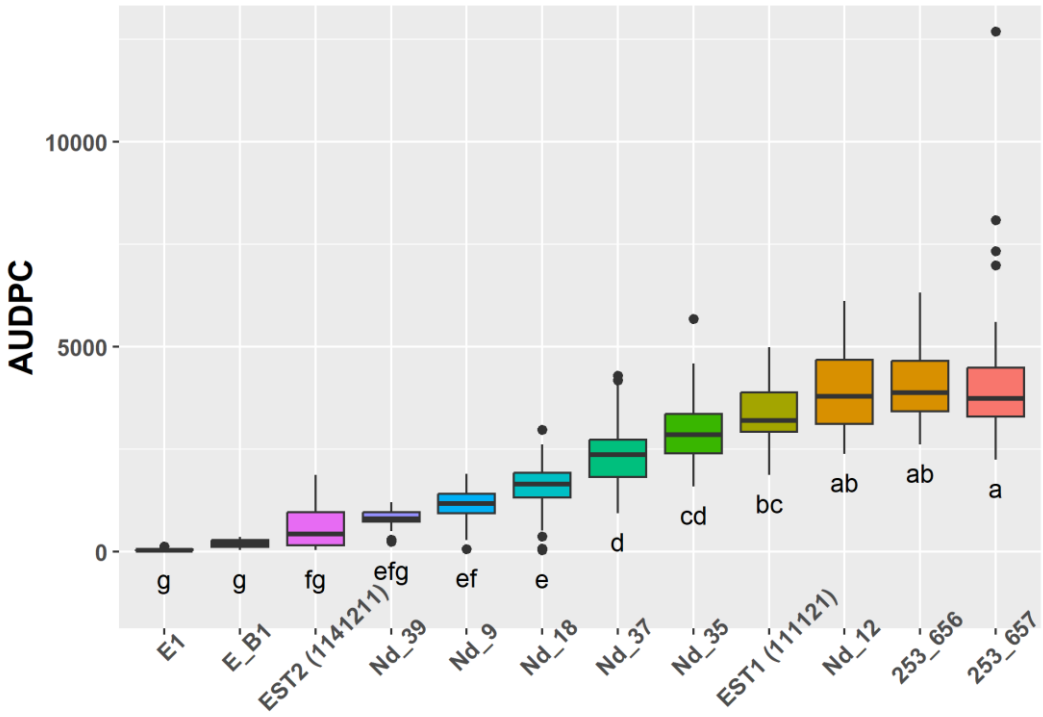

Figure 5. Box plot showing AUDPC of 12 N. ditissima isolates. Letters represent Duncan groups that correspond to statistically significant differences in virulence. Shared letters indicate no significant differences between isolates.

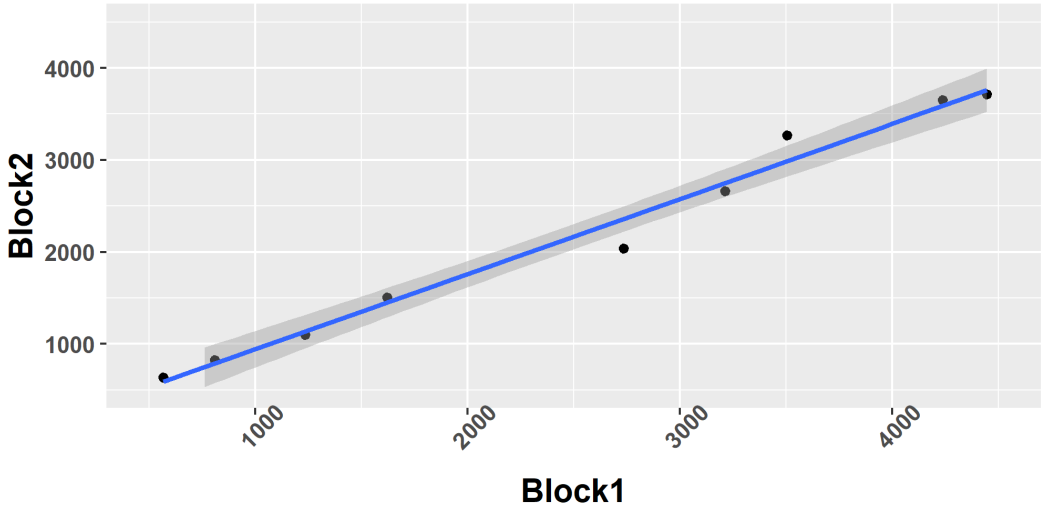

Figure 6. Plot showing block correlations for nine N. ditissima isolates with the blue line indicating the linear trend line and the grey area indicating the 95% confidence interval. E1, E_B1 and Est2 are not included in the block correlation estimate due a complete lack of infections in at least one block.

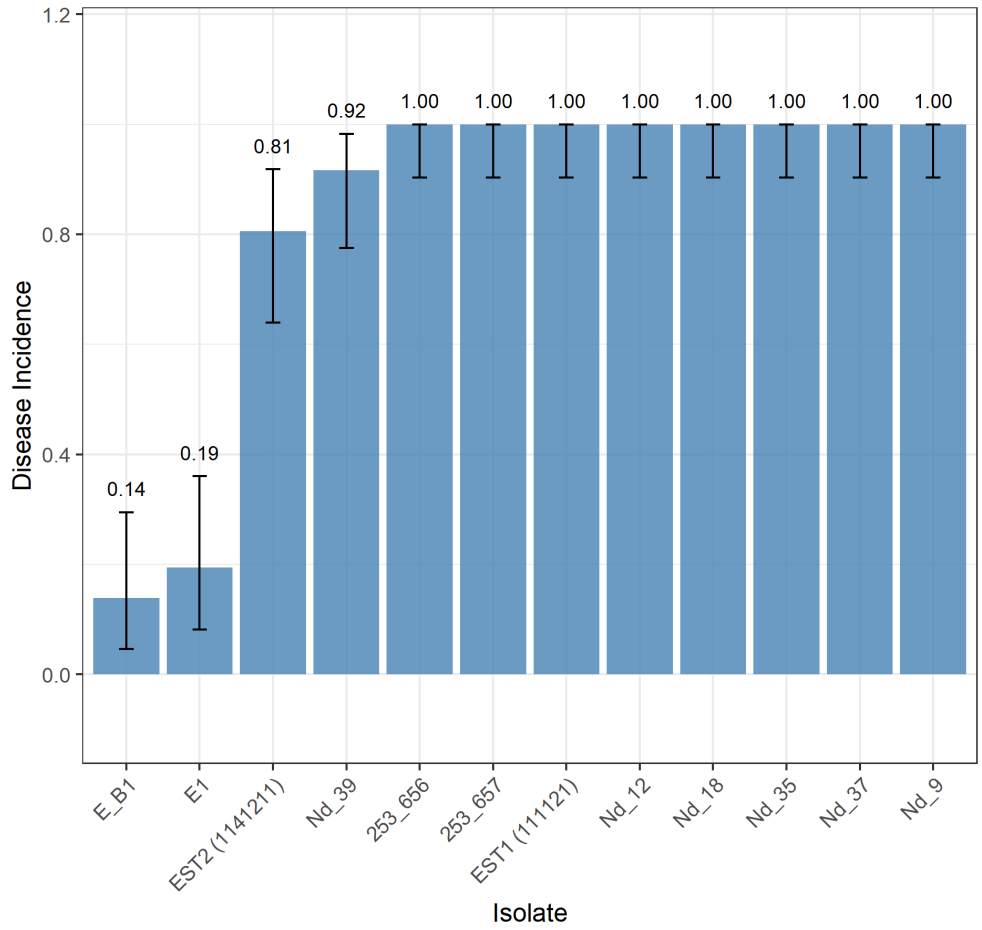

Figure 7. Bar diagram showing mean disease incidence of 12 N. ditissima isolates. Error bars indicate 95 % exact binomial confidence intervals.

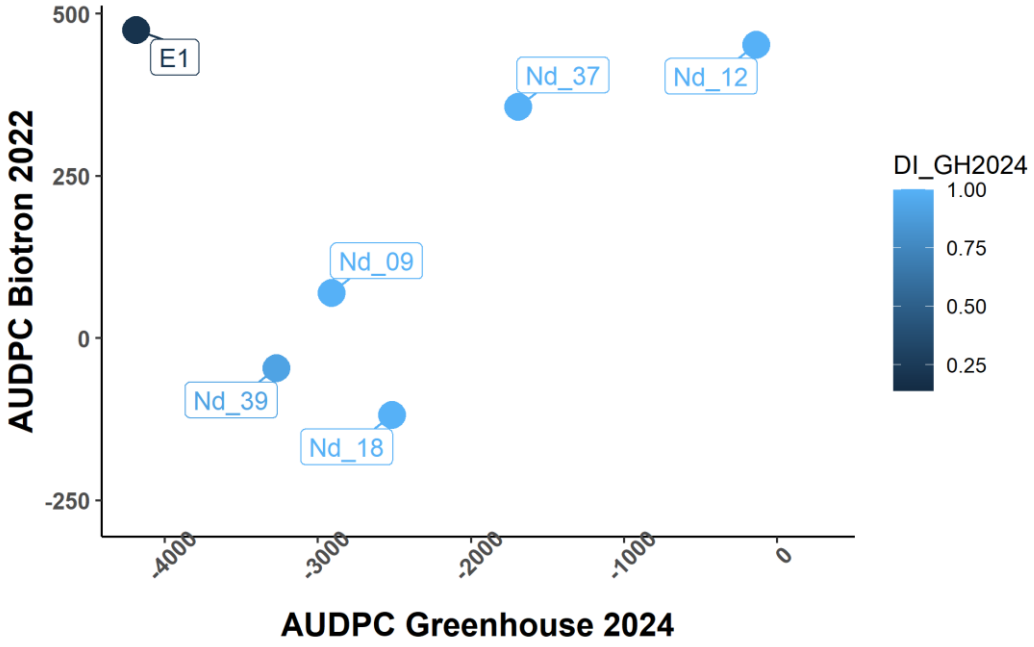

Figure 7. Plot of BLUE of AUDPC. Data from the glasshouse assay detailed in this report in the x-axis, and data from a biotron cut shoot assay (Garkava-Gustavsson 2024) performed in 2022 on the y-axis. Isolates are coloured according to disease incidence in the glass house assay detailed in this report.

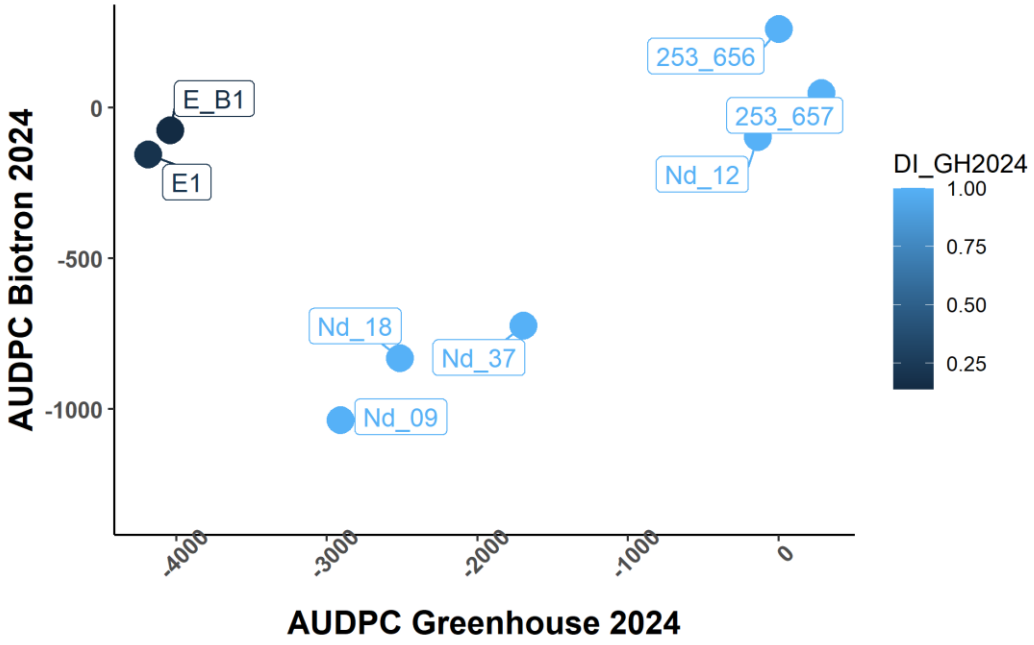

Figure 8. Plot of BLUE of AUDPC. Data from the glasshouse assay detailed in this report on the x-axis, and data from a biotron - cut shoot assay recently conducted at SLU (Garkava-Gustavsson 2024) on the y-axis. Isolates are coloured according to disease incidence in the glass house assay detailed in this report.

1.3.4 Population structure analysis

Analysis of SSR data

The SSR genotyping study resulted in the detection of two to six alleles per locus (Tab. 2), with the exception of GGT 39. This locus produced ambiguous results showing multiple weak peaks at varying fragment lengths (data not shown) and was therefore excluded from further analysis. Number of effective alleles varied between 1.17 for GGT23 and GGT44, and 2.88 for CAA3. Shannon's information index ranged from 0.28 to 1.32. Nei's genetic diversity was between 0.15 and 0.65. Unbiased diversity was between 0.15 and 0.68. A PCoA of the generated data (Fig. 10) showed signs of grouping along both axes with PC1 explaining 81.13 % and PC2 explaining 16.58 % of the variation. Visual assessment of the PCoA plot indicates two outlier groups: Nd_30, E_B1, and E_B2, which seem to form a distinct cluster separated from the other isolates along PC1. Nd_07, Nd_31, and Di_S2 seem to form a second group separated mainly by PC2. No statistical test was conducted to verify the significance of the observed groupings. Labelling of isolates according to virulence or pathogenicity values did not yield any perceived patterns. Labeling according to isolate origin (Fig. 10) did not show any clear signs of geographic influence. Nd_30 is from Brittany, France in 2015, while E_B1 and E_B2 come from the same orchard in Sweden in 2023. Nd_07 is from Jönköping, Sweden in 2013, Nd_31 is from the UK in 2015, and Di_S2 is from Lund, Sweden in 2023.

Table 2. SSR loci with corresponding number of alleles (Na), Number of effective alleles (Ne), Shannon's information index (I), Nei's genetic diversity (h) and unbiased diversity (uh)

Locus	Na	Ne	I	h	uh
CAA3	6	2.88	1.32	0.65	0.68
CAA11	5	2.73	1.28	0.63	0.66
GGT2	3	2.29	0.91	0.56	0.59
GGT3	2	1.86	0.65	0.46	0.48
GGT23	2	1.17	0.28	0.15	0.15
GGT44	2	1.17	0.28	0.15	0.15

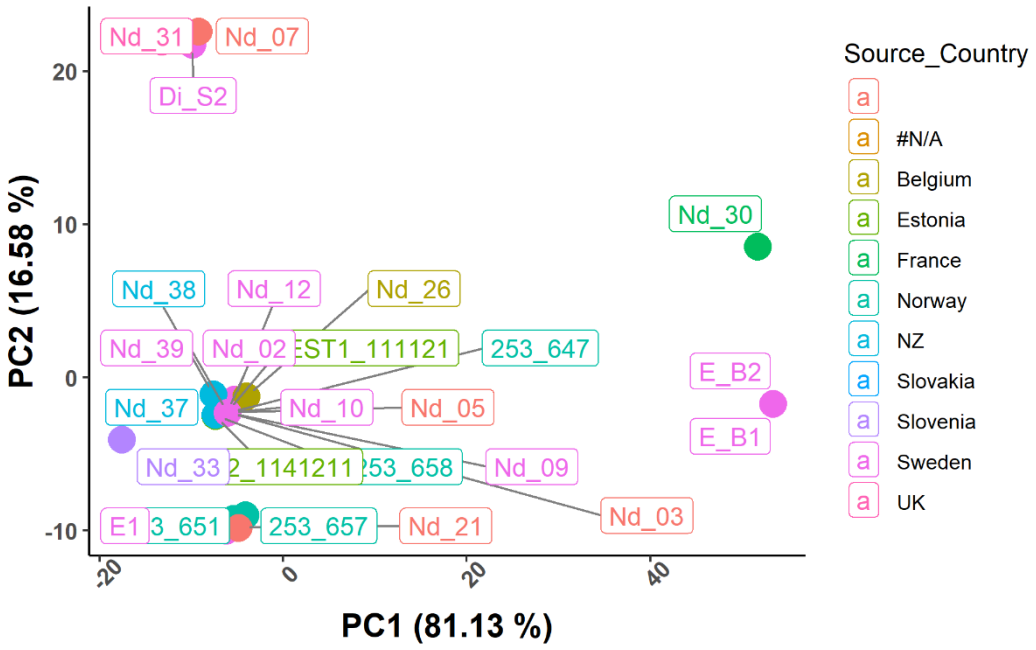

Figure 8. Plot showing PCoA of SSR genotypes for 25 isolates.

Analysis of WGS data

Analysis of IBD for 39 *N. ditissima* isolates revealed them to be mostly unrelated, with most pairs of isolates showing genome wide estimates of IBD close to 0. However, one pair of isolates (Nd_03 and Nd_05) had a pairwise IBD value of 0.97, indicating a clonal relationship. Based on this finding, one isolate from this clonal pair (Nd_05) was excluded from subsequent analysis.

For the remaining 38 isolates PCA showed that the first three PCs explained 4.74%, 3.63%, and 3.42% of the total genetic variation, respectively. The resulting plots with labelling according to host of origin and geographic origin seemingly indicate signs of grouping according to both host of origin, mainly along PC1 and geographic origin along PC2 and 3. Isolates Nd_36 and Nd_34 from *Fagus* hosts group closely (Fig. 11) despite being from USA and Slovakia respectively (Fig. 12). Isolate Nd_35 from *L. tulipifera* is also shifted from the main (*Malus*) cluster along PC1. In PC2 and -3 isolates Nd_37 and Nd_38 from New Zealand group farthest apart from the main cluster followed by Nd_30 from Brittany, France, close to Nd_31 and Nd_32 from the UK (Fig. 13). The Belgian isolates group in the middle of the plot. Swedish isolates Nd_04 and Nd_05 from Julita; Nd_06 and Nd_07 from Jönköping; Nd_08 and Nd_09 from Kivik; and Nd_21 and Nd_22 from Bjärred grouped regionally (Fig. 14). The isolates from Balsgård were dispersed across the plot. No statistical test was performed to verify statistical significance of the perceived groupings (Fig. 11-14).

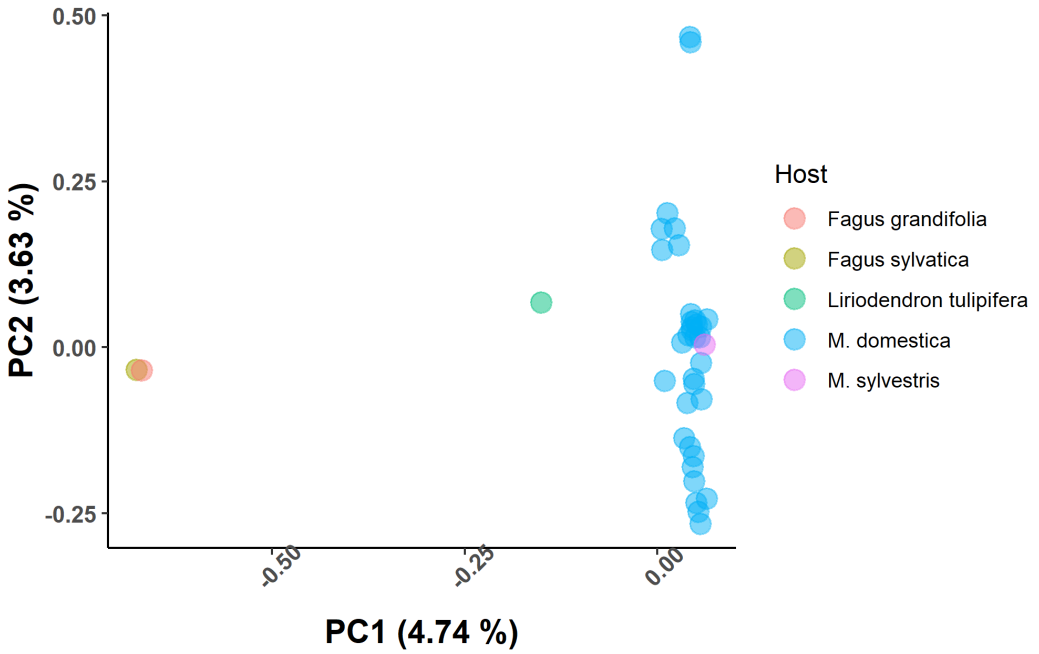

Figure 9. PCA showing PC1 and PC2 for 38 isolates coloured according to host of origin.

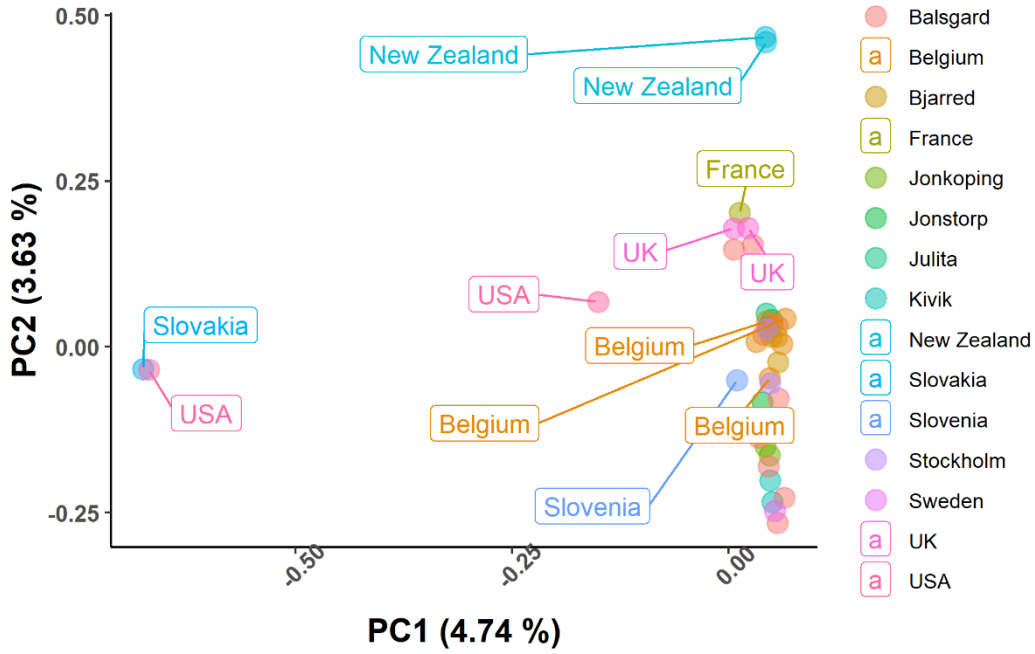

Figure 10. PCA showing PC1 and PC2 for 38 isolates coloured according to geographic origin with labels showing isolates of foreign origin.

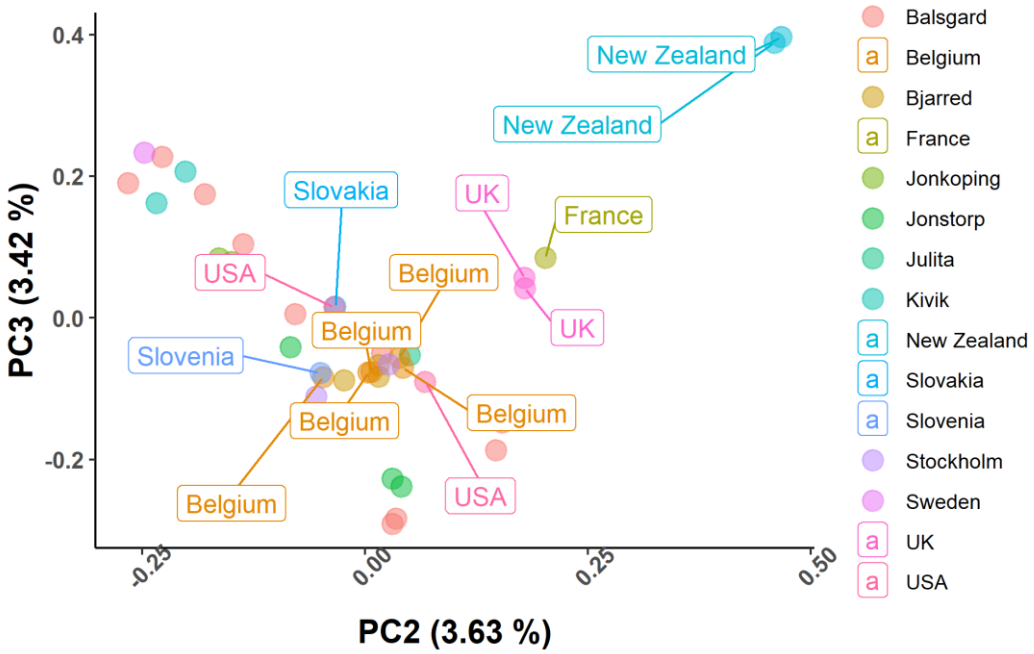

Figure 11. PCA showing PC2 and PC3 for 38 isolates with colouring according to geographic origin and labels showing isolates of foreign origin

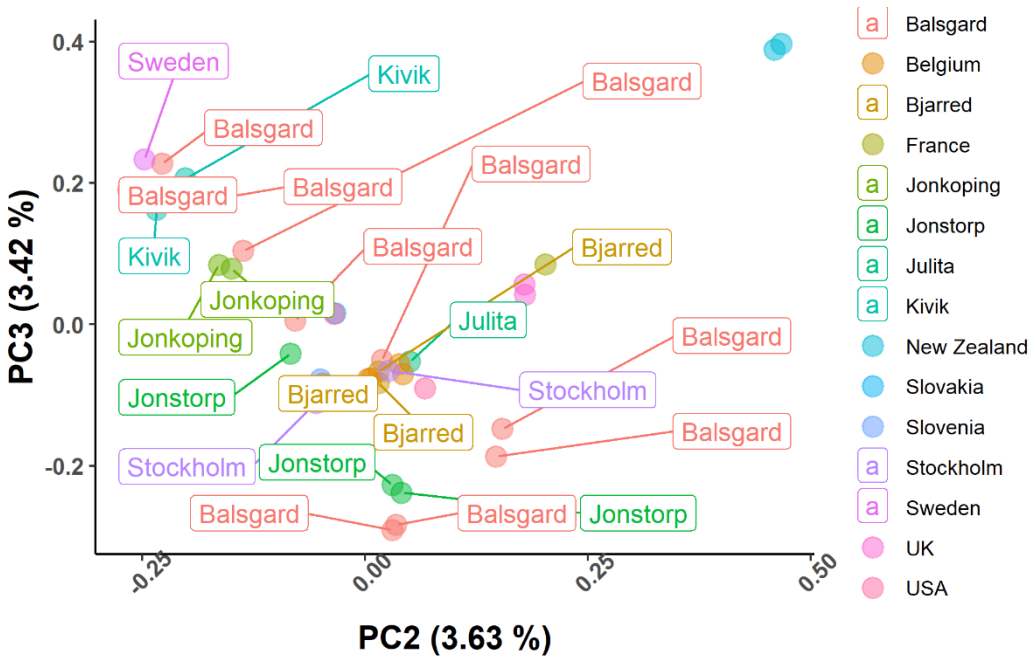

Figure 12. PCA showing PC2 and PC3 for 38 isolates coloured according to geographic origin and labels showing isolates of Swedish origin.

1.4 Discussion

Isolate identity

The qPCR assay showed peak fluorescence of 83°C for all isolates, differing slightly from results by Ghasemkhani et al. (2016b) reporting 82°C. The interpretation of the author is that the difference is likely due to an unknown experimental factor.

The β-tubulin locus is not expected to differentiate between closely related *Neonectria* spp. as Ghasemkhani et al. (2016b) used ITS sequences to verify the method and observed the resolution of the ITS locus to be insufficient for distinguishing *N. ditissima* from close relatives. That observation however was based on only a few samples of other species. The phylogenetic study detailed in this report suggest that ITS sequences might be able to differentiate between all species compared in this study apart from *N. ditissima* and *N. major*. Recent work has led to the development of a bioassay for *N. ditissima* identification which utilises parts of the ITS region (Elena et al. 2022; Harteveld et al. 2023).

The evidence supporting *N. major* as a distinct species lacks comparison with a diverse population of *N. ditissima* isolates from apple hosts (Castlebury et al. 2006). Zhao et al. (2011) combined the ITS, β-tubulin, EF-1α and RPB2 loci in an attempt of DNA-barcoding of *N.* major alongside 18 other *Neonectria* species. When RPB2 gene and EF-1α gene were combined, all examined species were identified as coherent groups, although with *N. major* and *N. ditissima* very close. The high ratio of intra- to interspecific variation observed in this study perhaps leaves the categorisation of *N. major* as a species an open question. Without a comprehensive study including larger numbers of *Neonectria* isolates from both *Alnus, Malus*, and other known hosts including tests of host compatibility and interspecific mating ability, the line between intraspecific population structure and speciation might be hard to pin down.

1.4.1 Virulence and pathogenicity

Halted growth of girdling lesions and interference between lesions located on the same tree, as described by Wenneker et al. (2017) seem to have contributed to an under-estimation of the most virulent isolates during this study. According to personal observations during data collection, the growth of distal lesions seemed to be influenced by a perceived loss of turgor in shoot tissues above large lesions. A gradual drying out of the shoot seemed to slow down the growth of the lesion closest to the tip, while the more basal lesions remained vigorous. For accurate phenotyping of highly virulent isolates, adaptations of the protocol could possibly include fewer inoculation sites per tree and earlier termination of experiment.

LGR, implemented as suggested by Wenneker et al. (2017) could perhaps solve the issues, if measurements are restricted to the period prior to lesion interference.

The close relationship between virulence and pathogenicity observed in this study may indicate a functional relationship between the two parameters; the inability of some isolates to cause disease may be caused by a low production of virulence factors rather than an inability to infect the host. *N. ditissima* has been isolated from surface sterilized symptomless tissue (Amponsah et al. 2014; Ghasemkhani et al. 2016b) but mycelial colonisation of unaffected was not histologically confirmed. If symptomless infection was confirmed by detection of mycelium in healthy plant tissue infected by non-virulent isolates, a functional relationship between virulence and pathogenicity could be inferred. To elucidate the mechanisms governing pathogenicity and virulence in the *N. ditissima – M. domestica* interaction, cytological and histological studies of the infection process as well as functional categorisation and mapping of virulence factors are necessary. Inoculation of non-virulent isolates on plants prepared with pre-induced necrosis, e.g. by exposure to heat or cold could perhaps provide clues to the importance of necrosis for fungal development.

The phenomenon of lost virulence in cultured fungal pathogens, as discussed by Scheper et al. (2019), Chang et al. (2020), and Breen et al. (2022) is highly relevant for this, and other similar studies. The phenotyped isolates can be formerly virulent. Such is the case with the isolate E1 which was previously used as a virulent reference isolate (Skytte af Sätra et al. 2023), while exhibiting low virulence in the current glasshouse assay. If the phenomenon is caused by epigenetic mechanisms e.g. methylation of virulence factors, the process may be reversed. In plants and animals, epigenetic reprogramming associated with germ cells and zygote formation have been well documented to have important implications for embryonic development (Kawashima & Berger 2014; Xavier et al. 2019). In fungi recent progress has shown that epigenetic reprogramming has important implications for sexual reproduction and development (Jeon et al. 2015). As epigenetic reprogramming is associated with gametogenesis and fertilisation (Xavier et al. 2019), further insights into possible epigenetic dynamics of weakened isolates might be gained from methylomic studies. Inoculating apple hosts with ascospores resulting from crosses between weak isolates could give insights into transgenerational inheritance of epigenetic effects.

1.4.2 Reproducibility of SSR markers

When compared to the alleles called by Ghasemkhani et al. (2016a) a lack of consistent allele matching between the studies (Appendix 3) prevented common analysis. The fragment lengths also differed from those of Marra & Corwin (2009), but the lack of matching with this study, also reported by Ghasemkhani et al. (2016a), could possibly be explained by genetic diversity. The isolates used by Marra & Corwin (2009) were all collected on black birch (*Betula nigra*) in West Rock Ridge State Park in New Haven, Connecticut, USA. With such geographic distance and lack of overlapping host of origin between the studies a low rate of similarity can be expected. The mis-match of alleles between the Ghasemkhani et al. (2016a) study and the study detailed in this report is more problematic as there are mis-matches between results obtained from a partly overlapping set of isolates (Appendix 1) and the isolates of both studies are collected on apple in Europe. In the overlapping set of nine isolates (Appendix 1) the most polymorphic loci (CAA 3, CAA 11, GGT 2) had between 0/9 and 2/9 matching alleles. The author has no suggestion of possible explanations for the lack of reproducibility observed between this study and that of Ghasemkhani et al. (2016a).

1.4.3 Population structure

Analysis of SSR data

The PCoA of the SSR genotypes showed indications of grouping. Although not statistically verified, the isolates seem to aggregate in four clusters with E_B1, E_B2 and Nd_30 separated from the rest of the isolates by PC1 and Nd_07, Nd_30 and Di_S2 forming a cluster separated along PC2. Isolates 253_651, 253_657, E_1 and Nd_21 form a cluster separated from the main cluster along PC2 in the negative direction. The conspicuous identical genotypes of E_B1 and E_B2 is a curious case. The isolates display a similar pattern of deviance in the ITS region. The similarity between the two isolates is not unexpected as they are collected from *M. domestica* 'Elise' on different trees in the same orchard. These isolates may therefore be closely related, or even clones, but the fact that they appear genetically different from others may be an example of regional diversity. E_B1 which is also represented in the glass house assay was one of the two least pathogenic. Overall, the clustering perceived in the PCoA does not seem to reflect geographic origin, and it does not appear to be clearly correlated with virulence or pathogenicity (not shown).

Analysis of WGS data

The PCA of WGS data shows an intriguing pattern with clustering of isolates

according to host of origin along PC1, and according to geographic origin along PC2 and 3. No statistical test was performed to verify the perceived clustering, but the striking closeness on the plot (Fig. 12) of two isolates from *Fagus* spp., and the perceived separation from the main cluster of an isolate from *Liriodendron* is compelling, especially given the fact that the isolates from *Fagus* hosts are from USA and Slovakia respectively. PC2 and 3 (Fig. 13) show interesting patterns of grouping with Belgian isolates in the centre and French isolates from Brittany grouping close to those from the UK. Swedish isolates group regionally to some extent, except for those from Balsgård. The dispersal of Balsgård isolates across the plot might be explained by the fact that Balsgård (recently shut down) was a national collection and centre for breeding and research of apple. The activities of collecting, propagating and distributing plant material from far and wide may increase the genetic diversity of the *N. ditissima* population at such facilities.

The indications of population structure presented in this study gives a contrasting image to the results presented by (Ghasemkhani et al. 2016a). The suggested explanation for weak population structure and low diversity provided by the authors was that gene flow from Dutch or Belgian fruit tree nurseries played a dominant role in shaping the structure of the Swedish population. The results of this study indicate that population structure might be detectable despite low between-orchard genetic variation. The use of SSR markers should perhaps be approached with caution with robust verification of reproducibility prior to employment in future studies. The study presented here may lack sample-size and -distribution sufficient to provide a comprehensive understanding of diversity and structure of the European *N. ditissima* population. The results does however give an indication that such a study is warranted. WGS data seems to be a viable option for future studies. Applying WGS in a larger study, using a collection of isolates from multiple orchards from various regions across Europe as well as from different species of wild or cultivated forest trees, would be an interesting prospect for deeper insights into the diversity of the species. Extending the research to include samples from related *Neonectria* spp. might also provide useful information regarding adaptations related to host specificity. Such knowledge might help elucidate critical mechanisms in the host – pathogen interaction, which could benefit resistance breeding.

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Popular science summary

Apple trees across Europe face a formidable enemy – European canker, a devastating disease caused by the fungus Neonectria ditissima. This disease creates distinctive canker lesions on tree branches and trunks, eventually killing parts of the tree and causing significant economic losses for apple growers, especially in rainy regions like Northern Europe. In Sweden, where strict environmental regulations limit chemical control options, European canker is considered the most serious threat to apple production.

Our research investigated this fungal pathogen to understand what makes some strains more dangerous than others and how diverse the pathogen population is across Europe. Fungal samples collected from different regions and hosts were tested in controlled conditions to see how aggressively they could infect apple trees. The results were striking – some fungal strains caused barely noticeable symptoms, while others aggressively colonized the trees, causing large cankers that quickly girdled shoots.

By analyzing the genetic makeup of these fungi using advanced DNA technologies, we discovered intriguing patterns. Fungi collected from the same geographic region often shared genetic similarities, but we also found that fungal strains from beech trees shared genetic similarieties although coming from different continents. This suggests that the fungus might adapt specifically to certain host plants.

The genetic diversity we observed challenges previous assumptions about how this pathogen spreads and evolves.

Our findings have important implications for apple breeding programs aimed at developing canker resistant varieties. Understanding the diversity and behavior of the pathogen will help breeders create more effective screening methods and potentially develop apple varieties that can better withstand European canker – a critical step toward more sustainable apple production in Sweden and throughout Northern Europe.

Appendix 1

Isolate	Species (previously proposed)	Isolated in (year)	Host of origin	Cultivar	Geographic origin	Glasshouse assay Bengtsson 2025	Culture and DNA extraction Bengtsson 2025	SSR-genotyping Ghasemkani et al. 2016	SSR-genotyping Bengtsson 2025	WGS-analysis Bengtsson 2025	ITS-sequencing Bengtsson 2025	ITS sequences retrieved from Ghasemkhani et al. 2016b	qPCR assay Bengtsson 2025 with peak florescense temperature
253_647	-	2023	Apple	Discovery	Stavanger, Norway	No	Yes	No	Yes	No	Yes	No	83
253_651	Neonectria ditissima	2023	Apple	Aroma	Kristiansand, Norway	No	Yes	No	Yes	No	Yes	No	83
253_656	-	2023	Apple	-	Lofthus, Norway	Yes	No	No	No	No	No	No	No
253_657	-	2023	Apple	Summerred	Gvarv, Norway	Yes	Yes	No	Yes	No	Yes	No	83
253_658	Neonectria ditissima	2023	Apple	Eden	Gvarv, Norway	No	Yes	No	Yes	No	Yes	No	83
Di_S2	Neonectria ditissima	2023	Apple	Discovery	Solnäs, Sweden	No	Yes	No	Yes	No	Yes	No	83
E_B1	-	2023	Apple	Elise	Brusås, Sweden	Yes	Yes	No	Yes	No	Yes	No	83
E_B2	-	2023	Apple	Elise	Brusås, Sweden	No	Yes	No	Yes	No	Yes	No	83
E1	Neonectria ditissima	2017	Apple	Elise	Sweden	Yes	Yes	No	Yes	No	Yes	No	83
EST1 (111121	-	2024	Apple	Krista	Suurna, Estonia	Yes	Yes	No	Yes	No	Yes	No	83
EST2 (114121	-	2024	Apple	Liivika	Suurna, Estonia	Yes	Yes	No	Yes	No	Yes	No	83
Nd_01	Neonectria ditissima		Apple	-	Jonstorp, Sweden	No	No	Yes	No	Yes	No	Yes	No
Nd_02	Neonectria ditissima	2013	Apple	Ingrid Marie	Jonstorp, Sweden	No	No	Yes	Yes	Yes	No	Yes	No
Nd_03	Neonectria ditissima	2013	Apple	Rubinstar	Jonstorp, Sweden	No	No	Yes	Yes	Yes	No	No	No
Nd_04	Neonectria ditissima		Apple	-	Julita, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_05	Neonectria ditissima	2013	Apple	Norrstack	Julita, Sweden	No	No	Yes	Yes	Yes	No	No	No
Nd_06	Neonectria ditissima	2013	Apple	Oranie	Jönköping, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_07	Neonectria ditissima	2013	Apple	James Grieve	Jönköping, Sweden	No	No	Yes	Yes	Yes	No	No	No
Nd_08	Neonectria ditissima		Apple	-	Kivik, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_09	Neonectria ditissima	2013	Apple	Ingrid Marie	Kivik, Sweden	Yes	No	Yes	Yes	Yes	No	No	No
Nd_10	Neonectria ditissima	2013	Apple	John Standish	Balsgård, Sweden	No	No	Yes	Yes	Yes	No	No	No
Nd_11	Neonectria ditissima	2013	Apple	Brite spur	Balsgård, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_12	Neonectria ditissima	2013	Apple	Pigeon	Balsgård, Sweden	Yes	Yes	Yes	Yes	Yes	Yes	No	83
Nd_13	Neonectria ditissima	2013	Apple	McIntosh	Balsgård, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_14	Neonectria ditissima	2013	Apple	Tompkins king	Balsgård, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_15	Neonectria ditissima	2013	Apple	Classic red delicious	Balsgård, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_16	Neonectria ditissima	2013	Apple	Beacon	Balsgård, Sweden	No	No	Yes	No	Yes	No	Yes	No
Nd_17	Neonectria ditissima		Apple	-	Balsgård, Sweden	No	No	Yes	No	Yes	No	Yes	No
Nd_18	Neonectria ditissima	2013	Apple	Freiherr von berlepsch	Balsgård, Sweden	Yes	No	Yes	No	Yes	No	No	No
Nd_19	Neonectria ditissima	2013	Apple	Gravensteiner	Balsgård, Sweden	No	No	Yes	No	Yes	No	No	No
Nd_20	Neonectria ditissima	2013	Apple	Gloster	Bjärred, Sweden	No	No	Yes	No	Yes	No	Yes	No
Nd_21	Neonectria ditissima	2013	Apple	Ingrid Marie	Bjärred, Sweden	No	No	Yes	Yes	Yes	No	No	No
Nd_22	Neonectria ditissima		Apple	-	Bjärred, Sweden	No	No	Yes	No	Yes	No	Yes	No
Nd_23	Neonectria ditissima	2014	Apple	Discovery	Belgium	No	No	Yes	No	Yes	No	No	No
Nd_24	Neonectria ditissima		Apple	-	Belgium	No	No	Yes	No	Yes	No	No	No
Nd_25	Neonectria ditissima	2014	Apple	Baron	Belgium	No	No	Yes	No	Yes	No	Yes	No
Nd_26	Neonectria ditissima	2014	Apple	Reinette	Belgium	No	No	Yes	Yes	Yes	No	No	No
Nd_27	Neonectria ditissima		Apple	-	Stockholm, Sweden	No	No	No	No	Yes	No	Yes	No
Nd_28	Neonectria ditissima	2014	Apple	Södermanlands	Stockholm, Sweden	No	No	No	No	Yes	No	No	No
Nd_29	Neonectria ditissima		Malus sylvestris	-	Belgium	No	No	No	No	Yes	No	No	No
Nd_30	Nectria galligena	1995	Apple	-	France	No	Yes	No	Yes	Yes	Yes	No	83
Nd_31	Nectria galligena	2015	Apple	-	UK	No	Yes	No	Yes	Yes	Yes	No	83
Nd_32	Neonectria ditissima		Apple	-	UK	No	No	No	No	Yes	No	Yes	No
Nd_33	Neonectria ditissima	2005	Apple	-	Slovenia	No	Yes	No	Yes	Yes	Yes	No	83
Nd_34	Neonectria ditissima	-	Fagus sylvatica	-	Slovakia	No	No	No	No	Yes	No	No	No
Nd_35	Neonectria ditissima		Liriodendron tulipifera	-	USA	Yes	No	No	No	Yes	No	No	No
Nd_36	Neonectria ditissima		Fagus grandifolia	-	USA	No	No	No	No	Yes	No	No	No
Nd_37	Nectria galligena	1987	Apple	Royal Gala	New Zealand,	Yes	Yes	No	Yes	Yes	Yes	No	83
Nd_38	Nectria galligena	-	Apple	-	New Zealand,	No	Yes	No	Yes	Yes	Yes	No	83
Nd_39	Neonectria ditissima	-	Apple	-	Sweden	Yes	Yes	No	Yes	Yes	Yes	No	83

Appendix 2

N. ditissima β-tubulin primers from Ghasemkani et al., (2016).							
Bt-fw135	5-CTCCAACACAACAACATTCG-3'						
Bt-rw284	5-AGTATCCCCGCACGTTAGAA-3'						
ITS-primers from Ihrmark et al. (2012)							
ITS1F: CTT GGT CAT TTA GAG GAA GTA A							
ITS4: TCC TCC GCT TAT TGA TAT GC							
SSR-primers from Marra & Corwin (2009)							
Locus	**GenBank no.**	**Primer name**	**Primer sequence (5'–3')**	**Microsatellite repeat motif**			
		NdCAA3f	CTTTCTAGCGGCCTGTTTTG	(CAA)8			
NditCAA3	EU853158	NdCAA3r	GACGAGGACAAACTGGTGGT				
		NdCAA11f	ACGGGGGATATACACAGCAG	(CAA)2 ... (CAA)4 ... (CAA)3 ... (CAA)3			
NditCAA11	EU853161	NdCAA11r	CTTGTCGAGGAGGTGGAGAG				
		NdGGT2f	GGACTACCACGACCCAAAGC	(GGC)6			
NditGGT2	EU853162	NdGGT2r	GTGTCTGTGGCGTATTCAGC				
		NdGGT3f	AGTCTCAATAGTTCCCCAAAGG	(GGT)5			
NditGGT3	EU853165	NdGGT3r	CGTGGCTGTTTGTTCATCC				
		NdGGT23f	GTTGTTGAGCTGGGTTGAGG	(GAG)2 ... (GAG)2			
NditGGT23	EU853164	NdGGT23r	TTTTCAGCCTTCCTTGTTGC				
		NdGGT39f	GTTTCTGGCGGCTATTGC	+/−AG; no recognizable motif			
NditGGT39	EU853167	NdGGT39r	GTCCATTCATTTAGCAGACTGG				
		NdGGT44f	CATCGCGGATTTATGTGG	(GGT)3			
NditGGT44	EU853169	NdGGT44r	TATCCACCGAGCAATTCTCC				

Appendix 3

Isolate	Study	CAA3	CAA11	GGT2	GGT3	GGT23	GGT39	GGT44
Nd_02	(Ghasemkani et al. 2016a)	295	218	187	191	215	208	183
	Bengtsson 2025	291	218	187	191	215	208*	186
		NO	YES	YES	YES	YES	YES	YES
Nd_03	(Ghasemkani et al. 2016a)	295	220	187	191	215	208	186
	Bengtsson 2025	291	218	191	191	215	208*	186
		NO	NO	NO	YES	YES	YES	YES
Nd_05	(Ghasemkani et al. 2016a)	269	218	193	191	215	208	186
	Bengtsson 2025	291	218	191	191	215	208*	186
		NO	YES	NO	YES	YES	YES	YES
Nd_07	(Ghasemkani et al. 2016a)	272	218	187	191	222	208	186
	Bengtsson 2025	291	164	187	208	215	208*	186
		NO	NO	YES	NO	NO	YES	YES
Nd_09	(Ghasemkani et al. 2016a)	295	235	187	208	215	290	186
	Bengtsson 2025	291	218	191	191	215	208*	186
		NO	NO	NO	NO	YES	NO	YES
Nd_10	(Ghasemkani et al. 2016a)	295	164	193	208	215	208	186
	Bengtsson 2025	291	218	191	191	215	208*	186
		NO	NO	NO	NO	YES	YES	YES
Nd_12	(Ghasemkani et al. 2016a)	298	218	187	191	215	208	186
	Bengtsson 2025	291	218	191	208	215	208*	186
		NO	YES	NO	NO	YES	YES	YES
Nd_21	(Ghasemkani et al. 2016a)	289	218	192	191	215	208	186
	Bengtsson 2025	291	235	187	191	215	208*	183
		NO	NO	NO	YES	YES	YES	NO
Nd_26	(Ghasemkani et al. 2016a)	292	238	187	208	215	208	186
	Bengtsson 2025	294	218	191	208	215	208*	186
		NO	NO	NO	YES	YES	YES	YES
	Matching between studies indicated by YES or NO							
	*Ambiguous results in Bengtsson 2025 study							

Data availability

ITS-sequence data generated during this study is available upon request from Jonas Skytte af Sätra (Jonas.skytte.af.satra@slu.se) or Larisa Gustavsson (Larisa.Gustavsson@slu.se).

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