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Vainio, Eeva J.

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Eeva J. Vainio¹, Sannakajsa M. Velmala¹, Pertti Salo², Seppo Huhtinen³ and Michael M. Müller¹

Defoliation of *Tilia cordata* trees associated with *Apiognomonia errabunda* infection in Finland

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Highlights

- Defoliation of *Tilia cordata* was investigated by fungal isolation from symptomatic leaf petioles and ITS sequence determination.
- The disease symptoms were associated with the presence of *Apiognomonia errabunda*.
- We report the first nucleotide sequences of *A. errabunda* from the Nordic countries.

Abstract

We investigated the causative agent of a disease outbreak affecting small-leaved limes (*Tilia cordata* Mill.) and resulting in darkening of the leaf petioles and excessive defoliation during summer 2016 in southern Finland. The fungal species composition of the symptomatic petioles was examined by culture isolation and molecular identification using ITS rDNA sequences, which revealed the most prevalent fungal species present in the petioles as *Apiognomonia errabunda* (Roberge) Höhn. Based on reviewing curated herbarium specimens deposited at the Universities of Helsinki and Turku, *A. errabunda* is native and widely distributed in small-leaved limes in Finland, and occasionally infects also other broadleaved trees, including *Quercus robur* L. and ornamental species of *Tilia* L. and *Fagus* L. The ITS sequence analysis conducted during this study revealed minor within-species polymorphisms similar to those observed earlier in the Central European and Russian populations of *A. errabunda*, and reports the first nucleotide sequences of this species from the Nordic countries.

Keywords small-leaved lime; fungal disease; anthracnose; ITS rDNA; direct PCR; Tiliaceae

Addresses ¹Natural Resources Institute Finland (Luke), Management and Production of Renewable Resources, P.O. Box 2, FI-00791 Helsinki, Finland; ²Finnish Museum of Natural History, Botanical Museum, P.O. Box 7, FI-00014 University of Helsinki, Finland; ³University of Turku, Herbarium, Biodiversity Unit, FI-20014 Turku, Finland

E-mail eeva.vainio@luke.fi

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1 Introduction

Tilia cordata Mill. (small-leaved lime) is a deciduous tree distributed widely in Central Europe, but lacking from the southernmost Mediterranean region as well as northern Fennoscandia (Radoglou et al. 2009). In Finland, it reaches its northern limit below latitude 63°44'N (Lampinen and Lahti 2016; Raino Lampinen, University of Helsinki, personal comment in 2017) and has a fragmented distribution on fertile soils. In the north, small-leaved limes are capable of producing mature seeds only during warm summers, and they propagate mainly by sprouting (Pigott and Huntley 1981). *T. cordata* trees are also commonly planted in private gardens, whereas common limes, i.e., *Tilia* × *vulgaris* Hayne (syn. *Tilia* × *europaea* L., hybrid of *Tilia cordata* and *Tilia platyphyllos* Scop. syn. *Tilia grandifolia* Ehrh.) are often planted in public parks and as streetside trees.

Tilia trees are considered to be relatively resistant to pathogens, but various pathogenic fungi commonly affect *T. cordata* trees suffering from anthropogenic disturbances in urban environments. In central Europe, the most common leaf spot diseases include *Mycosphaerella millegrana* (Cooke) J. Schröt. and *Apiognomonina errabunda* (Roberge) Höhn. (Snieškienė et al. 2012; Stravinskienė et al. 2015), and *Asteroma tiliae* F. Rudolphi and *Septoria tiliae* Westend. are also commonly detected (Snieškienė et al. 2012). Various sooty molds, such as *Aureobasidium pullulans* (de Bary) G. Arnaud, *Cladosporium herbarum* (Pers.) Link and *Leptoxylum fumago* (Woron.) R. C. Srivast. occur frequently on *T. cordata* leaves (Snieškienė et al. 2012). Several fungal pathogens like *Nectria cinnabarina* (Tode) Fr., *Chondrostereum purpureum* (Pers.) Pouzar and *Armillaria* spp. (Fr.) Staude infect *Tilia* branches, bark wounds and wood material.

In early June 2016, small-leaved limes at several sites in Southern Finland were reported to suffer from severe defoliation and darkening of the leaf petioles. In order to examine whether the symptoms were associated with known fungal pathogens of *T. cordata*, we investigated the fungal species composition of the symptomatic petioles by culture isolation and molecular identification using internal transcribed spacer (ITS) ribosomal DNA sequences. After identifying the most prevalent fungal species present in the leaf petioles as *A. errabunda*, the occurrence of this known leaf pathogen in Finland was reviewed based on curated museum specimens deposited at the Universities of Helsinki and Turku.

2 Material and methods

2.1 Sampling sites with *Tilia cordata* defoliation symptoms

Symptomatic *T. cordata* leaves were collected from three locations in southern and central Finland in early June, 2016: Kiikala (Salo, Varsinais-Suomi; 60°27'N, 23°35'E), Aulanko (Hämeenlinna, Kanta-Häme; 61°01'N, 24°27'E), and Akaa (Pirkanmaa; 61°07'N, 23°56'E). The sampling dates were 6 June 2016, 8 June 2016 and 8 June 2016, respectively. A second sampling was conducted at the Kiikala site in 1 August 2016. The sampling sites at Kiikala and Akaa are private gardens, and the Aulanko site is a protected forest park.

The most prominent symptoms were excessive leaf loss (Figs. 1A, C) and blackening of the leaf petioles (Fig. 1B). The Aulanko and Kiikala sites were inspected again in August, and the trees still suffered from defoliation as well as leaf anthracnose symptoms that had developed during the summer. At the Kiikala site, similar symptoms were reported already in 2015 and again in June 2017. At each sampling site, 3–12 neighboring *T. cordata* trees were severely affected by the disease.



Fig. 1. Disease symptoms caused by *Apiognomonina errabunda*. (A) Small-leaved lime trees suffering from defoliation in Aulanko, Hämeenlinna; (B) Leaves of *Tilia cordata* with darkened petioles collected from Kiikala, Salo; (C) leaf loss of small-leaved limes in Kiikala, Salo; (D) *A. errabunda* strain 3C2V representing the dominating morphotype retrieved from the leaf petioles cultured on MOS agar plate covered with a cellophane membrane. Pictures A, B and C were provided by Tapio Ykspetäjä and Seppo Lagom.

According to the weather data collected by the Finnish Meteorological Institute (<http://en.ilmatieteenlaitos.fi>), the mean temperature in May 2016 was 2.4–3.4 degrees above mean in the Hämeenlinna-Salo region with an average temperature of 10.1–10.4 °C in May during 1981–2010, while June temperatures were near average (<1 degrees above the mean temperature of 14.3–14.7 °C). The rainfall in June was slightly higher than normal (111–125% of mean June rainfall of 56.8–66.3 mm during 1981–2010), whereas May rainfall was 76% and 105% of average in Hämeenlinna and Salo, respectively (with mean June rainfall of 40.3 and 37.1 mm).

2.2 Direct polymerase chain reaction (PCR) from *Tilia cordata* leaf petioles

Small pieces of blackened leaf petioles were initially sampled with Harris Unicore™ sampling tool (Ø 1 mm) from five symptomatic *T. cordata* leaves (one from Kiikala and two from each of Aulanko and Akaa) and subjected to direct polymerase chain reaction (PCR) amplification with ITS primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990) using the dilution protocol of Phire Plant Direct PCR Kit (Thermo Fischer Scientific) as described earlier by Velmala et al. (2013). Resulting amplicons were extracted from 1% agarose gel supplemented with synergel (Diversified Biotech) and purified with the EZNA Biotek gel extraction kit (Omega Biotek) and subjected to sequencing in both directions with primers ITS1F and ITS4 at Macrogen Inc., Korea (<http://www.macrogen.com>).

2.3 Fungal cultures isolated from blackened *Tilia cordata* leaf petioles

A second batch of leaf samples was collected in 1 August 2016 from three diseased *T. cordata* trees at the Kiikala site and subjected to culture isolation. Blackened leaf petioles (N=20) were dispatched from the leaves and surface sterilized. In order to discard putative surface contaminants while preserving the viability of fungal mycelia residing within the petiole tissues, we tested five different surface sterilization protocols, each carried out using four petioles: (1) no surface sterilization, (2) 30 s incubation in 70% ethanol, (3) incubation of 30 s in 70% ethanol, 30 s in 5% NaOCl and 2× 30 s in 70% ethanol (4) incubation of 30 s in 70% ethanol, 60 s in 5% NaOCl and 2× 30 s in 70% ethanol (5) incubation of 30 s in 70% ethanol, 4 min in 5% NaOCl and 2× 30 s in 70% ethanol. The leaf petioles were then placed on malt extract agar and water agar plates (two and two petioles per treatment type) and incubated at 15 °C until the appearance of fungal hyphae. Fungal colonies emerging within three weeks of incubation were subcultured on modified orange serum (MOS) agar plates covered with cellophane membranes, incubated in ambient light at room temperature and morphotyped.

Representative isolates from each morphotype were selected for ITS sequence analysis using direct PCR with the Phire II Hot Start DNA polymerase as described above, but using a small amount of mycelia scraped directly from the membrane-covered MOS agar plates. According to the manufacturer (Thermo Fischer Scientific), the DNA polymerase used has an error rate of 1.14×10^{-5} .

2.4 Internal transcribed spacer (ITS) sequence analysis

Global sequence alignments were constructed using Geneious R10 version 10.0.8 (Biomatters Ltd.) and MAFFT alignment algorithms. Sequence comparisons were conducted using NCBI BlastN (nucleotide collection) and the UNITE database (Köljalg et al. 2013).

2.5 Herbarium collections

Herbarium specimens deposited at the Universities of Helsinki (LUOMUS) and Turku were revisited to investigate the earlier occurrences of *A. errabunda* in Finland. The survey included also specimens deposited using the current or past anamorph names of the fungus (e.g., *Gloeosporium tiliae* Oudem, *Gloeosporium umbrinellum* Berk. & Broome, *Discula umbrinella* (Berk. & Broome) M. Morelet; see MycoBank at <http://www.mycobank.org> and Index Fungorum at <http://www.indexfungorum.org>).

3 Results

3.1 Fungal morphotypes

Altogether 31 fungal isolates were retrieved from the 20 leaf petioles collected from the Kiikala sampling site (1–3 distinct colonies from each petiole; 5, 7, 6, 8 and 5 colonies per sterilization treatment protocol 1–5, respectively). The majority of these cultures (N=25) formed light gray velutinous mycelial colonies on MOS plates, part of them with concentric dark rings or lobed colony margins (Fig. 1D). The remaining six cultures represented mostly dark grey spotted or moldlike morphotypes. Four of these moldlike cultures were obtained from petioles plated without surface sterilization.

Table 1. ITS sequence information.

Sequences determined during this study		Most similar sequences in the NCBI GenBank database					
Sample ID	Location	GenBank accession	GenBank accession	Host tree/sample source	Country (if known)	Fungal species	BlastN identity (query cover)
Akaa5 ¹	Akaa	KY927826	DQ313554	<i>Tilia cordata</i>	Novgorod, Russia	<i>Apiognomonita errabunda</i>	100% (100%; 501/501)
2C2 ²	Kiikala	KY927827	AJ293871	<i>Quercus</i> sp.	unpublished	<i>Fusicoccum quercus</i>	100% (100%; 501/501)
3C2V ²		KY927828					
4B1 ²		KY927829					
4C1 ²		KY927830					
5C1 ²		KY927831					
5C2 ²		KY927832					
Akaa4 ¹	Akaa	KY927822	DQ313524	<i>Chamaenerion angustifolium</i>	Switzerland	<i>Apiognomonita errabunda</i>	100% (100%; 501/501)
Aul3 ¹	Hämeenlinna	KY927821	AY853204	<i>Quercus</i> sp.	Netherlands	<i>Fusicoccum quercus</i>	100% (100%; 501/501)
4D2 ²	Kiikala	KY927823	AJ293872	<i>Quercus</i> sp.	unpublished	<i>Fusicoccum quercus</i>	100% (100%; 501/501)
5B1 ²		KY927824	AJ293873	<i>Quercus</i> sp.	unpublished	<i>Fusicoccum quercus</i>	100% (100%; 501/501)
4C2 ²		KY927825	AJ293874	<i>Quercus</i> sp.	unpublished	<i>Fusicoccum quercus</i>	100% (100%; 501/501)
			FJ820749	Air sample	Germany	Uncultured fungus	100% (100%; 501/501)
5A1 ²	Kiikala	KY927820	DQ313524	<i>Chamaenerion angustifolium</i>	Switzerland	<i>Apiognomonita errabunda</i>	99% (100%; 501/502)
1C2 ^{2,3}	Kiikala	KY927833	KT896641	<i>Ips typographus</i>	Finland	<i>Monilinia</i> sp.	99% (92%; 442/443)
1D2 ^{2,3}	Kiikala	KY927834	KF859919	Canadian National Mycological Herbarium (DAOM 166439)	unpublished	<i>Botrytis cinerea</i>	100% (100%; 487/487)
4B2 ^{2,3}	Kiikala	KY927835	LN850753	<i>Capsicum annum</i>	Cuba	<i>Cladosporium</i> sp.	100% (100%; 491/491)

¹Direct PCR product from leaf petiole²Cultured isolate. The first number of the sample code corresponds to the leaf petiole treatment used (1–5)³Sequence determined in one direction only

3.2 Internal transcribed spacer (ITS) sequences analysis

Using direct PCR, we obtained amplification products of the expected size from two leaf petiole samples from Akaa and one sample from Aulanko. The corresponding high quality ITS sequences covered the entire ITS1–5.8S rDNA–ITS2 region of 501 nt. No amplification products were obtained by direct PCR from the Kiikala sample, whereas ITS sequences were successfully determined from all the 13 cultured isolates from Kiikala selected for sequence analysis.

Based on ITS sequence analysis, the most common morphotype constituting 81% of the retrieved cultures from Kiikala was identified as *A. errabunda*. This species constituted 20%, 86%, 100%, 88%, and 100% of the cultures obtained using surface sterilization protocols 1–5, respectively. The other morphotypes observed more than once were identified as *Botrytis* sp. P. Micheli, *Cladosporium* sp. Link and *Monilinia* sp. Honey (Table 1).

There were three different ITS sequence variants representing *A. errabunda* among the Kiikala isolates, two of which were also detected by direct PCR in Aulanko and/or Akaa (Table 1). These sequence variants were highly similar and contained only 1–2 differing nucleotide sites corresponding to 99.6–99.8% overall sequence identity. One sequence variant detected in Akaa and Kiikala showed 100% identity to those of *A. errabunda* isolates from *T. cordata* in Novgorod, Russia. Another sequence variant found at all three sampling sites shared 100% identity with the ITS sequence of *A. errabunda* isolated from fireweed (*Chamaenerion angustifolium* L. Scop.) in Switzerland (Sogonov et al. 2007). There was also one unique sequence variant with an insertion of a single G nucleotide after position 48 of the ITS2 sequence (GenBank accession KY927820). All sequences lacked the A insertion at sequence position 182 characteristic of *Apiognomonina veneta* (Sacc. & Speg.) Höhn. (Sogonov et al. 2007). The sequences determined in this study were deposited in GenBank under the accession numbers KY927820–KY927835.

3.3 Previous observations of *Apiognomonina errabunda* in Finland based on herbarium specimens

There were a total of 27 and 6 curated specimens of *A. errabunda* in the herbaria of the Universities of Helsinki and Turku, respectively. Detailed information is reported in Supplementary file S1, available at <https://doi.org/10.14214/sd.7749>, which lists the *A. errabunda* specimens of the University of Helsinki with corresponding URLs at the Finnish Biodiversity Information Facility (FinBIF; <https://www.laji.fi>). Specimens of the University of Turku are described at the Research portal Mkk Kotka (TUR, <http://mus.utu.fi/TFU.62600>; TUR, <http://mus.utu.fi/TFU.77828>; TUR, <http://mus.utu.fi/TFU.107022>; TUR, <http://mus.utu.fi/TFU.116689>; TUR, <http://mus.utu.fi/TFU.153601>; TUR, <http://mus.utu.fi/TFU.172978>). The collection sites of herbarium specimens and the leaf samples collected during this study are shown in Fig. 2.

The oldest available museum specimen of *A. errabunda* in Finland dates from 1870 and was identified by P. A. Karsten as *Gnomonia petiolicola* (Fuckel) P. Karst. Based on re-examining this sample, Monod (1983) later identified it to be *A. errabunda* (Suppl. file S1). During the following decades, this species has been detected at several locations in southern and southwestern Finland, and the northernmost available samples originate from Jyväskylä (62°14'N, 25°45'E). The most common host species is *T. cordata*, but there are also single samples collected from *T. platyphyllos*, *Quercus robur*, *Tilia* × *vulgaris* and *Fagus sylvatica* L. (the latter two are non-native tree species used as ornamentals in Finland). Most samples have been named according to the anamorph state of *A. errabunda*, namely *Gloeosporium tiliae* (Suppl. file S1).



Fig. 2. Historical records of *Apiognomonina errabunda* in Finland and the sampled disease outbreaks on small-leaved lime in 2016. The 2016 disease outbreaks were observed in three locations (red dots). Sampling sites of *A. errabunda* specimens in the herbaria of University of Helsinki (LUOMUS) and University of Turku date from 1870 to 2004 and are plotted with the following symbols indicating host tree species: gray = *Tilia cordata*, black = *Tilia × europaea* or white dots = other or unknown tree species. The northern border of the distribution area of *Tilia cordata* in Finland is given according to Lampinen and Lahti (2016).

4 Discussion

In this study, we identified *Apiognomonia errabunda* as the most probable cause of the disease symptoms observed in small-leaved lime trees in early summer 2016. This fungal species was the most common culture morphotype isolated from blackened leaf petioles, and the only species detected using direct PCR amplification. The observed symptoms also supported *A. errabunda* as the causative agent: reported disease symptoms include brown leaf spots with dark margins as well as defoliation in early summer, followed by wilting and death of branches (Horst 2001). Since Karsten's early observations (Karsten 1873), the disease has been described by its anamorph name *Gloeosporium tiliae* in Finnish plant pathology textbooks (Liro 1924; Rauhala 1958), which mention this species to be common. Pohjakallio (1963) describes the disease on lime and oak trees using the name *Discula quercina* (Cooke) Sacc. (= *Gloeosporium umbrinellum*). *A. errabunda* has a wide geographical distribution in the temperate Northern Hemisphere including central and Eastern Europe and North America, and occurs on several important forest trees, such as *Quercus* spp. L., *Fagus sylvatica*, *Populus tremula* L. and *Castanea sativa* Mill. (Sogonov et al. 2007). However, the occurrence of this fungal pathogen in Finland has not been systematically reviewed in scientific literature before this report.

Moreover, prior to this study there were no GenBank records reporting nucleotide sequences of *A. errabunda* from any of the Nordic countries, although it has been recorded to occur also in Sweden and Denmark (Sogonov et al. 2007; Eriksson 2014; Sundelin et al. 2015). The ITS sequence analysis conducted during this study revealed minor within-species polymorphisms similar to those observed earlier in the Central European and Russian populations of *A. errabunda*. Thus, one of the common sequence variants detected in this study is identical with the *A. errabunda* sequence described by Sogonov et al. (2007) from Novgorod, Russia. This sequence variant originates from *T. cordata*, and represents the taxonomical clade formerly classified as *Apiognomonia tiliae* (Rehm) Höhn. (Sogonov et al. 2007; see also the description by Dr. Paul Cannon from the Mycology Department at Kew (<http://fungi.myspecies.info/>)). However, the second sequence variant observed in Finland clusters outside this "tiliae clade" of *A. errabunda* (Sogonov et al. 2007). It should be noted that the latter sequence was also 100% identical with unpublished ITS sequences assigned to *Fusicoccum quercus* Oudem. (Table 1). According to the MycoBank database (<http://www.mycobank.org>), *F. quercus* may be a synonym of *Apiognomonia errabunda*, and the fungus causes bark cankers on oak trees (Ragazzi et al. 2003). However, Sogonov et al. (2007) conclude that the unpublished sequences assigned as *F. quercus* (AY853206-14 and AJ293872-5; see Table 1) actually represent *A. errabunda* and not *F. quercus* (the anamorphic state of *Botryosphaeria* Ces. & De Not.). Overall, only four polymorphic sites have been reported in the ITS1-5.8S-ITS2 region of *A. errabunda* (Sogonov et al. 2007).

From an epidemiological point of view, it may be noteworthy that in 2016 the noticeable disease symptoms were observed soon after a massive amount of aphids drifted via southwestern winds into Finland in May 15th (Huusela-Veistola 2016). The rarer fungal morphotypes isolated from the leaf petioles during this study (*Botrytis* sp. and *Cladosporium* sp.) have been described to cause sooty mold in *Tilia* and are typically associated with aphid infestation (Snieskienė et al. 2012).

We conclude that the disease symptoms observed in early summer 2016 in *T. cordata* can be associated with the presence of *A. errabunda*, which is a native and widely distributed species in Finland causing occasional disease outbreaks. Future studies are needed to investigate the host range and epidemiology of *A. errabunda* in the Nordic countries. At the Kiikala sampling site, there were five healthy common lime trees (*Tilia* × *vulgaris*) growing next to twelve small-leaved lime trees, all of which were affected by the disease in 2015 and 2016 (Seppo Lagom, pers. comm. 2016).

Moreover, the vast majority of Finnish museum specimens originated from *T. cordata*, and there were only single occurrences of the disease on common lime or large-leaved lime (*Tilia grandifolia* = *T. platyphyllos*). These observations suggest that our native species *T. cordata* may be more susceptible to *A. errabunda* than the hybrid limes commonly planted as park and streetside trees.

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Total of 20 references.

Supplementary files

S1.xlsx; *Apiognomonina errabunda* specimens of the University of Helsinki, Excel table, available at <https://doi.org/10.14214/sf.7749>.