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Neofabraea kienholzii, a novel causal agent of grapevine trunk diseases in Hungary

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Abstract Recently, more and more new fungal pathogens have been described as causal agents of grapevine trunk diseases (GTDs), which lead to increasingly significant economic losses in viticulture worldwide. The genus *Neofabraea* consists of species mainly known as important plant pathogens causing perennial canker and bull's eye rot, a common postharvest disease of apple (*Malus domestica*) and pear (*Pyrus communis*) fruits. *Neofabraea kienholzii* also causes lesions on pome fruits and canker on woody tissues, but its pathogenicity has not been demonstrated on grapevine yet. In 2015, two strains, identified as

N. kienholzii based on ITS sequence data, were isolated from vines showing symptoms of GTDs in Hungary. For an unambiguous taxonomic placement of the isolates, four loci (ITS, LSU, TUB2 and RPB2) were amplified and sequenced. The phylogenetic analysis confirmed that the two isolates represent *N. kienholzii*. Pathogenicity tests performed on potted grapevines, shoots, and canes confirmed the virulence of these fungi. Their growth and sporulation on different media were also investigated. To the best of our knowledge, this is the first proof of *N. kienholzii* might cause symptoms on *Vitis* and might have a role in GTDs.

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Grapevine trunk diseases (GTDs) cause great economic losses in viticulture worldwide, due to the cost of replacement of dead grapevines that can exceed 1.5 billion dollars per year (Hofstetter et al. 2012). The most important and severe diseases include eutypa dieback, esca complex and Petri disease, botryosphaeria dieback, phomopsis dieback, and black foot disease (de la Fuente Lloreda et al. 2016). However, examining the mycota of healthy and diseased plants of vineyards and rootstock, a high diversity of fungal taxa was found (Hofstetter et al. 2012). In Hungary, although vineyards have been monitored for GTDs since 2015, still little is known about the potential pathogens. Recently, the Tokaj Wine Region – a historically and economically important territory for winemaking in the north-eastern

part of the country – was investigated to evaluate the occurrence of GTDs, focusing on the environmental factors affecting the incidence of *Diplodia seriata*, a pathogen involved in botryosphaeria dieback (Kovács et al. 2017). Two newly detected fungi have been also described: *Seimatosporium vitis* (Váczy 2017), an accompanying species of different main pathogens of GTDs, and *Dothiorella omnivora* (Váczy et al. 2018), a causal agent of botryosphaeria dieback.

The genus *Neofabraea* (Helotiales, Ascomycota) includes eight species (Johnston et al. 2004; Chen et al. 2016). These species represent fungal pathogens isolated from various plant species including apple and pear, on which *N. kienholzii* is known to cause perennial canker and bull's eye rot (Wenneker et al. 2017). While the economic significance of perennial canker is difficult to estimate, the post-harvest bull's eye rot was reported to cause 5–50% yield losses depending on the examined vintage and crop variety (Garipey et al. 2005). Although some strains of *N. malicorticis* have been isolated from healthy grapevines (Hofstetter et al. 2012) and a single isolate of *N. kienholzii* was isolated from *Vitis* trunk with GTD (Baranek et al. 2018), we are not aware of any reports of proof for *Neofabraea* causing GTD symptoms of *Vitis* cultivars.

In 2015, approximately 500 grapevines were monitored in 25 vineyards in five wine regions in Hungary and the number of GTD symptomatic plants was determined. In order to identify the causal agents, fungi were isolated from the trunks and characterized using general morphological and molecular tools based on sequencing of the internal transcribed spacer region (ITS) (Schoch et al. 2012). During these studies, two isolates showing >97% internal transcribed spacer (ITS) rDNA sequence similarity with *Neofabraea kienholzii* were chosen for further investigations. In this study, in order to characterize the two isolates obtained from GTD-affected vines in Hungary and putatively identified as *N. kienholzii*, we aimed to: (i) provide an unambiguous taxonomic placement of the isolated strains by multi-locus molecular phylogenetic analyses; (ii) study their morphology, growth and sporulation on different media; and (iii) carry out pathogenicity tests on grapevines.

Woody tissue samples were collected from grapevine plants showing typical GTD symptoms, e.g., foliar discoloration, stunted shoots, apoplexy (Mugnai et al. 1999; Sosnowski et al. 2007; Kuntzmann et al. 2010), from five wine regions (Eger, Neszemély, Pécs, Szekszárd, and Villány) in Hungary in 2015. Five thin

discs were cut from asymptomatic and necrotic parts of the trunk. Bark tissue was removed, and the discs were surface-sterilized in 1% chloramine B solution for 5 min, rinsed in sterile distilled water and dried between two layers of paper towels. Five small pieces were cut from the discs, placed on potato dextrose agar (PDA, Sigma-Aldrich, Germany) and incubated at 23–25 °C. The growth of the fungi was monitored for four weeks and in order to obtain pure cultures, the individual colonies were transferred separately to new plates containing PDA. The isolates were identified based on their morphological and molecular properties.

Two strains showing colony morphology similar to that of *Neofabraea* species were isolated from trunks showing discoloration of leaves and necrotic lesions in the woody tissues. The strain T15377 was found in a 13-year-old Chardonnay' vine from Neszemély Wine Region in North West Hungary (Fig. 1a). Besides this fungus, *Diplodia seriata* and *Eutypa lata* were also detected in the trunk. The strain T15671 was isolated from a 14-year-old Kékfrankos' from Eger Wine Region in North Hungary (Fig. 1b), together with *Phaeoconiella chlamydospora* and *Phaeoacremonium minimum*. In order to determine the species similar to *Neofabraea*, molecular characterization of the strains was performed. DNA was extracted from lyophilized non-sporulating cultures using DNeasy Plant Mini Kit (Qiagen, Germany) following manufacturer's instructions. The ITS and the partial 28S rDNA gene (LSU) were amplified and sequenced with ITS1 and ITS5 (White et al. 1990), and LR0R (Rehner and Samuels 1994) and LR5 (Vilgalys and Hester 1990) primer pairs, respectively. For further molecular phylogenetic characterization, partial β -tubulin (TUB2) and RNA polymerase II second largest subunit (RPB2) genes were amplified with the Bt-T2m-Up and Bt-LEV-Lo1 (de Jong et al. 2001), and RPB2-5F2 (Sung et al. 2007) and RPB2-7cr (Liu et al. 1999) primer pairs, respectively. The ITS, LSU, TUB2, and RPB2 sequences of the T15377 and T15671 strains were deposited in NCBI GenBank (Table 1). Both strains have been deposited at CBS-KNAW Culture Collection (Westerdijk Fungal Biodiversity Institute, Netherlands) (Table 1).

Alignments of our sequences with sequences of related species obtained from GenBank (Table 1) were assembled using E-INS-i method of the online MAFFT version 7 (Kato and Standley 2013). The alignments were checked and edited in MEGA6 (Tamura et al. 2013). Multi-locus phylogenetic Bayesian analysis was

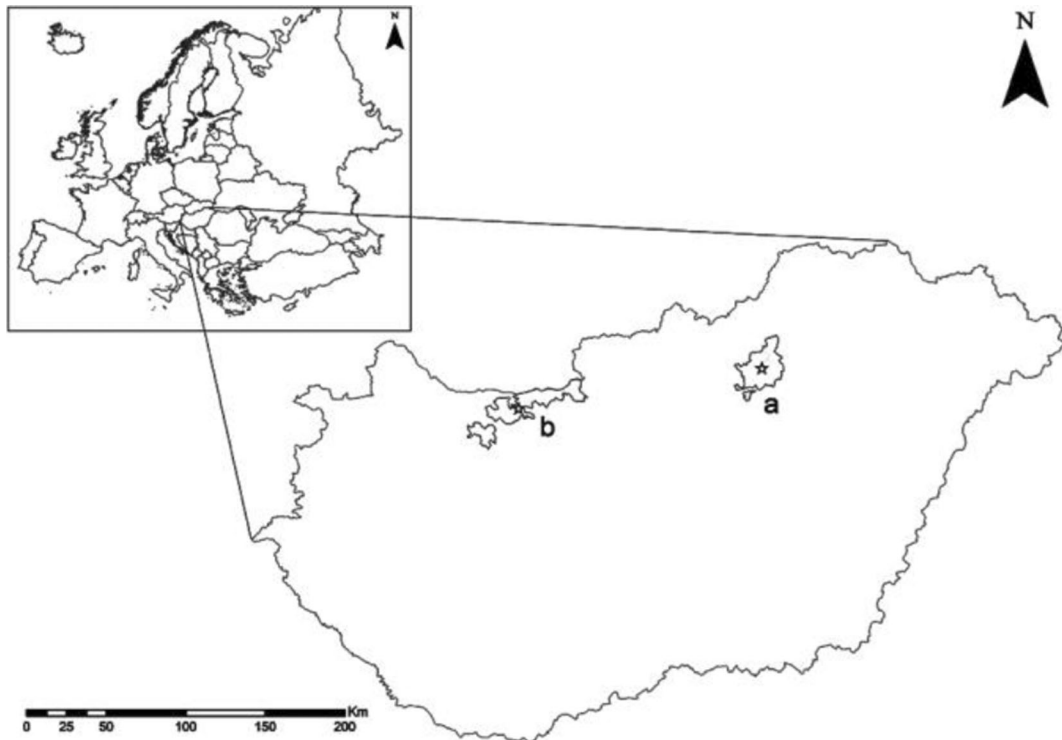


Fig. 1 Map indicating the origins of *Neofabraea kienholzii* isolates in Hungary. **a** Strain T15377 was collected in Eger Wine Region, **b** T15671 was collected in Neszmély Wine Region

performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the GTR + G nucleotide substitution model. Four Markov chains were run for 10,000,000 generations sampled every 1000 generations with a burn-in value set at 4000 sampled trees. Topological convergence was checked by AWTY (Nylander et al. 2008). The maximum likelihood (ML) phylogenetic analysis was carried out with the raxmlGUI v. 1.3 (Silvestro and Michalak 2012) implementation of RAxML (Stamatakis 2014). The GTR + G nucleotide substitution model was used with ML estimation of base frequencies and a ML bootstrap analysis with 1000 replicates was used to test the support of the branches. The phylogenetic trees were visualized and edited using MEGA6 (Tamura et al. 2013).

Morphology and sporulation of the two isolates were examined on PDA, malt extract agar (MEA, Sigma-Aldrich, Germany), oatmeal agar (OA, Sigma-Aldrich, Germany), and water agar (WA, agar from Sigma-Aldrich, Germany) plates, with four technical replicates for each condition. Plates were incubated at 23–25 °C and colony diameter was measured every day for 14 days. Conidia were obtained on OA and their size was determined with ImageJ software (Schneider et al.

2012) using a Bürker chamber as reference, measuring 100–100 conidia of each strain. Microscopic examinations were done by Alpha BIO-5f (Alpha) microscope equipped with an Arctam-500MI camera (Artray).

Pathogenicity tests were performed on young shoots, 1-year-old canes, and potted Blauburger' grapevines. Four shoots, canes, or potted plants were inoculated with agar plugs containing actively growing mycelium of the T15377 or T15671 strains and without mycelium, as control. Young shoots were surface sterilized with 70% alcohol, wounded with sterile scalpel and a 5 mm diameter PDA plug was placed on the wound and fixed with Parafilm M®. Shoots were kept in water at RT. During pruning, one cane of several vines was left to carry out pathogenicity tests. Canes were surface sterilized; a 5 mm diameter hole was drilled in each one and were inoculated as described above. Potted grapevines were inoculated following the same method as for the one-year-old canes. Symptoms were monitored after 14 days in case of young shoots, 5 months in case of canes, and two months in case of potted grapevines.

The T15377 and T15671 strains were re-isolated from the grapevine vascular tissues according to the subsequent protocol: about 2 cm above and below the inoculation

Table 1 List of isolates included in the phylogenetic analysis

Species	Isolate	GenBank accession number				Host plant	Country	References
		ITS	LSU	TUB2	RPB2			
<i>Neofabraea actinidiae</i>	CBS 121403 ^T	KR859079	KR858870	KR859285	KR859319	<i>Actinidia deliciosa</i>	New Zealand	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>N. actinidiae</i>	CBS 194.69	KR859080	KR858871	KR859286	KR859320	<i>Ilex verticillata</i>	Netherlands	(Chen et al. 2016)
<i>N. inaequalis</i>	CBS 326.75 ^T	KR859081	KR858872	KR859287	KR859321	<i>Chamaecyparis</i> sp.	France	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>N. kienholzii</i>	CBS 126461 ^T	KR859082	KR858873	KR859288	KR859322	<i>Malus domestica</i> cv. Fuji	USA	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>N. kienholzii</i>	CBS 318.77	KR859083	KR858874	KR859289	KR859323	<i>Aucuba japonica</i>	Netherlands	(Chen et al. 2016)
<i>N. kienholzii</i>	TI15377 = CBS 144251	MG989275	MT449925	MH005032	MH005030	<i>Vitis vinifera</i> cv. Chardonnay	Hungary	this study
<i>N. kienholzii</i>	TI15671 = CBS 144252	MG989276	MT449926	MH005033	MH005031	<i>Vitis vinifera</i> cv. Kékfrankos	Hungary	this study
<i>N. krawtzevii</i>	CBS 102867	KR859084	KR858875	AF281459	KR859324	<i>Populus x berolinensis</i>	Norway	(de Jong et al. 2001; Chen et al. 2016; Marin-Felix et al. 2017)
<i>N. malicorticis</i>	CBS 122030 ^{NT}	KR859086	KR858877	KR859291	KR859326	<i>Malus</i> sp.	USA, Oregon	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>N. malicorticis</i>	CBS 102863	KR859085	KR858876	KR859290	KR859325	apple tree canker	Canada, British Columbia	(Chen et al. 2016)
<i>N. perennans</i>	CBS 102869 ^T	KR859087	KR858878	AF281473	KR859327	apple tree canker	Canada, British Columbia	(de Jong et al. 2001; Chen et al. 2016; Marin-Felix et al. 2017)
<i>N. perennans</i>	CBS 453.64	KR859089	KR858880	AF281474	KR859329	–	UK	(Chen et al. 2016)
<i>N. perennans</i>	CBS 275.29	KR859088	KR858879	KR859292	KR859328	<i>Malus sylvestris</i>	–	(Chen et al. 2016)
<i>P. caliginosa</i>	CBS 124806 ^T	KR859090	KR858881	KR859293	KR859330	<i>Eucalyptus caliginosa</i>	Australia, New South Wales	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>Parafabraea eucalypti</i>	CBS 124810 ^T	KR859091	KR858882	KR859294	KR859331	<i>Eucalyptus globulus</i>	Australia	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>Phyctema vagabunda</i>	CBS 109875	KR859275	KR859069	AY064702	KR859346	<i>Fraxinus americana</i>	USA, Michigan	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>Ph. vagabunda</i>	CBS 304.62	KR859276	KR859070	KR859310	KR859347	<i>Malus sylvestris</i>	South Africa	(Chen et al. 2016)
<i>Ph. vincetoxici</i>	CBS 123727 ^T	KR859278	KR859072	KR859312	KR859349	<i>Vincetoxicum officinale</i>	Czech Republic	(Chen et al. 2016; Marin-Felix et al. 2017)

Table 1 (continued)

Species	Isolate	GenBank accession number				Host plant	Country	References
		ITS	LSU	TUB2	RPB2			
<i>Ph. viticetoxici</i>	CBS 102469	KR859277	KR859071	KR859311	KR859348	<i>Paeonia</i> sp.	New Zealand	(Chen et al. 2016)
<i>Pseudofabrea citricarpa</i>	CBS 130297 ^T	KR859279	KR859073	KR859313	KR859350	<i>Citrus unshiu</i>	China	(Chen et al. 2016; Marin-Felix et al. 2017)
<i>Ps. citricarpa</i>	CBS 130532	KR859280	KR859074	KR859314	KR859351	shoot of <i>Citrus unshiu</i>	China	(Chen et al. 2016)
<i>Ps. citricarpa</i>	CBS 130533	KR859281	KR859075	KR859315	KR859352	leaf of <i>Citrus unshiu</i>	China	(Chen et al. 2016)

^T and ^{NT} indicate ex-type and ex-neotype strains, respectively

¹ CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands

² ITS: internal transcribed spacers and intervening 5.8S nrDNA; LSU: partial 28S nrDNA; TUB2: partial β -tubulin gene; RPB2: partial RNA polymerase II second largest subunit

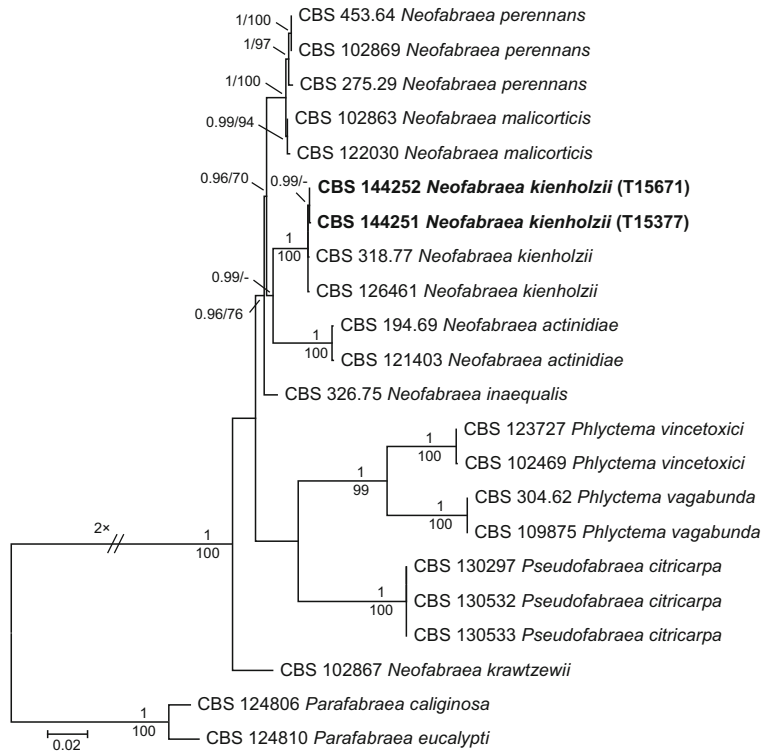
point discs were cut from the shoots and canes, were surface sterilized in sodium hypochlorite (4% available chlorine *w/v*), rinsed in 70% alcohol and dried. Potted grapevines were cut vertically. Five pieces per replicate were placed on PDA and plates were incubated at 23–25 °C for one week, until the colonies appeared. The re-isolated strains were verified by sequencing of their ITS region.

According to the multi-locus phylogenetic analysis, both isolates from Hungary (T15377 and T15671) belonged to the genus *Neofabrea* and formed a well-supported clade with *N. kienholzii* isolates (Fig. 2). The difference in the ITS, LSU, TUB2 and RPB2 sequences of our isolates and other *N. kienholzii* strains CBS 318.77 and CBS 126461 (ex-type) was 0–0.2%, 0%, 0–0.3% and 0–0.1%, respectively. The results of the molecular phylogenetic analyses unambiguously show that the two isolates are *N. kienholzii*.

After being kept at 23–25 °C for 14 days, the colony diameter of the two strains showed significant difference on MEA (30 and 23.5 mm), OA (42 and 39.5 mm), and WA (34.5 and 18.5 mm), but not on PDA (24 mm). Colonies on PDA were salmon or cream-colored covered with white radiating mycelium, white or greyish aerial mycelium in the center and wavy margin. On MEA, the strains showed similar morphologies as detected on PDA, but with smooth margin. Colonies on OA were yellowish, waxy, on WA cream-colored, on both media lacking the white cottony mycelium. Sporulation was detected at 14 days post inoculation (dpi) on OA. Microconidia were one-celled, oblong-ellipsoidal, and weakly to strongly curved of both strains (Fig. 3b). Slight variation was observed in the size of conidia in case of the examined strains – 5–8.5 \times 2.5–3.5 μ m of T15377, 4–7 \times 2–3.5 μ m of T15671 – and they were slightly bigger than the microconidia described for the same species before (Spotts et al. 2009; Wenneker et al. 2017). Macroconidia – rarely occurred – were one-celled, oblong-ellipsoidal, sometimes weakly curved (Fig. 3c), measuring 13 \times 3 μ m of each isolate. Conidiogenous cells of the colonies grown on OA forming macroconidia were also observed (Fig. 3a).

The virulence of the two isolates was confirmed by artificial inoculations under laboratory and field conditions. White mycelial mass appeared on the young shoots (Fig. 4a), suggesting the growth of the fungus on grapevine tissues. Cross section of one-year-old canes showed linear brown vascular coloration (Fig. 4b, d). Brown coloration was seen around the inoculation point in vertically cut

Fig. 2 Maximum Likelihood (RAxML) tree of concatenated ITS, LSU, TUB2 and RPB2 sequences of representative species of *Neofabraea* and related genera. Bayesian posterior probabilities (≥ 0.90) are shown above branches and before slashes, ML bootstrap support values (≥ 70) are shown below branches and after slashes. Strains introduced in this study are shown in bold. *Parafabraea eucalypti* and *P. caliginosa* were used as outgroups. The scale bar indicates expected changes per site per branch



potted vines too (Fig. 4c). *Neofabraea kienholzii* was successfully re-isolated from the inoculated grapevines (Fig. 4e), and sequences of the ITS regions were identical to those of the previously described strains. Control plants remained symptomless.

According to morphological characteristics and the results of multi-locus molecular phylogenetic analyses, the two isolates examined causing typical symptoms of GTDs

belong unambiguously to the species *N. kienholzii*. The helotialean genus *Neofabraea* represents a taxonomically challenging taxon that currently comprises eight species with diverse host preferences, including the type species of *Phlyctema*, *P. vagabunda*, which is the asexual morph of *N. alba* [current name: *N. vagabunda*] (Verkley 1999; Johnston et al. 2014; Chen et al. 2016). The first species – *N. malicorticis* – was described as a causal agent of apple

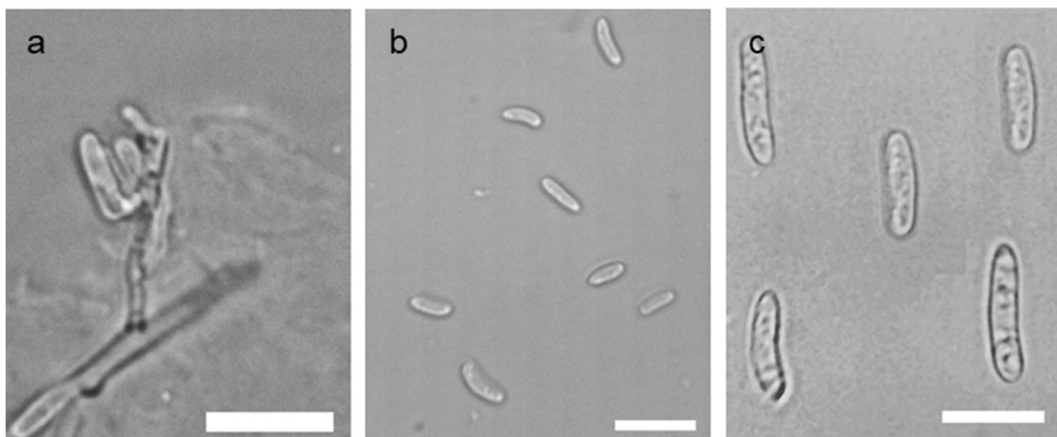


Fig. 3 Macro- and microconidia production of two Hungarian *Neofabraea kienholzii* isolates, induced on OA. **a** Conidiogenous cells of the strain T15377. **b** Microconidia of the strain T15377. **c** Composite photo of macroconidia of T15377 and T15671 strains. Scale bars: 10 μm



Fig. 4 Grapevine cv. Blauburger' inoculated with Hungarian *Neofabraea kienholzii* isolates. **a** Infection of young shoot at 14 dpi with white mycelium mass. **b, d** Healed wound on a one-year-old cane after removal of the mycelial disc and cross section

showing brown vascular coloration (arrows). **c** Infection of potted grapevine. Symptoms appeared as dark coloration of the vascular tissue around the inoculation point. **e** *N. kienholzii* isolated from grapevine wood growing on PDA

anthracnose (Jackson 1913). In 1939, *N. populi* [current name: *N. krawtzevii* (Verkley 1999)] was isolated from poplar cankers and characterized (Thompson 1939), and was detected in Southern Finland also on poplar trees (Kasanen et al. 2002). Later, other fungal pathogens were reported causing perennial canker, bull's eye rot of apple and pear fruits, and described as *Neofabraea* or *Pezicula alba*, *N. malicorticis*, and *N. perennans* (Kienholz 1939; Guthrie 1959; Verkley 1999, and Abeln et al. 2000). The phylogenetic relationship among these taxa was first clarified by de Jong et al. (2001), suggesting the existence of *N. malicorticis*, *N. perennans*, *N. alba*, and an undetermined *Neofabraea* species. Regarding possible natural habitats, *Neofabraea* species have been detected in soil DNA sequences from tropical forests in Vietnam and Madagascar (Tedersoo et al. 2014) as well as from xerotherm oak forests in Hungary (Geml 2019), the latter being climatically ideal for grapevine cultivation. Currently, new species were isolated from different perennial plants: *N. actinidiae* from kiwifruit (*Actinidia deliciosa*) (Johnston et al. 2004), *Cryptosporiopsis kienholzii* (syn. *Neofabraea kienholzii*) from apple and pear (Spotts et al. 2009; Wenneker et al. 2017), *N. brasiliensis* from apple (Crous et al. 2015), *N. inaequalis* from *Chamaecyparis* sp. and *N. actinidiae* on winterberry (*Ilex verticillata*) (Chen et al. 2016). In subsequent morphological and phylogenetic analyses of the genus *Neofabraea*, *Pezicula* and some

related genera, species were identified (Chen et al. 2016; Marin-Felix et al. 2017). During a survey seeking for the causing agents of esca disease, Hofstetter et al. (2012) obtained four isolates of *N. malicorticis* from healthy grapevines, although no connection to GTDs was established. So far, *N. kienholzii* has been known to cause bull's eye rot on apple and pear fruits accompanied with branch cankers (Spotts et al. 2009, Wenneker et al. 2017) and recently this species has been found on Japanese laurel (*Aucuba japonica*) as well (Chen et al. 2016). Although Baranek et al. (2018) reported a single *N. kienholzii* isolate from *Vitis*, no sequence data of that isolate is available, nor was any connection shown to GTDs.

Our study demonstrates, that *N. kienholzii* can infect grapevine causing brown vascular coloration in the canes, a characteristic symptom of GTDs. This observation supports the hypothesis that apple and pear trees that harbor fungi associated with dieback symptoms could serve as inoculum source of pathogens causing GTDs (Cloete et al. 2011). Furthermore, Gramaje et al. (2016) suggest that fungi associated with GTDs can infect other woody crops, such as pome and stone fruits, nut crops and olive trees as well. Because *N. kienholzii* already causes important damages on apple and pear, in time this pathogen may get more attention in viticulture, too. With this new report of a fungal genus causing disease on grapevine, our results contribute to the better understanding of the complex problem of GTDs.

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