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Diversity of *Botryosphaeriaceae* Species Associated with Grapevine Trunk Diseases in the Czech Republic

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Abstract: During a study of *Botryosphaeriaceae* species associated with grapevine trunk diseases in the Czech Republic, a collection of 22 *Botryosphaeriaceae*-like strains were isolated from four cultivars (Blaufränkisch, Pálava, Pinot Noir, and Welschriesling) in four distinct vineyards. Based on morphology and DNA sequence data (ITS, *tub2*, and *tef*), four species were identified: *Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata*, and *Neofusicoccum parvum*. These species are reported for the first time from grapevine in the Czech Republic. Relationships between vascular lesions and particular species were highlighted in this study. *Diplodia seriata* was the most frequently isolated species, present in all four sampled cultivars, while *D. mutila* was the least frequent, present only in 'Pálava'. The cultivar Pinot Noir was the most tolerant host for *Botryosphaeriaceae* fungi.

Keywords: *Botryosphaeriaceae*; grapevine trunk diseases; phylogeny; taxonomy; *Vitis vinifera*

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1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the Czech Republic's most valuable fruit crops. In 2021, registered vineyards covered an area of 16,360 hectares, producing 90,060 tonnes annually, with an estimated market value of $77,162,000 USD [1]. During the last few decades, an increased incidence of grapevine trunk diseases (GTDs) has been reported in grape-producing countries worldwide [2,3], with estimated economical loses exceeding 1 billion dollars annually [4].

The *Botryosphaeriaceae* family comprises a diverse group of cosmopolitan fungi, responsible for dieback and canker diseases in various woody hosts, including grapevines [5]. More than 26 different *Botryosphaeriaceae* species have been associated with Botryosphaeria dieback of grapevine [6]. External symptoms of Botryosphaeria dieback on grapevine include leaf spots, leaf wilting, fruit rots, perennial cankers, cordon dieback, and sudden plant mortality, while internal wood symptoms manifest as wedge-shaped necroses and dark lines beneath the bark [7].

Plants are usually infected by fungal spores that colonize the plants through winter pruning wounds. Besides infection through pruning wounds, the presence of latent infections caused by *Botryosphaeriaceae* fungi has been well documented in nurseries during the grapevine propagation process [8–11]. It was confirmed that *Botryosphaeriaceae* fungi can live within their host as endophytes or latent pathogens that become pathogenic when their hosts are exposed to stress conditions [12,13].

Due to a lack of studies, very little is known about the incidence of *Botryosphaeriaceae* pathogens in Czech vineyards. Thus, the aim of this study was to provide a comprehensive overview of the *Botryosphaeriaceae* fungi responsible for Botryosphaeria dieback in the Czech Republic.

2. Materials and Methods

2.1. Collection and Isolation

Plant material displaying symptoms of dieback (Figure 1) and asymptomatic material, in the case of a young 3-year-old vineyard, were collected from four commercial vineyards located in the South Moravia region of the Czech Republic with the permission of landowner (Table 1). The field observation and sampling were performed in July 2019. In total, 40 grapevines (ten plants per vineyard) were sampled and immediately transported to the laboratory of Mendeleum–Institute of Genetics, Mendel University, the Czech Republic, for further processing. Trunks and arms were debarked using a sterile scalpel and cut longitudinally and transversely to identify the type and location of internal wood necrosis. Bark-less wood tissues were subjected to surface sterilization. From each tissue, wood fragments, approx. 1 cm^3, were cut and surface sterilized with 1% sodium hypochlorite for ten minutes and then rinsed three times with sterile distilled water, following protocols previously described [14]. The disinfected wood fragments were cut into small chips of 5 × 2 mm and aseptically transferred onto Petri dishes (five chips per plate) containing potato dextrose agar (PDA, HiMedia, Mumbai, India) supplemented with 0.5 g/L streptomycin sulfate (Sigma–Aldrich, St. Louis, MO, USA). The plates were incubated at 25 °C in the dark for four weeks, and fungal growth was checked every two days. Newly developed mycelia were immediately transferred to new PDA plates and purified using hyphal tip isolation [15]. All fungal isolates were deposited in MEND-F, Fungal Culture Collection of Mendeleum, Mendel University in Brno, the Czech Republic.

Table 1. Sampled localities and sampling characterization.

Sampling	Locality	Sampling Year	Age of the Vineyards	Sampled Vines (n)	Cultivar
1.	Klentnice (48°51′27.4″ N 16°39′04.9″ E)	2019	30	10	Pálava *
2.	Pavlov (48°51′49.1″ N 16°39′23.0″ E)	2019	30	10	Blaufränkisch **
3.	Maliny (48°49′36.6″ N 16°37′29.9″ E)	2019	30	10	Pinot Noir **
4.	Maliny (48°49′34.9″ N 16°37′23.6″ E)	2019	3	10	Welschriesling *

Note: * white varieties, ** red varieties.

2.2. Morphology

Botryosphaeriaceae-like isolates were selected according to the keys provided in the study by Phillips et al. [5]. Culture characteristics were determined on PDA incubated for 7 days at 25 °C in the dark. Water agar plates (WA, HiMedia, Mumbai, India) with double autoclaved pine needles were incubated for 1–3 weeks at 25 °C with exposure to near-UV light to induce sporulation.

2.3. DNA Extraction and Amplification

Genomic DNA was extracted from 7-day-old mycelium grown on PDA at 25 °C in darkness using a NucleoSpin DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. To confirm the identity of the fungal species, fragments of three genes were amplified: internal transcribed spacer region (ITS), beta-tubulin (*tub2*), and translation elongation factor 1-alpha (*tef*). PCR was performed utilizing G2 Flexi DNA polymerase (Promega, Madison, USA), and the primers are listed in Table 2, following protocols previously described [16,17]. Resulting products were purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. Subsequently, the purified products were sequenced from both ends using the Sanger method at Eurofins Genomics (Ebersberg, Germany).

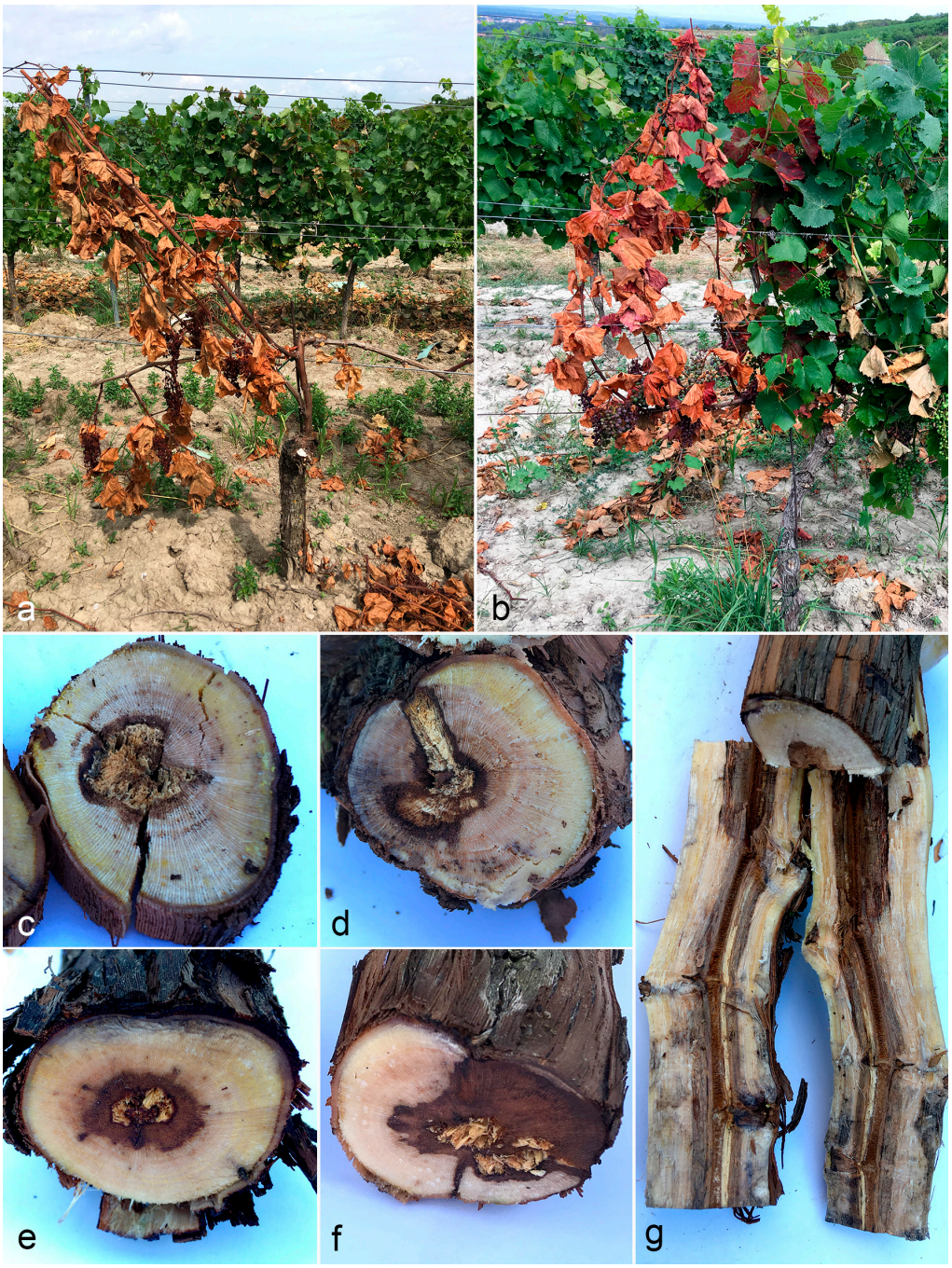

Figure 1. Typical symptoms of sampled plants. (**a**,**b**) Apoplexy. (**c**–**g**) Internal wood necroses.

Table 2. Primers used for PCR amplification and sequencing.

Locus	Primer	Primer DNA Sequence (5′-3′)	Reference
ITS	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	[18]
tef	EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	[19]
tub2	T1 Bt2b	AACATGCGTGAGATTGTAAGT ACCCTCAGTGTAGTGACCCTTGGC	[20] [21]

Note: ITS, internal transcribed spacer; *tef*, translation elongation factor 1-alpha; *tub2*, beta-tubulin.

2.4. Phylogenetic Analyses

To identify the isolates, newly generated DNA sequences, together with those retrieved from GenBank, were subjected to phylogenetic analyses (Table 3). The dataset of each gene was aligned separately using the MAFFT v. 7 employing the European Bioinformatics Institute platform (EMBL-EBI, https://www.ebi.ac.uk, accessed on 1 February 2023) [22]. Obtained alignment was manually checked and edited when necessary, using Geneious Prime® (v.2023.0.1., Biomatters Ltd., Auckland, New Zeland). Concatenated dataset was built in Sequence Matrix v.1.8 [23], and the missing information sites were denoted by a question mark. The combined (ITS, *tub2*, and *tef*) dataset was subjected to Maximum Likelihood (ML) analyses. Phylogenetic trees were constructed using IQ-TREE 2 [24], running 1000 bootstrap replicates. The best model for ML analyses was selected according to the Akaike Information Criterion (AIC). Bayesian analyses (BI) employed MrBayes v. 3.2.7 [25,26]. The BI analyses included four parallel runs of 50 M generations starting from a random tree topology, every 1000 generations were sampled, and the first 25% of the trees were discarded as the 'burn-in'. The most suitable substitution model was determined separately for each locus using jModelTest v. 2.1.7 [27]. Trees were visualized in iTOL v. 6.7 [28] and edited in Adobe Illustrator CC 2019. The resulting trees of both methods shared a similar topology; thus, we decided to present ML trees with support values of both methods–bootstrap (BS) and posterior probabilities (PP) labelled at the nodes. Values below 0.85 (PP) and 75% (BS) support are not shown or indicated with a hyphen. The alignments and corresponding trees are available on Figshare (10.6084/m9.figshare.22837472).

Table 3. Fungal species and barcodes used in phylogenetic analyses.

Species	Strain	Host	Geographic Origin	ITS	*tub2*	*tef*
Botryosphaeria agaves	CBS 133992^T	*Agave* sp.	Thailand	JX646791	JX646841	JX646856
B. corticis	CBS 119047^T	*Vaccinium corymbosum*	United States	DQ299245	EU673107	EU017539
B. dothidea	CBS 115476^T	*Prunus* sp.	Switzerland	AY236949	AY236927	AY236898
B. dothidea	CAA859	*Quercus ilex*	Portugal	MK940302	MT309378	MT309403
B. dothidea	CAA938	*Quercus suber*	Portugal	MT237173	MT309379	MT309401
B. dothidea	CAA860	*Quercus suber*	Portugal	MK940295	MT309380	MT309402
B. dothidea	**MEND-F-0386**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987974**	**OQ994785**	**OQ994763**
B. dothidea	**MEND-F-0385**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987975**	**OQ994786**	**OQ994764**
B. dothidea	**MEND-F-0379**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987976**	**OQ994787**	**OQ994765**
B. fabicerciana	CBS 127193^T	*Eucalyptus* sp.	China	HQ332197	KF779068	HQ332213
B. fusispora	MFLUCC 10–0098^T	*Entada* sp.	Thailand	JX646789	JX646839	JX646854
B. pseudoramosa	CERC2001^T	*Eucalyptus* sp.	China	KX277989	KX278198	KX278094
B. qingyuanensis	CERC2946^T	*Eucalyptus* sp.	China	KX278000	KX278209	KX278105
B. ramosa	CBS 122069^T	*Eucalyptus camaldulensis*	Australia	EU144055	KF766132	EU144070
B. rosaceae	CGMCC 3.18007^T	–	China	KX197074	KX197101	KX197094
B. wangensis	CERC2298^T	*Cedrus deodara*	China	KX278002	KX278211	KX278107
Diplodia africana	CBS 120835^T	*Prunus persica*	South Africa	EF445343	KF766129	EF445382
D. alatafructa	CBS 124931^T	*Pterocarpus angolensis*	South Africa	FJ888460	MG015799	FJ888444
D. corticola	CBS 112546^T	*Quercus ilex*	Spain	AY259090	EU673117	EU673310
D. corticola	CBS 112549	*Quercus suber*	Portugal	AY259100	DQ458853	AY573227
D. corticola	CAA862	*Eucalyptus globulus*	Portugal	MK940298	MT309381	MT309410
D. corticola	CAA865	*Pinus pinaster*	Portugal	MK940296	MT309382	MT309411
D. corticola	CAA870	*Quercus ilex*	Portugal	MK940303	MT309383	MT309408
D. corticola	CAA875	*Quercus suber*	Portugal	MK940297	MT309384	MT309409
D. corticola	CAA499	*Eucalyptus globulus*	Portugal	MG015741	MG015800	MG015723
D. corticola	CDFA519	*Quercus* sp.	United States	GU799472	GU799466	GU799469
D. insularis	CBS 140350^T	*Pistacia lentiscus*	Italy	KX833072	MG015809	KX833073
D. insularis	CAA890^T	*Eucalyptus globulus*	Portugal	MK940299	MT309385	MT309406
D. intermedia	CAA147^T	*Malus pumila*	Portugal	GQ923857	MG015811	GQ923825
D. mutila	CBS 136014	*Populus alba*	Portugal	KJ361837	MG015815	KJ361829
D. mutila	CBS 230.30	*Phoenix dactylifera*	United States	DQ458886	DQ458849	DQ458869
D. mutila	CAA507	*Fraxinus ornus*	Portugal	MG015746	MG015816	MG015728
D. mutila	CBS 121862	*Pyrus communis*	Netherlands	KX464093	KX464799	KX464567
D. mutila	CAA891	*Eucalyptus globulus*	Portugal	MK940300	MT309386	MT309407
D. mutila	**MEND-F-0366**	*V. vinifera* 'Palava'	Czechia	**OQ987977**	**OQ994788**	**OQ994766**

Table 3. *Cont.*

Species	Strain	Host	Geographic Origin	ITS	*tub2*	*tef*
D. mutila	**MEND-F-0381**	*V. vinifera* 'Palava'	Czechia	**OQ987978**	**OQ994789**	**OQ994767**
D. pseudoseriata	CBS 124906^{T}	*Blepharocalyx salicifolius*	Uruguay	EU080927	MG015820	EU863181
D. quercivora	CBS 133852	*Quercus canariensis*	Tunisia	JX894205	MG015821	JX894229
D. rosacearum	CBS 141915^{T}	*Eriobotrya japonica*	Italy	KT956270	MG015823	KU378605
D. sapinea	CBS 393.84^{T}	*Pinus nigra*	Netherlands	DQ458895	DQ458863	DQ458880
D. sapinea	CAA892	*Pinus pinaster*	Portugal	MK940292	MT309387	MT309404
D. sapinea	CAA903	*Quercus suber*	Portugal	MK940312	MT309388	MT309405
D. scrobiculata	CBS 109944^{T}	*Pinus greggii*	Mexico	DQ458899	DQ458867	DQ458884
D. seriata	CBS 112555^{T}	*Vitis vinifera*	Portugal	AY259094	DQ458856	AY573220
D. seriata	**MEND-F-0367**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987979**	**OQ994790**	**OQ994768**
D. seriata	**MEND-F-0370**[a]	*V. vinifera* 'Welschriesling'	Czechia	**OQ987980**	**OQ994791**	**OQ994769**
D. seriata	**MEND-F-0383**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987981**	**OQ994792**	**OQ994770**
D. seriata	**MEND-F-0365**[a]	*V. vinifera* 'Welschriesling'	Czechia	**OQ987982**	**OQ994793**	**OQ994771**
D. seriata	**MEND-F-0363**	*V. vinifera* 'Palava'	Czechia	**OQ987983**	**OQ994794**	**OQ994772**
D. seriata	**MEND-F-0368**	*V. vinifera* 'Blaufränkisch'	Czechia	**OQ987984**	**OQ994795**	**OQ994773**
D. seriata	**MEND-F-0372**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987985**	**OQ994796**	**OQ994774**
D. seriata	**MEND-F-0369**[a]	*V. vinifera* 'Welschriesling'	Czechia	**OQ987986**	**OQ994797**	**OQ994775**
D. seriata	**MEND-F-0382**	*V. vinifera* 'Blaufränkisch'	Czechia	**OQ987987**	**OQ994798**	**OQ994776**
D. seriata	**MEND-F-0371**[a]	*V. vinifera* 'Welschriesling'	Czechia	**OQ987988**	**OQ994799**	**OQ994777**
D. seriata	**MEND-F-0378**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987989**	**OQ994800**	**OQ994778**
D. subglobosa	CBS 124132^{T}	*Fraxinus excelsior*	Spain	DQ458887	DQ458852	DQ458871
Endomelanconiopsis microspora	CBS 353.97^{T}	Soil	Papua N. Guinea	EU683655	KX464893	EU683636
Neofusicoccum arbuti	CBS 116131	*Arbutus menziesii*	United States	AY819720	KF531793	KF531792
N. arbuti	CBS 117090	*Arbutus menziesii*	United States	AY819724	KF531794	KF531791
N. australe	CMW6837^{T}	*Acacia* sp.	Australia	AY339262	AY339254	AY339270
N. australe	CAA919	*Eucalyptus globulus*	Portugal	MK940294	MT309395	MT309423
N. australe	CAA434	*Eucalyptus globulus*	Portugal	KT440913	KX505927	KT440973
N. australe	CAA455	*Eucalyptus globulus*	Portugal	KT440915	KX505928	KT440975
N. batangarum	CBS 124924^{T}	*Terminalia catappa*	Cameroon	FJ900607	FJ900634	FJ900653
N. cordaticola	CMW14124	–	–	EU821925	EU821865	EU821895
N. cordaticola	CBS 123634	*Syzygium cordatum*	South Africa	EU821898	EU821838	EU821868
N. cryptoaustrale	CMW23785^{T}	*Eucalyptus* sp.	South Africa	FJ752742	FJ752756	FJ752713
N. cryptoaustrale	LM03	*Pistacia lentiscus*	–	KX505912	KX505930	KX505903
N. cryptoaustrale	BL34	*Vitis vinifera*	–	KJ638328	KX505931	KX505904
N. eucalypticola	CBS 115679^{T}	*Eucalyptus grandis*	Australia	AY615141	AY615125	AY615133
N. eucalyptorum	CBS 115791^{T}	*Eucalyptus grandis*	South Africa	AF283686	AY236920	AY236891
N. eucalyptorum	CAA932	*Eucalyptus globulus*	Portugal	MK940311	MT309396	MT309422
N. eucalyptorum	CAA511	*Eucalyptus globulus*	Portugal	KX505907	KX505919	KX505896
N. eucalyptorum	CAA709	*Eucalyptus globulus*	Portugal	KT440941	KX505920	KT441001
N. eucalyptorum	CAA713	*Eucalyptus globulus*	Portugal	KT440943	KX505921	KT441003
N. kwambonambiense	CBS 123639	*Syzygium cordatum*	South Africa	EU821900	EU821840	EU821870
N. kwambonambiense	CAA755	*Eucalyptus globulus*	Portugal	KT440946	KX505917	KT441006
N. kwambonambiense	CMW14155	–	–	EU821923	EU821863	EU821893
N. lumnitzerae	CMW41469^{T}	*Barringtonia racemosa*	South Africa	KP860881	KP860801	KP860724
N. luteum	CBS 110299^{T}	*Vitis vinifera*	Portugal	AY259091	DQ458848	KX464688
N. luteum	CAA935	*Eucalyptus globulus*	Portugal	MK940305	MT309397	MT309418
N. luteum	CAA628	*Fraxinus excelsior*	Portugal	KX505911	KX505929	KX505902
N. luteum	CMW9076	–	–	AY236946	AY236922	AY236893
N. mangiferae	CBS 118531^{T}	*Mangifera indica*	Australia	AY615185	AY615172	DQ093221
N. mangroviorum	CMW41365^{T}	*Avicennia marina*	South Africa	KP860859	KP860779	KP860702
N. mediterraneum	CBS 121718	*Eucalyptus* sp.	Greece	GU251176	GU251836	GU251308
N. mediterraneum	CAA002	*Pistacia vera*	United States	EU017537	KX505925	KX505900
N. mediterraneum	SPA9	*Pistacia lentiscus*	–	KX505910	KX505926	KX505901
N. nonquaesitum	IMI500168	*Vaccinium corymbosum*	–	JX217819	KX505918	KX505895
N. occulatum	CBS 128008^{T}	*Eucalyptus grandis*	Australia	EU301030	EU339472	EU339509
N. parvum	CMW9081^{T}	*Populus nigra*	New Zealand	AY236943	AY236917	AY236888
N. parvum	CAA940	*Eucalyptus globulus*	Portugal	MK940304	MT309399	MT309421

Table 3. *Cont.*

Species	Strain	Host	Geographic Origin	ITS	*tub2*	*tef*
N. parvum	CMW9080	–	–	AY236942	AY236916	AY236887
N. parvum	CAA322	*Malus pumila*	Portugal	KX505906	KX505916	KX505894
N. parvum	**MEND-F-0375**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987990**	**OQ994801**	**OQ994779**
N. parvum	**MEND-F-0376**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987991**	**OQ994802**	**OQ994780**
N. parvum	**MEND-F-0377**	*V. vinifera* 'Blaufränkisch'	Czechia	**OQ987992**	**OQ994803**	**OQ994781**
N. parvum	**MEND-F-0374**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987993**	**OQ994804**	**OQ994782**
N. parvum	**MEND-F-0373**	*V. vinifera* 'Blaufränkisch'	Czechia	**OQ987994**	**OQ994805**	**OQ994783**
N. parvum	**MEND-F-0384**	*V. vinifera* 'Pinot Noir'	Czechia	**OQ987995**	**OQ994806**	**OQ994784**
N. pistaciarum	CBS 113084	–	United States	KX464187	KX464999	KX464713
N. pistaciicola	CBS 113089^{T}	*Pistacia vera*	United States	KX464199	KX465014	KX464727
N. ribis	CBS 115475^{T}	*Ribes* sp.	United States	AY236935	AY236906	AY236877
N. ribis	CBS 121.26	*Ribes* sp.	–	AF241177	AY236908	AY236879
N. umdonicola	CMW14106	–	–	EU821899	EU821839	EU821869
N. umdonicola	CMW14058	–	–	EU821904	EU821844	EU821874
N. vitifusiforme	B8	*Vitis vinifera*	–	KC469638	KC884951	KC884948
N. vitifusiforme	B9	*Vitis vinifera*	–	KX505908	KX505923	KX505898

Notes: ^{T} ex-type strain. ^{a} indicates strain originated from asymptomatic plant. Newly obtained strains and newly generated sequences are highligted in bold. CBS, Westerdijk Fungal Biodiversity Institute, Netherlands; CGMCC, China General Microbiological Culture Collection; CMW, the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria; IMI, CABI Bioscience, Eggham, the UK; MEND-F, fungal culture collection of Mendeleum, Mendel University in Brno, the Czech Republic; MFLUCC, culture collection of Mae Fah Luang University, Thailand.

3. Results

3.1. Fungal Isolation

In total, 204 isolates were obtained from the 40 sampled plants. A preliminary morphological characterization revealed 22 isolates that displayed morphological and growth characteristics consistent with the *Botryosphaeriaceae* family.

3.2. Phylogenetic Analyses

Molecular identification was performed on the 22 representative isolates, and their identity confirmed employing three-gene based (ITS, *tub2*, *tef*) phylogenetic analyses. The dataset consisted of sequences from 106 isolates (Table 3), including the outgroup *Endomelanconiopsis microspora* (CBS 353.97^{T}). The combined dataset contained a total of 1259 characters, including alignment gaps. Among these characters, 822 were conserved, 351 provided informative data for parsimony analysis, and 86 were unique. Detailed results for each individual gene dataset, along with the corresponding models used, can be found in Table 4. The ML/BI analyses (Figures 2 and 3) placed 11 isolates in group with the type strain of *D. seriata* (CBS 112555) with strong support of 91/0.99 (BP/pp); six isolates formed a fully supported clade with the type strain (CMW 9081) and three other *Neofusicoccum parvum* strains; three isolates were placed in group with the type strain (CBS 115476) and three other strains of *Botryosphaeria dothidea* with robust 97/1.0 (BP/pp) support; finally, two isolates were displayed in a well-supported clade 98/0.95 (BP/pp) with the type strain (CBS 121862) and three other strains of *D. mutila*.

Table 4. Detailed characteristics of phylogeny datasets.

Locus	No. of Sequences	No. of Characters	Parsimony-Informative	Constant	Unique	BI Model
ITS	134	503	113	366	24	GTR + I + G
tef	134	336	143	150	43	HKY + G
tub2	123	420	95	306	19	GTR + G

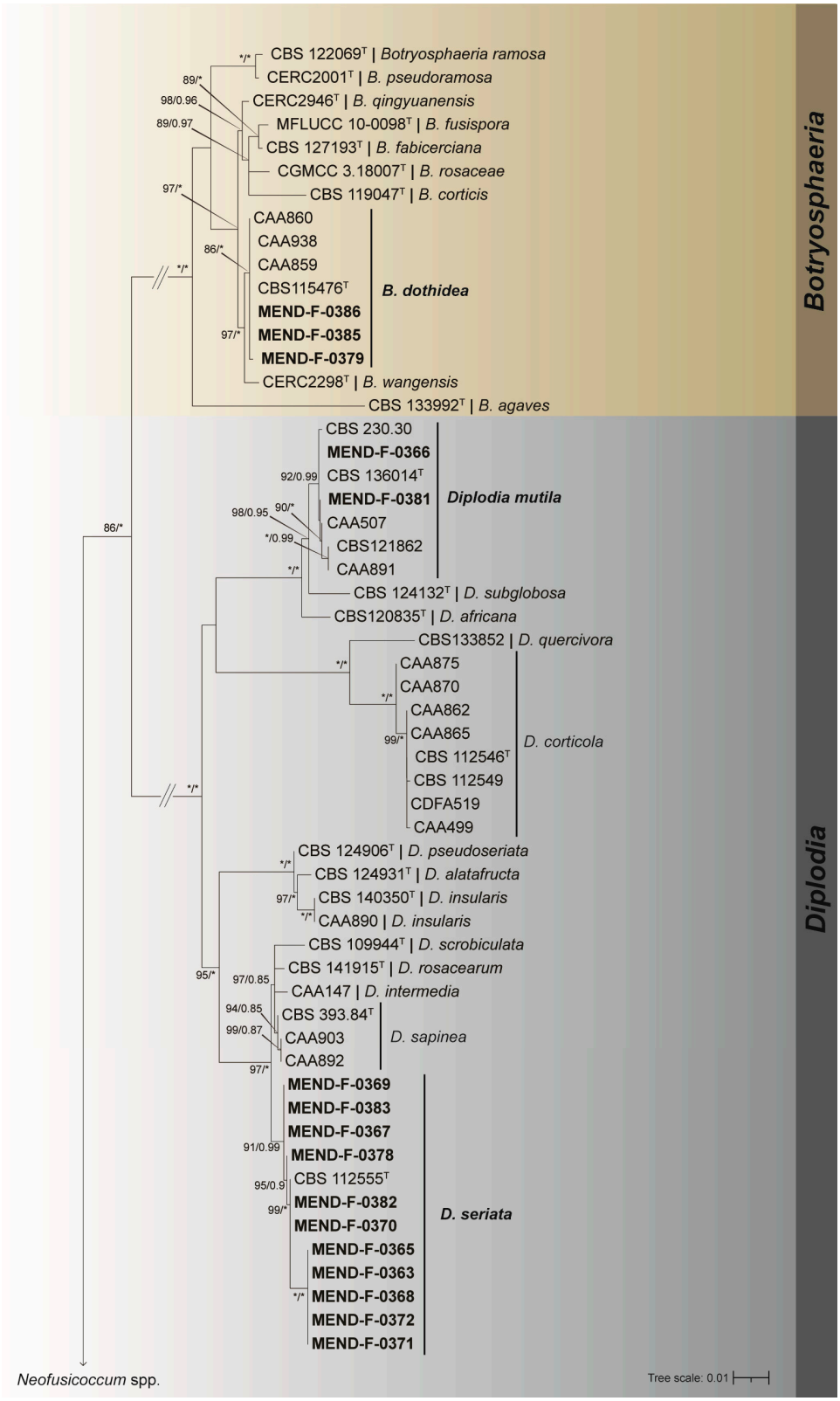

Figure 2. Maximum likelihood tree generated from the combined (ITS, *tef*, and *tub2*) *Botryosphaeriaceae* dataset. Support values of both methods–bootstrap (BS) and posterior probabilities (pp) labelled at the nodes. Values below 75% (BS) and 0.85 (pp) support are not shown or indicated with a hyphen. Asterisk represents full support. Strains obtained in this study are highlighted in bold. ^{T} indicates ex-type strain. The tree continues in Figure 3.

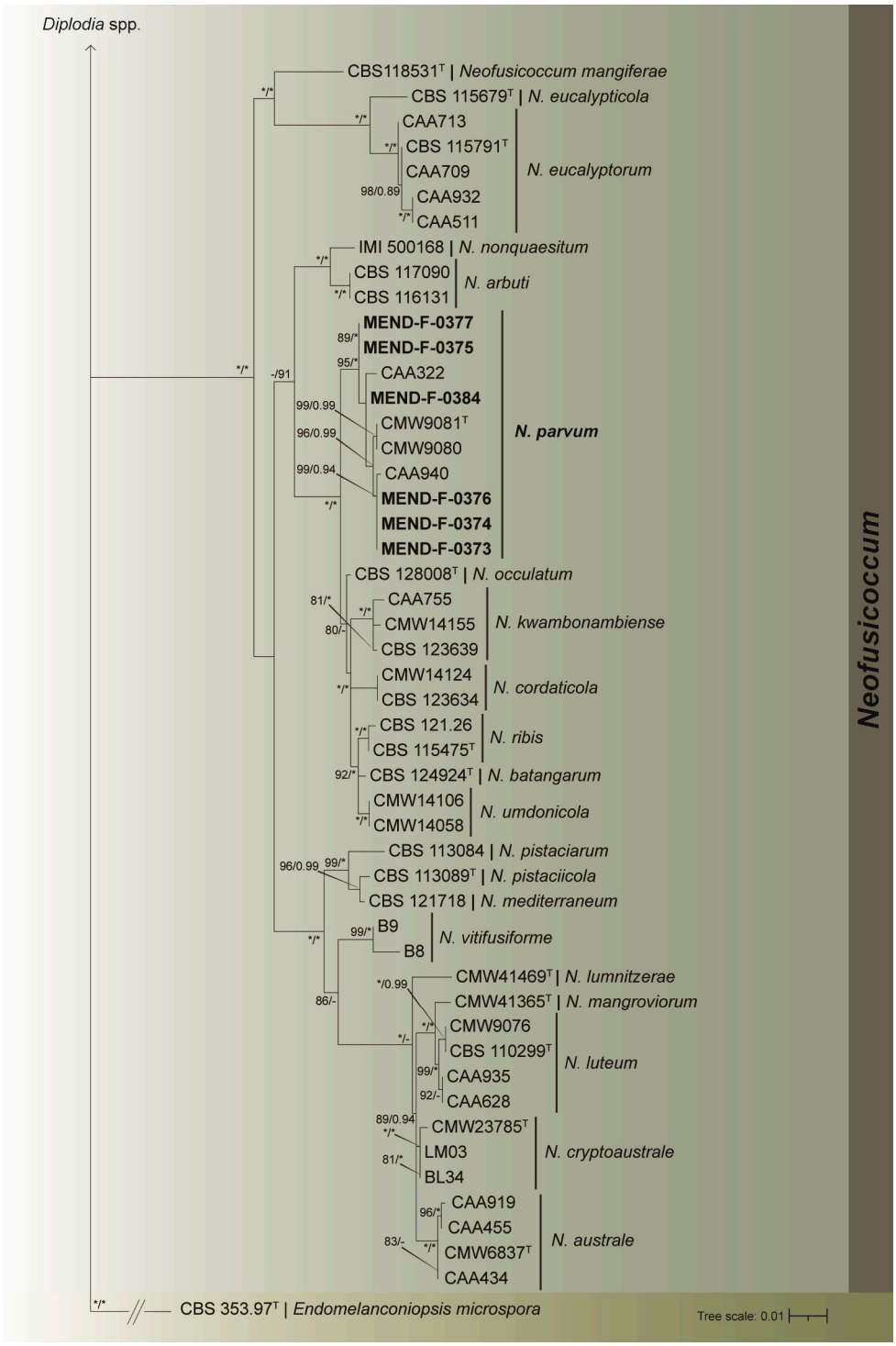

Figure 3. Maximum likelihood tree generated from the combined (ITS, *tef*, and *tub2*) *Botryosphaeriaceae* dataset. Support values of both methods–bootstrap (BS) and posterior probabilities (pp) labelled at the nodes. Values below 75% (BS) and 0.85 (pp) support are not shown or indicated with a hyphen. Asterisk represents full support. Strains obtained in this study are highlighted in bold. ^{T} indicates ex-type strain. *Endomelanconiopsis microspora* strain CBS 353.97^{T} served as an outgroup.

3.3. Species Diversity in Different Grapevine Varieties and Wood necrosis

Diplodia seriata was the most frequently isolated species (11 isolates), present in all four sampled varieties, followed by *N. parvum* (n = 6) isolated from both red varieties, *B. dothidea* (n = 3) detected only in cf. Pinot Noir, and *D. mutila* (n = 2) detected only in cf. Pálava.

Wood necroses associated with specific pathogens are displayed in Figure 4. Three different shapes of inner necrosis were observed in transverse sections of trunk and arm from symptomatic grapevines: black spots (BS); black sectorial necrosis (BSN); black central necrosis (BCN). *Botryosphaeriaceae* isolates were inhabiting mostly the BSN (35%), followed by BS and BCS with 31% and 17%, respectively. The remaining 17% of the obtained *Botryosphaeriaceae* isolates originated from asymptomatic wood tissues from the young Welschriesling vineyard.

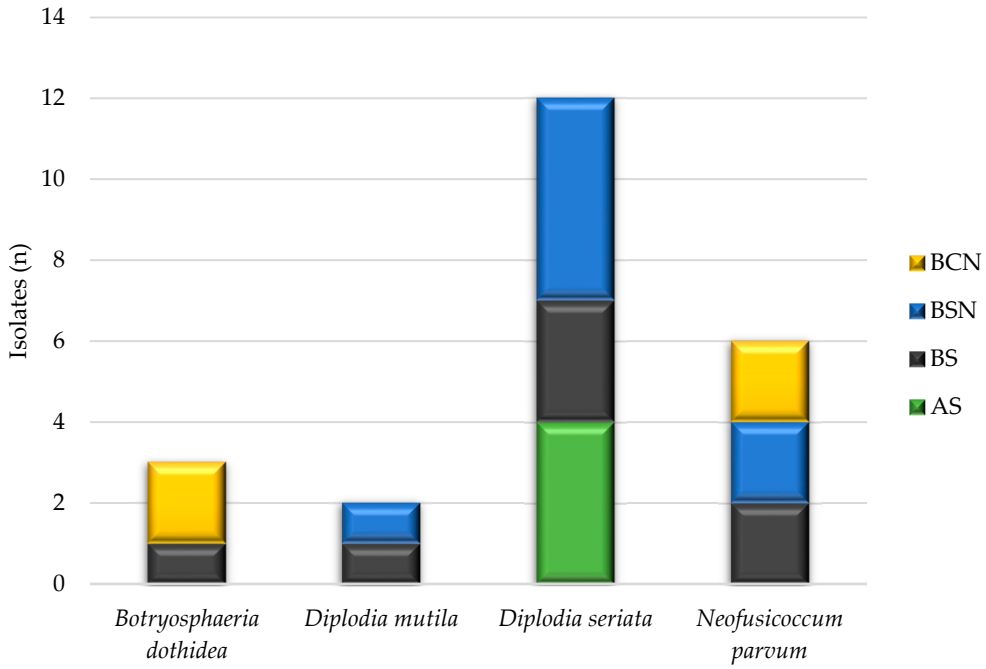

Figure 4. The association between the *Botryosphaeriaceae* isolates and the type of wood necrosis. AS = asymptomatic; BS = black spots; BSN = black sectorial necrosis; BCN = black central necrosis.

4. Discussion

This study provides the initial comprehensive evaluation of the occurrence of *Botryosphaeriaceae* species in grapevines within Czech vineyards. Among 22 *Botryosphaeriaceae* strains obtained, four species belonging to the three genera were detected, among which *Diplodia seriata* De Not. comprised 50%, *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers, and A.J.L. Phillips 27%, *Botryosphaeria dothidea* (Moug.) Ces. and De Not. 14%, and *Diplodia mutila* (Fr.) Mont. 9%. These species have already been isolated from grapevines worldwide and their pathogenicity has been confirmed [29–35]. The most isolated species in the Czech Republic was *D. seriata*. This finding is in accordance with previous studies that have identified *D. seriata* as the predominant fungus associated with the decline of mature vines in Iran [36], Mexico [37], Hungary [38], and Tunisia [39].

In our study, the pathogen *D. seriata* was also isolated from the asymptomatic material from the young (3-year-old) vineyard, suggesting latent infection from propagation process in grapevine nursery. This result is consistent with previous studies that reported infection by *Botryosphaeriaceae* fungi in grapevine nurseries. Fourie et al. reported the presence of latent infection caused by *Botryosphaeriaceae* fungi in rootstock mother plants in South Africa [40]. Aroca et al. reported presence of three *Botryosphaeriaceae* fungi in grapevine propagation material in Spain, namely, *Botryosphaeria dothidea*, *Diplodia seriata*, and *Neofusicoccum parvum* [41]. Eichmeier et al. also reported the presence of the same three *Botryosphaeriaceae* fungi in young grapevine seedlings in Spain [42].

To the best of our knowledge, only two studies have been performed to date on detection of GTDs in the Czech Republic. The initial investigation was conducted by a study of Baranek et al. [43]. The authors examined two grapevine cultivars, namely, 'Chardonnay' and 'Cabernet Sauvignon', and identified a total of 21 fungal taxa. Among these taxa,

only one species, *Botryosphaeria dothidea*, was classified under the *Botryosphaeriaceae* family. Subsequently, an incidence of *Dactylonectria torresensis*, a causal agent of black-foot disease, was reported from Czech vineyards [44].

Multiple *Botryosphaeriaceae* species do not have specificity in host range and have the ability to transition from their original indigenous hosts to agricultural crops cultivated in proximity [45]. Excluding grapevine, two *Botryosphaeriaceae* spp. were recently reported causing dieback of highbush blueberry from the Czech Republic, namely, *Lasiodiplodia theobromae* and *Neofusicoccum parvum* [46,47].

5. Conclusions

This study provided an investigation of the *Botryosphaeriaceae* fungi associated with GTDs in four Czech vineyards. Four pathogenic *Botryosphaeriaceae* spp. have been identified based on phylogenetic analyses, and a correlation between fungal isolates, grapevine cultivar, and type of wood necroses was described in this study. The detection of the pathogen *Diplodia seriata* in young asymptomatic grapevine plants represents an urgent matter for Czech viticulture. Producing healthy propagation material is an essential requirement. We propose incorporating molecular detection techniques into nurseries to reveal hidden fungal infection. We also highly recommend implementing preventative treatment during the grapevine propagation process using hot water treatment [48], novel nanomaterials [49], or phenolic compounds [50].

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Data Availability Statement: Newly generated sequences were deposited in the NCBI GenBank database under the accession numbers shown in Table 3. The alignments and corresponding trees are available on Figshare (10.6084/m9.figshare.22837472).

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