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Diaporthe toxicodendri sp. nov., a causal fungus of the canker disease on *Toxicodendron vernicifluum* in Japan

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Abstract

We describe for the first time the fungus *Diaporthe toxicodendri* sp. nov., which causes canker disease on the stems and twigs of *Toxicodendron vernicifluum*. We conducted a phylogenetic analysis using combined multigene sequence data from the rDNA internal transcribed spacer sequence and partial genes for calmodulin, histone H3, beta-tubulin, and translation elongation factor 1-alpha. The results indicate that *D. toxicodendri* occupies a monophyletic clade with high support. Although 10 species are phylogenetically closely related to *D. toxicodendri*, morphological characteristics of size of alpha conidia and lacking of beta and gamma conidia support the distinction of this fungus from those closely related species. No sexual morphic structures have yet been found for the species. The pathogenicity of this species was confirmed by the inoculation test to *T. vernicifluum*.

Key words – Anacardiaceae – canker disease – Diaporthales – *Phomopsis* – taxonomy

Introduction

Toxicodendron vernicifluum (Stokes) F.A. Barkley is a deciduous tree belonging to the family Anacardiaceae. The tree has economic and cultural importance as its resin is used to make lacquer (Miyamoto & Kakuda 2008). Recently, outbreaks of a canker disease have occurred at *T. vernicifluum* plantations in Hokkaido, Aomori, and Iwate Prefectures of northern Japan (Tabata pers. obs., Takemoto et al. 2014). Fungal species of the genus *Phomopsis* (Sacc.) Bubák have frequently been detected in lesions of the diseased trees, but the causal agent has not previously been identified (Takemoto et al. 2014).

The genus name *Phomopsis* has been used for the asexual morphs of *Diaporthe* species Nitschke (Diaporthales, Ascomycota). However, due to recent changes of the International Code of Nomenclature for algae, fungi, and plants, the sexual and asexual morphs of a single species must now have the same name (Hawksworth et al. 2011, Wingfield et al. 2012). The name *Diaporthe* over *Phomopsis* was proposed for this group due to its prior use (Udayanga et al. 2012, Gomes et al. 2013), and here we follow this suggestion.

The genus *Diaporthe* includes many important pathogens that cause dieback and canker diseases on a wide variety of woody and herbaceous plants (Rehner & Uecker 1994, Udayanga et

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al. 2011, Gomes et al. 2013). Large numbers of *Diaporthe* species and their asexual morphs have been described, but their taxonomy is confused. A taxonomic revision of the species and a new delimitation of the genus has been proposed, based on analyses of multi-locus DNA sequence data (e.g. Gomes et al. 2013, Udayanga et al. 2014a, b, Dissanayake et al. 2017). Udayanga et al. (2012) reassessed the species in *Diaporthe* using multi-locus phylogenetic analysis and proposed the phylogenetic species recognition should be applied to this genus. Gomes et al. (2013) also supported to adopt the phylogenetic species recognition for *Diaporthe* by multi-locus phylogeny using 243 *Diaporthe* isolates. Udayanga et al. (2014a, b) promoted this species recognition and resolved species boundaries of taxonomically confused groups, that are *D. eres* species complex and species on Citrus together with related *Diaporthe* species. In the most recent phylogenetic study, Dissanayake et al. (2017) revealed taxonomic status of 171 *Diaporthe* species used available ex-type isolates by multi-locus phylogenetic analysis. Several new species have been described according to this delimitation, especially from Asia (Tan et al. 2013, Gao et al. 2014, 2016, Fan et al. 2015, Udayanga et al. 2015, Du et al. 2016, Tanney et al. 2016). However, many unknown and/or ambiguous species from all over the world are still waiting to be defined and described.

Three *Diaporthe* and 2 *Phomopsis* species were listed in Japan in 1917 (Shirai & Miyake 1917). Later, Hara (1954) identified 42 more species of *Diaporthe* and *Phomopsis* from various woody and herbaceous plants. However, some of these may have been misidentified. Kobayashi (1970) studied the Japanese Diaporthaceae fungi and recognized 19 species in the *Diaporthe* genus, providing detailed morphological descriptions and illustrations. Thereafter, several additional *Diaporthe* or *Phomopsis* species have been reported in fruit trees and agricultural crops in Japan (Kajitani & Kanematsu 2000, Kishi 1998, Katsumoto 2010). Among these, a species of diaportalean fungus associated with *Toxicodendron* was reported as *Diaporthe spiculosa* (Westend.) Nitschke (Kobayashi 1970). However, *D. spiculosa* lacked the asexual morphic state and it was different from the *Diaporthe* spp. reported as *Phomopsis* by Takemoto et al. (2014).

The aims of the present study are to clarify the taxonomic position of the causal agent of canker disease on *Toxicodendron vernicifluum* using combined multi-gene sequences as in recent studies (e.g., Gomes et al. 2013, Udayanga et al. 2014b) as well as morphological characteristics and to confirm the pathogenicity of this fungus.

Materials & Methods

Fungal isolation

Eight samples were collected from the stems and twigs of *Toxicodendron vernicifluum* in the plantations at Hokkaido, Aomori, and Iwate Prefectures in northern Japan. Isolates were obtained from the samples using single conidia or hyphae.

For single conidial isolation, spore masses were picked from the samples, suspended in 500 µl distilled water, and streaked onto the 1% malt extract agar (MA) plates. Single hyphae germinated from single conidia were then transferred to 2% MA plates.

For single hyphal isolation, tissue fragments were punched out from visible lesions on collected twigs using a 4 mm cork borer. The fragments were immersed in 70% ethanol for 30s and in sodium hypochlorite solution (1% available chlorine) for 3 min, rinsed twice in sterile distilled water, and blotted dry on sterile filter paper for 15 min. Each fragment was placed on the surface of 1% MA plate. The plates were incubated at 15°C in the dark and observed intermittently under a dissecting microscope. Any single hypha growing from a fragment was isolated and transferred to a 2% MA plate and maintained.

The pure cultures were used for culture characterization, optimal growth temperature assessment, molecular phylogenetic analysis, and inoculation. Isolates obtained in this study were deposited in the Forestry and Forest Products Research Institute (FFPRI) culture collection at Tsukuba, Japan, or author's culture collection (AYC). The specimens collected in this study were deposited in the Herbarium of Forest Mycology and Pathology (TFM) of FFPRI.

Table 1 Isolate and GenBank accession numbers used in this study.

Species	Isolate [a]	GenBank accession number [b]				
		ITS	CAL	HIS	EF-1α	BT
D. ampelina	CBS 114016 [T]	AF230751	AY745026	–	AY745056	JX275452
D. betulicola	CFCC 51128 [T]	KX024653	KX024659	KX024661	KX024655	KX024657
D. carpini	CBS 114437	KC343044	KC343286	KC343528	KC343770	KC344012
D. detrusa	CBS 109770	KC343061	KC343303	KC343545	KC343787	KC344029
D. fibrosa	CBS 109751	KC343099	KC343341	KC343583	KC343825	KC344067
D. impulsa	CBS 114434	KC343121	KC343363	KC343605	KC343847	KC344089
D. juglandicola	CFCC 51134 [T]	KU985101	KX024616	KX024622	KX024628	KX024634
D. padi var. *padi*	CBS 114649	KC343170	KC343412	KC343654	KC343896	KC344138
D. rostrata	CFCC 50062 [T]	KP208847	KP208849	KP208851	KP208853	KP208855
D. scobina	CBS 251.38	KC343195	KC343437	KC343679	KC343921	KC344163
D. thunbergii	MFLUCC 100576 [T]	JQ619893	JX197440	–	JX275409	JX275449
D. toxicodendri sp. nov.	FFPRI420984	**LC275189**	**LC275197**	**LC275205**	**LC275213**	**LC275221**
	FFPRI420985	**LC275190**	**LC275198**	**LC275206**	**LC275214**	**LC275222**
	FFPRI411163	**LC275191**	**LC275199**	**LC275207**	**LC275215**	**LC275223**
	FFPRI420987 [T]	**LC275192**	**LC275200**	**LC275208**	**LC275216**	**LC275224**
	FFPRI420990	**LC275193**	**LC275201**	**LC275209**	**LC275217**	**LC275225**
	FFPRI420991	**LC275194**	**LC275202**	**LC275210**	**LC275218**	**LC275226**
	FFPRI411164	**LC275195**	**LC275203**	**LC275211**	**LC275219**	**LC275227**
	FFPRI411165	**LC275196**	**LC275204**	**LC275212**	**LC275220**	**LC275228**
D. woolworthii	CBS 148.27	KC343245	KC343487	KC343729	KC343971	KC344213

[a] Ex-type or ex-epitype isolates are marked by T.
[b] Sequences obtained in this study are shown in bold.

Morphological observations

Samples were dissected and sectioned under a stereomicroscope using flame-sterilized scalpels and tweezers. Fungal structures were mounted in Shear's fluid (Chupp 1940) on glass slides and observed under a differential interference contrast microscope (Leica DM2500, Leica microsystems Inc.). Fifteen pycnidia and more than 150 conidia were selected randomly and measured to calculate the averages and ranges.

In order to determine the optimal temperatures for growth in culture, 3 isolates (FFPRI420984, FFPRI420985, and FFPRI420987) were selected. Sterile cork borers (5 mm diam.) were used to cut disks from the margins of colonies that had been growing actively on potato dextrose agar (PDA) for one month, and the disks were transferred to the centers of fresh 90 mm PDA plates. Incubations were carried out in the dark at 5−30°C with 5°C intervals. Three replications were conducted for each isolate. The average diameter of each culture was measured at 3, 5, 7, and 14 days or until the mycelial growth reached the edges of the plates.

DNA extraction, PCR amplification, and sequencing

The 8 isolates were cultured on PDA for two weeks before sampling for molecular phylogenetic analyses. DNA was extracted from mycelial samples using the Prepman® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacture's protocol.

The following sequences were used in the analysis: the internal transcribed spacer (ITS) region of the ribosomal RNA genes and portions of the calmodulin (CAL), histone H3 (HIS), translation elongation factor 1-alpha (EF-1α), and β-tubulin (BT) genes. The sequences were amplified by the polymerase chain reaction (PCR) using the primers ITS5 and ITS4 (White et al. 1990) for the ITS; CAL563F (Udayanga et al. 2014b) and CL2A (O'Donnell et al. 2000) for CAL; CYLH3F (Crous et al. 2004) and H3-1b (Glass & Donaldson 1995) for HIS; EF1-728F and EF1-986R (Carbone & Kohn 1999) for EF-1α; and T1 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) for BT. PCR reactions were set up using the GoTaq® Green Master Mix (Promega, Madison, WI, USA). Amplifications were performed in a BioRad iCycler (Bio-Rad Laboratories, Hercules, CA, USA) following the protocols of Gomes et al. (2013) for HIS and Udayanga et al. (2014) for ITS, CAL, EF-1α, and BT.

The PCR products were separated by electrophoresis in 1% agarose gels stained with ethidium bromide and visualized under UV light. The products were purified using the ExoSAP-IT PCR Product Cleanup kit (Affymetrix Japan, Tokyo, Japan) and sequenced using the BigDyeTM Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems) with both forward and reverse primers. The sequences were analyzed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), assembled and edited using BioEdit ver. 7.2.5 (Hall 1999), and then deposited in GenBank (Table 1).

Phylogenetic analyses

We made two datasets (dataset I and II) to the estimate phylogenetic position of current fungus in the *Diaporthe*. Dataset I was composed of the combined multi-locus sequence data from the ITS, BT, EF-1α, and CAL sequences. The sequences were combined and aligned with reference sequences obtained from GenBank. The reference sequences had been used in most recent phylogenetic study of *Diaporthe* (Dissanayake et al. 2017). Dataset II was composed of the combined multi-locus sequence data from the ITS, CAL, HIS, EF-1α and BT of closely related species to current fungus estimated by the phylogenetic analysis of dataset I (Table 1). In both dataset, alignments were performed using the online version of MAFFT 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standly 2013) with the G-INS-i option. Sequences were manually edited when necessary using BioEdit ver. 7.2.5 (Hall 1999).

The phylogenetic trees were generated by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses. The best fit evolutionary model was determined via the corrected Akaike informative criterion (AICc) (Akaike 1974, Sugiura 1978) for MP analysis and the Bayesian information criterion (BIC; Schwarz 1978) for BI analysis using the Kakusan 4 nucleotide substitution model selection program (Tanabe 2007, 2011).

The MP analysis was performed using PAUP* version 4.0b10 software (Swofford 2002) with the heuristic search and step-wise addition options and 1000 replications. All characters had equal weight and gaps were treated as missing data. The branch-swapping algorithm was tree bisection and reconstruction. The best tree was automatically selected using the Kishino-Hasegawa likelihood test (Kishino & Hasegawa 1989) that is part of the PAUP* software. The tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated. Bootstrap analyses were performed using a heuristic search algorithm with 10,000 random addition replicates and nearest-neighbor interchange branch swapping. The ML analysis was performed using RAxML 8.0.2 software (Stamatakis 2014) with the GTR + Gamma model of evolution and 1,000 bootstrap replicates. The BI analysis was performed using MrBayes5D v.3.1.2.2012.12.13 (Tanabe 2008), which is a modified version of MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003), to estimate the posterior probabilities of tree topologies with Markov Chain Monte Carlo (MCMC) searches. The BI analysis was performed for 10,000,000 generations and trees were sampled every 500 generations. Convergence of the MCMC procedure was assessed by calculating the effective sampling size using Tracer 1.6 (Rambaut et al. 2014). The first 2001 trees were discarded as burn-in. The support of nodes was tested based on posterior probabilities obtained from a 50% majority rule consensus after deleting the trees in the burn-in period. *Diaporthella corylina* Lar.N. Vassiljeva and *Diaporthe scobina* Nitschke and *D. thunbergii* Udayanga, X.Z. Liu & K.D. Hyde were used for the outgroup in dataset I and II, respectively.

Inoculation

Four to Nine yr-old of ten *Toxicodendron vernicifluum* trees (1.5 to 3.4 m high, 1.6 to 3 cm in diam. at breath height) were used. They were planted in the plantation of Ninohe City. Two Isolates, FFPRI420984 and AYC128-1, were grown on 90 mm PDA plate at 25°C for two weeks and used for inocula.

Each tree was inoculated with two isolates and control on 26 May 2017. The bark plugs (5 mm diam.) were triplicatedly removed by using sterile cork borer and replaced with each inoculum. The inoculation was made at the heights of about 0.6, 0.9 and 1.2m on the stem of each tree.

Inoculum discs were placed into the wounds. Each inoculation point was then covered with plastic film (Parafilm) and adhensive tape.

Inoculated trees were cut down and examined four weeks after inoculation. The bark was peeled and the maximum extent of lesions was measured, and results were expressed as the range, average and standard deviation of necrotic lesions of 30 inoculation points in ten trees. Seven to nine pieces of bark were cut, transferred onto 1% MA plates and incubated at 20°C for 3 wk. to confirm the presence of the inoculated fungi. The length and width of the lesions were analyzed by the Steel-Dwass test ($P < 0.05$) to assess the significant differences among the two inocula and the control.

Results

Sample collection and Morphological characteristics

We found 1–4 y old *T. vernicifluum* trees diseased with canker in Hokkaido, Aomori, and Iwate Prefectures and identified the asexual morph of *Diaporthe* in the diseased parts of the trees (Fig 2A). This fungus was frequently isolated from pycnidia produced on stems and twigs, and also from bark tissues in canker-diseased lesions. The pycnidia were scattering over the stems and twigs and produced conspicuous yellowish cream colored conidial masses on their tips when mature (Fig 2B). We used these pycnidia for observation of morphological characters. This fungus was characterized by lacking beta and gamma conidia (Fig 2G). Sexual morph structures were not found on natural substrates and were not observed even after twigs with pycnidia collected at Iwate Pref. were incubated on artificial media.

Phylogenetic analyses

The dataset I was contained the sequences from our 8 isolates with 179 available sequences including 171 ex-type sequences used by Dissanayake et al. (2017). This dataset was composed of 2608 sites (ITS = 545, BT = 970, EF-1α = 634, CAL = 459) including gaps. The result of this analysis indicated that this fungus grouped in monophyletic clade in *Diaporthe* and closely related to 10 species (data not shown, but this alignment file and phylogenetic tree were deposited in TreeBASE: S20847).

The dataset II used in this study was composed of 2523 sites (ITS = 476, CAL = 482, HIS = 500, EF-1α = 358, BT = 707) including alignment gaps. We analyzed the sequences from our 8 isolates with 12 other available sequences, including the outgroup. This combined multigene dataset was deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S20847) as S20847. In this dataset, 1884 sites were constant, and there were 313 variable sites that were uninformative. The MP analysis was performed used the remaining 326 parsimony informative characters, and one parsimonious tree was obtained (TL = 961, CI = 0.7919, RI = 0.8241, RC = 0.6526, HI = 0.2081). The results of the ML analysis are shown in Fig. 1.

The phylogenetic analyses showed that this fungus is closely related to 10 other *Diaporthe* species: *D. impulsa* (Cooke & Peck) Sacc., *D. ampelina* (Berk. & M.A. Curtis) R.R. Gomes, Glienke & Crous, *D. fibrosa* (Pers.) Fuckel, *D. detrusa* (Kunze) Fuckel, *D. carpini* (Pers.) Fuckel, *D. padi* var. *padi* G.H. Otth, *D. juglandicola* Q. Yang, *D. rostrata* C.M. Tian, X.L. Fan & K.D. Hyde, *D. woolworthii* (Peck) Sacc., and *D. betulicola* C.M. Tian & Z. Du. However, this fungus was clustered in a distinct clade from the 10 other species with highly supported values (ML / MP / BI = 100 / 100 / 1) (Fig.1).

Taxonomy

Diaporthe toxicodendri Y. Ando, Masuya et Tabata, sp. nov. Fig. 2

Index Fungorum number: IF553325; Facesoffungi number: FoF03868

MycoBank Number: MB821711.

Etymology – from genus name of host.

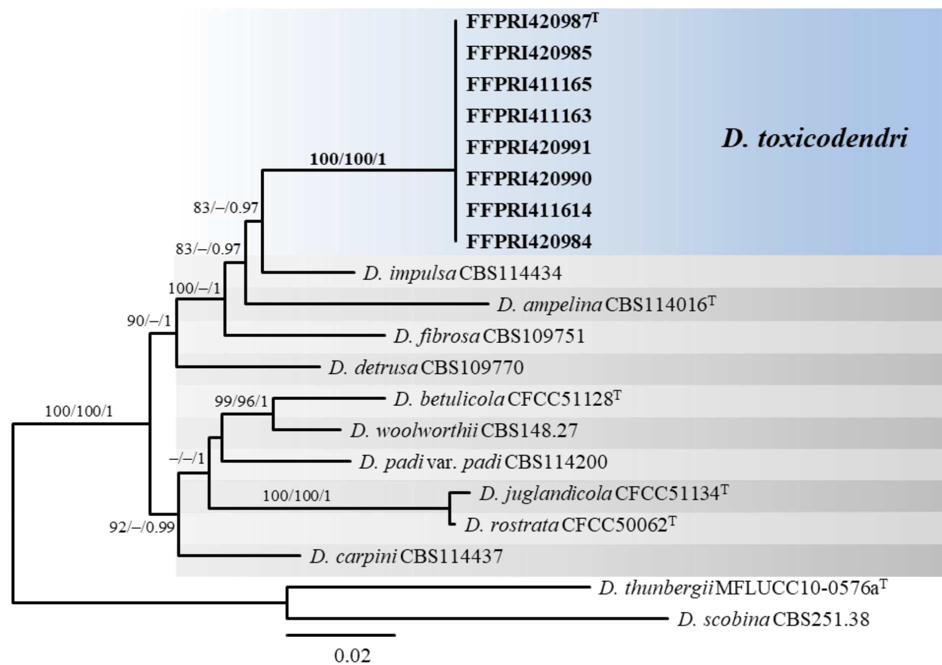

Figure 1 – The maximum likelihood tree based on the combined ITS, CAL, HIS, EF-1α, and BT genes dataset (composed of 20 OTUs and 2523 sites including gaps) of *D. toxicodendri* and closely related *Diaporthe* species. The support values for nodes are shown: left, ≥75 maximum likelihood bootstrap; middle, ≥75 maximum parsimony bootstrap; right, ≥0.95 bayesian inference. T: Ex-type or ex-epitype isolate. The isolates of the current study are shown in bold. Outgroup are used *D. scobina* (CBS 251.38) and *D. thunbergii* (MFLUCC 100576).

Sexual morph: unknown. Asexual morph on the stems and twigs of *T. vernicifluum*: *Conidiomata* pycnidial (Fig 2C-E), brown to dark brown, subglobose to obrate, immersed, scattered, up to 1 mm diam, solitary, embedded in tissue, erumpent at maturity, with an elongated, black neck less than 200 µm long, cream conidial droplets exuding from central ostiole. *Locules* 462–985 × 126–278 µm (ave. = 638 × 194 µm, n = 15) (Fig 2E), undivided, obrate to conoid. *Conidiophores* 13–30(–35) × 1.5–3.5(–4.0) µm (ave. = 21.4 × 2.5 µm, n = 150), hyaline (Fig 2F), smooth, 0–1 septate, rarely branched, cylindrical to ampulliform, densely aggregated, straight. *Conidiogenous cells* (8.0–)8.5–19.5(–23.5) × 1.5–3.5 µm (ave. = 13.9 × 2.1 µm, n = 150), phialidic, hyaline to pale brown at the base, cylindrical, terminal, slightly tapering towards apex. *Paraphyses* absent. *Alpha conidia* 8.5–13(–14) × 2.5–4 µm (ave. = 10.9 × 3.1 µm, n = 200), hyaline (Fig 2G), aseptate, smooth, ellipsoid to oblong, straight to variously curved, tapering towards both ends. *Beta* and *Gamma conidia* not seen.

Culture characteristics – Colonies on PDA (Fig. 2H) covering the entire 90 mm plate after 7 days in the dark at 25°C; surface canescent with abundant white compact aerial mycelium; reverse white to canescent at first, then becoming pale brown to dark brown towards the center. The optimal growth temperature is 25°C with growth in the range 5°C to 30°C.

Material examined – JAPAN, Joboji, Ninohe, Iwate Pref., on the stem of *Toxicodendron vernicifluum*, 26 May 2011, coll. M. Tabata (holotype: TFM FP-10740, ex-type culture: FFPRI420987). JAPAN, Joboji, Ninohe, Iwate Pref., on the stem of *T. vernicifluum*, 24 May 2011, coll. M. Tabata (TFM FP-10739, living culture FFPRI420985); JAPAN, Joboji, Ninohe, Iwate Pref., on the twig of *T. vernicifluum*, 24 June 2013, coll. H. Masuya (TFM FP-10743); JAPAN, Joboji, Ninohe, Iwate Pref., on the twig of *T. vernicifluum*, 6 May 2016, coll. H. Masuya, (TFM FP-10744, living culture FFPRI411165).

Known distribution – Japan (Hokkaido, Aomori, and Iwate-Prefectures)

Known host – *Toxicodendron vernicifluum* (Anacardiaceae)

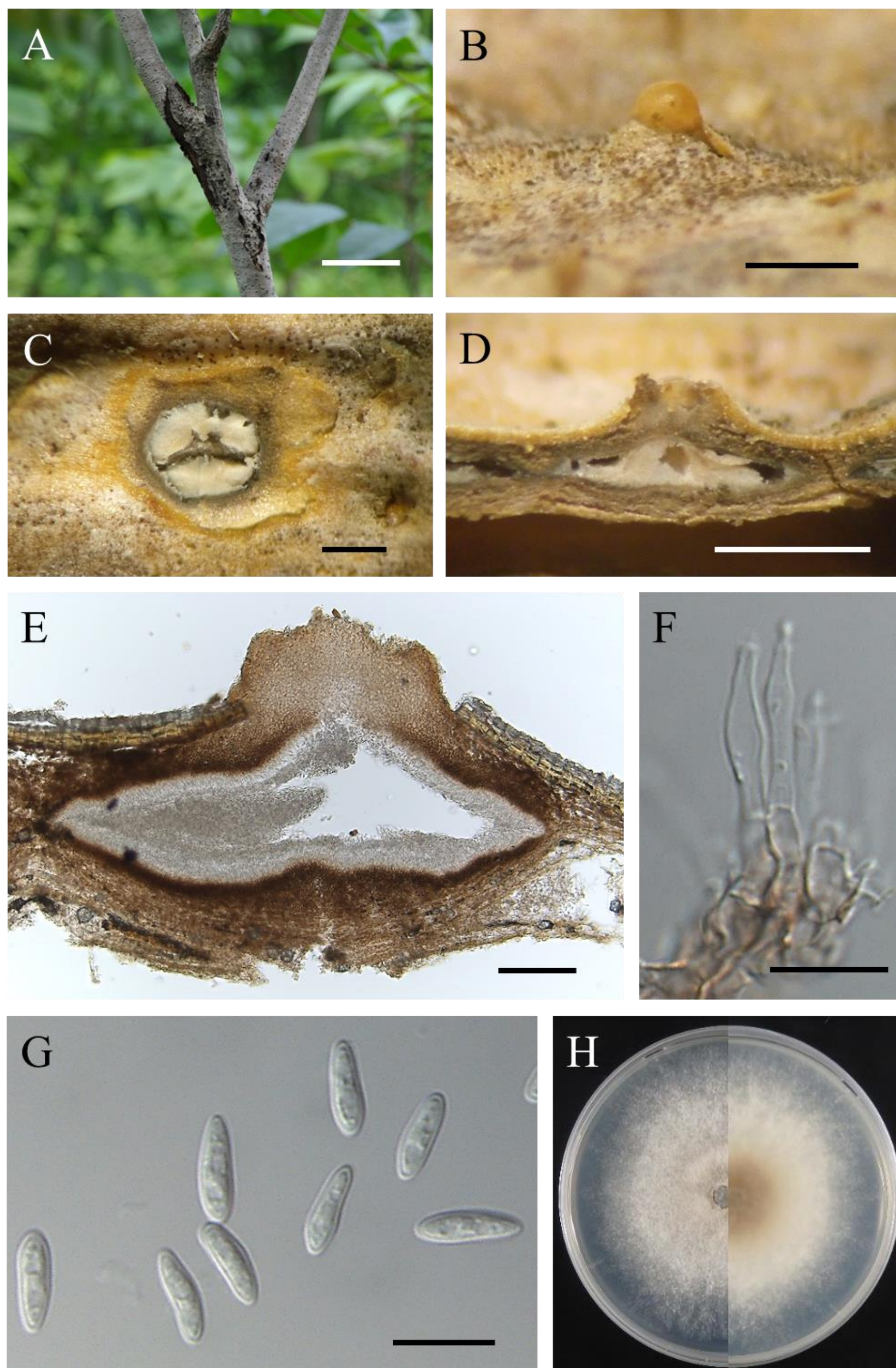

Figure 2 – *Diaporthe toxicodendri*. A: Disease symptom on *Toxicodendron vernicifluum*. B: Pycnidial exudates (TFM FP-10739). C: Horizontal section of the pycnidium (TFM FP-10739). D–E: Longitudinal section of pycnidium (TFM FP-10739). F: Conidiophores (TFM FP-10744). G: Conidia (TFM FP-10740). H: Culture morphology incubated after 7 days in 25 °C, darkness (left: surface, right: reverse) (FFPRI420987). – *Scale bars*: B–D = 0.5 mm, E = 100 µm, F–G = 10 µm.

Inoculation

All the inoculated point of *D. toxicodendri* isolates showed spindle shaped necrotic lesions in the inner barks, while there were almost no necrotic lesions in those of the mock inoculation (control). Compared to the length and width of the lesions in the bark of control, two *D. toxicodendri* isolates were formed necrotic lesions with significantly differences (Table 2). Moreover, it was indicated the significantly differences between two isolates. It was suggested that

virulence of *D. toxicodendri* was different between the isolates. *Diaporthe toxicodendri* was re-isolated from the lesions of all inoculated trees.

Table 2 Dimensions of necrotic lesions in the inner bark of *T. verniciffluum* inoculated with *D. toxicodendri* isolates.

Inoculum	Length of necrotic lesions (mm) [a]	Width of necrotic lesions (mm) [a]
FFPRI420984	8.4 – 22.9 (11.7 ± 3.3) b	7.4 – 12.7 (8.8 ± 1.3) b
AYC128-1	9.7 – 54.3 (18.7 ± 10.2) c	6.8 – 15.9 (10.3 ± 1.9) c
Control	7.8 – 10.8 (8.9 ± 0.7) a	6.3 – 8.4 (7.6 ± 0.5) a

[a] Values indicate Min. – Max. (Ave. ± SD) followed by the different letter are significantly different (P = 0.05) by the Steel-Dwass test.

Discussion

In this study we describe *Diaporthe toxicodendri* as a novel species based on both morphological characteristics and phylogenetic analyses using combined multigene sequences. The phylogenetic analyses showed this species is closely related to 10 species of *Diaporthe* (Fig. 1). These closely related species are known as pathogen of leaf spot, canker or dieback disease and widely or narrowly distributed in northern hemisphere (Gomes et al. 2013). Most of these species have been described from Europe or North America, three novel species were recently introduced in this group from China (Fan et al. 2015, Du et al. 2016, Yang et al. 2017). Although 6 of these species (*D. impulsa*, *D. fibrosa*, *D. detrusa*, *D. carpini*, *D. padi* var. *padi*, and *D. woolworthii*) were included in phylogenetic analysis by Gomes et al. (2013), the sequences from type material are unavailable. The epi- or neotypification for these species are needed in further study. In the morphological comparison with these closely related species (Table 3), *D. impulsa*, *D. ampelina*, *D. betulicola*, and *D. detrusa* could be distinguished from *D. toxicodendri* based on the existence of beta conidia. These 4 species are known to produce beta conidia on natural substrates and/or culture medium (Wehmeyer 1933, Gomes et al. 2013, Du et al. 2016). On the other hand, *D. toxicodendri* did not form conidiomata after twigs were placed on culture media. *D. toxicodendri* could also be distinguished from *D. fibrosa* (Saccardo 1882) and *D. rostrata* (Fan et al. 2015), which have wider alpha conidia than those of *D. toxicodendri*, and *D. toxicodendri* form large size of alpha conidia in comparison to *D. junlandicola* (Yang et al. 2017). The remaining 3 species, *D. woolworthii*, *D. padi* var. *padi*, and *D. carpini*, were provided using putatively named strains by Gomes et al. (2013), and were difficult to distinguish from *D. toxicodendri* by morphology. However, *D. toxicodendri* was clearly differentiated from these species in the phylogenetic analysis with high supported values (Fig. 1).

Different *Diaporthe* species associated with Anacardiaceae plants can have wide host ranges or be host specific (Gomes et al. 2013). According to the U.S. National Fungus Collections Database (United States Department of Agriculture, https://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm), a total of 25 *Diaporthe* species have been isolated from Anacardiaceae plants worldwide. Most of these species produce smaller alpha conidia than *D. toxicodendri* and others lack alpha conidia or produce beta conidia (Spegazzini 1910, Wehmeyer 1933, Srivastava et al. 1966, Kobayashi 1970, Sutton 1980, Uecker & Kuo 1992, Punithalingam 1993, Gomes et al. 2013, Udayanga et al. 2014b). One *Diaporthe* species associated with Anacardiaceae plant according to Gomes et al. (2013), *Diaporthe* sp. 7, was not included in the phylogenetic analyses in this study. However, *D. toxicodendri* is distantly related to this species. Therefore, the known *Diaporthe* species associated with Anacardiaceae are not closely related to *D. toxicodendri*.

Currently, over 80 *Diaporthe* species have been identified in Japan (Katsumoto 2010) but this list includes species that need re-examination with molecular data. Japanese *Diaporthe* isolates from fruit trees, agricultural crops, and forest trees are deposited in the National Institute of Agrobiological Sciences (NIAS) Genebank (Tsukuba, Ibaraki, Japan). ITS sequence data for 207

Diaporthe isolates, including 33 identified and unidentified species, are provided at the NIAS Genebank website (https://www.gene.affrc.go.jp/databases-micro_search.php). However, the *D. toxicodendri* ITS sequences did not match the sequences of any isolate in that database. It appears

Table 3 The comparison of size of alpha and beta conidia of *Diaporthe toxicodendri* and closely related species.

Species	Host (family)	Alpha conidia (µm)	Beta conidia (µm)	Reference
D. toxicodendri	*Toxicodendron vernicifluum* (Anacardiaceae)	8.5–13(–14) × 2.5–4.0	-	This study
D. impulsa	*Sorbus. americana*, *S. aucuparia* (Rosaceae)	15–27 × 2.5–5	10–15 × 1–1.5	Wehmeyer (1933)
D. ampelina	*Vitis vinifera* (Vitaceae)	(7–)9.5–10.5(–13) × (1.5–)2–3(–3.5)	20–25 × 0.5–1	Gomes et al. (2013)
D. betulicola	*Betula albosinensis* (Betulaceae)	10–14.5(15) × 1.5–2.5	17–24 × 0.5–1(–1.5)	Du et al. (2016)
D. detrusa	*Berberis microphylla*, *B. spathulata*, *B. vulgaris* (Berberidaceae) *Mahonia aquifolia* (Berberidaceae)	8–17 × 2.5–3(–5)	11–33 × 1–1.5	Wehmeyer (1933)
D. fibrosa	*Prunus spinose* (Rosaceae), *Rhamnus cathartica*, *R. frangula* (Rhamunaceae), *S. aucuparia* (Rosaceae)	11 × 5	-	Saccardo (1882)
D. rostrata	*Juglans mandshurica* (Juglandaceae)	(8–)8.5–11.5(–12) × 4–5(–5.5)	-	Fan et al. (2015)
D. juglandicola	*J. mandshurica* (Juglandaceae)	(7.5–)8–9(–9.5) × 2.5–3(–3.2)	-	Yang et al. (2017
D. woolworthii	*Ulmus americana* (Ulmaceae)	10	-	Saccardo (1882)
D. padi var. *padi*	*P. padus* (Rosaceae)	9–11 × 3	-	Wehmeyer (1933)
D. carpini	*Carpinus betulus* (Betulaceae)	12 × 3–4	-	Saccardo (1882)

-: not seen

that species consistent with *D. toxicodendri* have not previously been reported in Japan, although this may be due to confusion in the species identification of available isolates.

Takemoto et al. (2014) isolated several *Diaporthe* species (as *Phomopsis* species) from *T. verniciffluum*, and we have isolated *D. toxicodendri* predominantly from canker-diseased lesions in *T. verniciffluum*. In this study, we have confirmed the pathogenicity of the fungus to *T. verniciffluum* (Table 2). The result of inoculation suggested that this fungus had different virulence between the isolates. Further studies are needed to assess the aggressiveness of *D. toxicodendri* and to identify resistant varieties of *T. verniciffluum* for future cultivation.

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