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Athelia rolfsii infects dichondra in Taiwan

Yuan-Min Shen[1*], Yi-Jia Lin[1, 2], Chia-Chin Hsu[1], Tung-Chin Huang[1]

[1] Taichung District Agricultural Research and Extension Station, Dacun, Changhua, Taiwan
[2] Taiwan Agricultural Research Institute, Wufeng, Taichung, Taiwan
[*] Corresponding author, Dr. Yuan-Min Shen; E-mail: shenym@tdais.gov.tw

Abstract

In May 2018, mycelia and sclerotia were discovered on blighted *Dichondra donelliana* plants at Dacun, Changhua County, Taiwan. A southern blight fungus was isolated and identified as *Athelia rolfsii* based on the morphological and molecular characteristics. Koch's postulates were fulfilled by pathogenicity tests conducted on *D. donelliana* and *D. repens* and the identification of the plants was supported by the ITS, *rbcL*, and *matK* sequences. To our knowledge, this is the first record of *D. donelliana* as a host of *A. rolfsii* worldwide.

Keywords: *Sclerotium rolfsii*, dichondra, ground cover, molecular identification, Taiwan, horticulture

Introduction

Plant species of dichondra (*Dichondra* spp.), also known as ponyfoots, are creeping perennial herbs native to America, New Zealand and Australia [7]. There are 15 accepted *Dichondra* species [7] and some of the cultivated plants have been spread around the world, mainly in tropical to warm-temperate regions [1, 12, 42]. The plants are used as ground covers [14, 15, 18, 38], mulching [17], lawns [1, 10], green roofs [5], and traditional medicines [1, 31, 42]. Furthermore, dichondra could be a big scale item in horticulture [1].

In 2017, a dichondra was introduced to Taichung District Agricultural Research and Extension Station (TDARES) in Dacun Township, Changhua County, Taiwan as a ground cover and for horticultural therapy. In May 2018, some of the dichondra plants were blighted and the dying plants were grouped in circular patches about 30 cm in diameter (Fig. 1A). The disease incidence was around 1%. White cottony mycelia and white-to-brown small (0.4-2 mm) sclerotia were formed on and near the surfaces of the stems (Fig. 1B). The disease on dichondra is considered to be southern blight caused by a *Sclerotium*-like pathogen. Before our current study, the pathogen remains unreported on dichondra in this region. In this study, the pathogen and the host plants used in the experiments are identified and pathogenicity tests are demonstrated.

Materials and Methods

Sample collection and morphological observation of the pathogen and plants

Diseased dichondra was collected at the newly refurbished garden in TDARES. The stem tissues were surface-disinfested in 0.6% NaOCl (Clorox) for 1 min, rinsed with sterile distilled water,

Fig. 1. Dichondra plants with disease symptoms in the field. (A) Diseased plants grouped in circular patches. White arrows indicate the diseased areas. (B) Mycelia and sclerotia. Scale bar indicates 1 cm in length.

and transferred to 2% water agar (WA) plates. The mycelial tips from WA were subsequently cultured on potato dextrose agar (PDA) plates. The growth of the colony was determined by placing a 5-mm mycelial plug on PDA and incubating under 28℃ for 3 days in dark. The colony diameters were measured in two perpendicular directions and the average was calculated from 5 replicates. The mycelia were observed under a Leica DM2500 light microscope (Leica Microsystems, Wetzlar) under 400× magnification. Sclerotia were produced by incubating the fungal isolate on PDA under 20℃ for 2 weeks.

A representative fungal culture was deposited in Bioresource Collection and Research Center in Hsinchu, Taiwan under BCRC number FU30952. The morphological classification of the plants referred to Tharp and Johnston [36] and the website of The Jepson Herbarium (http://ucjeps.berkeley.edu/). Two voucher plant specimens were deposited at the herbarium of the National Museum of Natural Science (TNM) in Taichung, Taiwan.

Pathogenicity tests

In May 2018, dichondra plants from the same source as that of the diseased plants (designated as dichondra sp1 here) were transplanted in 3-inch nursery pots in a greenhouse. After 15 days, the plants were inoculated with the fungal isolate BCRC FU30952. The inoculum was prepared by incubating the isolate on sterilized rice grains (rice: water: dextrose = 200 g: 200 g: 4 g) in an orchid flask for more than 20 days in an ambient environment. Five plants were inoculated with the fungus-colonized rice grains (about 10 g/ plant) directly on the base of each plant. Five plants inoculated similarly with sterile rice grains served as negative controls. The experiment was repeated by inoculated 5 plants with reduced inoculum (10 grains/plant). Reduced amount of sterile rice grains applied to 5 plants served as negative controls. To fulfill Koch's postulates, symptomatic stem tissues were surface-disinfested as the previous described isolation method. The re-isolated fungi were cultured on PDA and compared to the original isolate.

Since another dichondra with smaller leaves naturally grown in TDARES, the plants (designated as dichondra sp2 here) were transplanted in the greenhouse and further used for pathogenicity tests. In September 2018, the plants of dichondra sp2 in 3-inch nursery pots were inoculated with freshly prepared fungus-colonized rice grains as previously mentioned. The inoculum amount of 10 g/ plant was applied to five plants and reduced inoculum (10 grains/ plant) was inoculated to another five plants. The same amount of sterile rice grains treated to equal number of plants used as negative controls. Re-isolation of the fungus was conducted to fulfill Koch's postulates.

Molecular identification

Total genomic DNA of the fungus and the two plants (dichondra sp1 and sp2) were extracted with a Plant Genomic DNA Extraction Miniprep Kit (Viogene, New Taipei), according to the manufacturer' s instructions. Partial internal transcribed spacer (ITS) and large subunit ribosomal DNA (LSU rDNA) sequences of the fungus were amplified with primer combinations ITS1/ITS4 [43] and LR0R/ LR6 [24, 39], respectively. Partial internal transcribed spacer (ITS), chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*), and chloroplast maturase K gene (*matK*) sequences of the plants were amplified using primer pairs ITS1/ITS4, rbcL-1F (5'-ATGTCACCACAAACAGAAAC-3') / rbcL-1460R (5'-TCCTTTTAGTAAAAGATTGGGCCGAG-3') [11, 26, 28], and matK-390F (5'-CGATCTATTCATTCAATATTTC-3')/ matK-1326R (5'-TCTAGCACACGAAAGTCGAAGT-3') [41], respectively. The PCR conditions were as follows: Initial denaturation at 95℃ for 2 min, followed by 35 cycles of 30 s at 95℃ for denaturation, 30 s for primer annealing at 55℃ for ITS, LSU rDNA, and *rbcL*, 50℃ for *matK*, and 2 min at 72℃ for DNA elongation, followed by a final elongation period of 5 min at 72℃. The amplicons were directly

TABLE 1. GenBank accessions of fungi used in this study

Species	Isolate[1]/Reference[2]	GenBank accessions	
		ITS	LSU
Athelia rolfsii (Sclerotium rolfsii)	**BCRC FU30952/ this study**	**MN380239**	**MN368290**
A. rolfsii	-[3]/ Cating et al. 2009	GQ358518	
A. rolfsii	NAGC7T1/ Le et al. 2012	HQ895919	
A. rolfsii	NAGC4T2/ Le et al. 2012	HQ895962	
A. rolfsii	BCRC 30230/ Shen et al. 2014	KJ677121	
A. rolfsii	KACC 47820/ Kwon et al. 2015	KP257581	
A. rolfsii	Sc-03/ Huang et al. 2017	KX186998	
A. rolfsii	CBS 191.62/ Vu et al. 2019	MH858139	
S. delphinii	S-58/ Okabe et al. 2003	AB075314	
S. delphinii	ZM130296/ Zhang et al. 2014	KJ145328	
S. coffeicola	CBS115.19/ Okabe et al. 2003	AB075319	
Sclerotinia sclerotiorum	BCRC 34830/ Shen et al. 2012	JQ653934	

[1] The isolate in this study is marked in bold.
[2] References [3, 16, 20, 21, 25, 29, 30, 40, 44].
[3] -: not available.

TABLE 2. GenBank accessions of plants used in this study

Species	Voucher[1]/ Reference[2]	GenBank accessions		
		ITS	*rbcL*	*matK*
***Dichondra donelliana* (dichondra sp1)**	**TNM S205110/ this study**	**MN380240**	**MN379752**	**MN379754**
***D. repens* (dichondra sp2)**	**TNM S205111/ this study**	**MN380241**	**MN379753**	**MN379755**
D. argentea	GBIF7416938/ Martínez-Blancas et al. 2018		MH028853	MH037666
D. brachypoda	-[3]/ Stefanovic et al. 2002		AY101022	
D. carolinensis	-/ McNeal et al. 2007	EU330328		EU330287
D. carolinensis	Abbott 18777/ -		KJ773457	KJ772729
D. carolinensis	FLAS 240847/ -			MH551805
D. donelliana	JEPS118007/ Thornhill et al. 2017	MF964068	MF963293	MF963681
D. micrantha	H. Schaefer 2008 225/ Schaefer et al. 2011		HM849950	HM850895
D. occidentalis	-/Stefanovic et al. 2002		AY101023	
D. repens	PS1537MT01/ -	FJ980434		
D. repens	DIRE G2 P.BC PM 1 of 3/ -.	KY700304		
D. repens	DIRE G2 P.BC PM 2 of 3/ -	KY700305		
D. repens	DIRE DP 3 of 3/ -	KY700306		
D. repens	CHR:688766/ -		KT626692	
Nicotiana tabacum	Clarkson 006 BM/ Chase et al. 2003	AJ492448		
N. tabacum	-/ Shinozaki et al 1986		Z00044	Z00044

[1] The vouchers in this study are marked in bold.

[2] References [4, 22, 23, 28, 32, 34, 37].

[3] -: not available.

sequenced from both ends using ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California) at Tri-I Biotech, Inc., New Taipei. The sequences were deposited in GenBank and analysed by the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/).

Phylogenetic analyses were performed by maximum likelihood (ML) method using MEGA X software [19]. The fungal and plant sequences obtained in this study and the reference sequences available in GenBank (Table 1, Table 2) were trimmed and aligned using MUSCLE strategy [9]. The ML phylogenetic trees estimated from the ITS, *rbcL*, and *matK* sequences were generated with Tamura 3-parameter model [35] and 1,000 bootstrap replications.

Results

The fungus and the host plants

The fungal isolate formed white, cottony mycelia. The average diameter of the colonies was 5.6 cm under 28℃ after 3 days incubation. The hyphae had typical clamp connections (Fig. 2A). Brown, spherical to irregular-shaped sclerotia on PDA (Fig. 2B) were 1.4 – 3.7 mm in width and 1.9 – 5.0 mm in length.

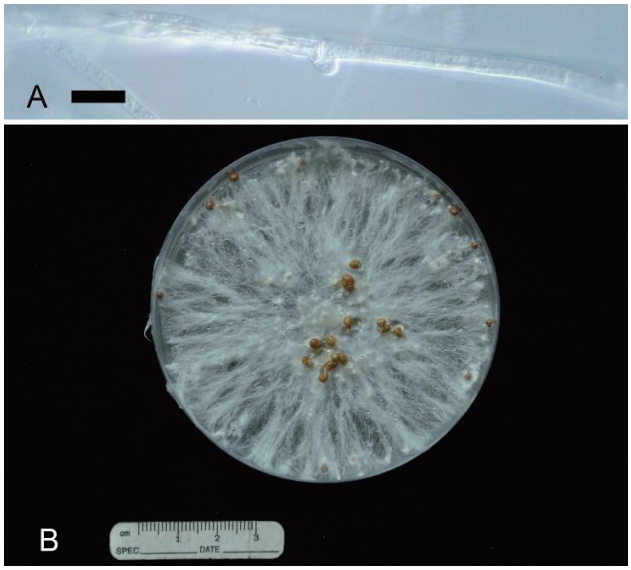

Fig. 2. *Athelia rolfsii* BCRC FU30952. (A) Clamp connection of hyphae. Scale bar indicates 20 μm in length. (B) Fungal colony on PDA after 2 weeks of incubation, showing white mycelium and brown sclerotia.

The dichondra (sp1) with southern blight symptoms newly grown in TDARES had suborbicular to reniform leaf blades, mostly 0.9 – 2.4 cm broad. The leaf surfaces were not strongly contrasted

and not silvery. The stems were 0.7 – 1.2 mm thick, with dense hairs, internodes 1.3 – 3.2 cm long.

The dichondra (sp2) naturally grown in TDARES used in the pathogenicity tests had suborbicular to reniform leaf blades, mostly 0.5 – 1.4 cm broad. The leaf surfaces were not strongly contrasted and not silvery. The stems were 0.4 – 0.7 mm thick, with sparse hairs, internodes 0.6 – 2.0 cm long.

Pathogenicity tests

All the dichondra sp1 plants inoculated with the fungus exhibited rot and blight symptoms at 4 days post inoculation (dpi) (Fig. 3A). White mycelia and sclerotia were formed near the rotten stems. Most of the infected plants were toppled and died at 8 dpi whereas those treated with sterile rice grains were symptomless. All the plants inoculated with reduced inoculum showed blight symptoms which was less severe at 8 dpi, while the control plants remained healthy. White fungal colonies resemble the original isolate was re-isolated from the artificially inoculated plants in the two experiments, satisfying Koch's postulates.

In the pathogenicity test using dichondra sp2, all the plants treated with 10 g of inoculum each plant had rot and blight symptoms at 4 dpi (Fig. 3B). All the plants then became deteriorated and died. Treated with reduced inoculum, the plants in one pot exhibited blight symptoms at 4 dpi and the plants had the disease symptoms in every pot at 14 dpi. All the controls remained symptomless at 14 dpi. Koch's postulates were fulfilled followed the completion of re-isolation of the pathogen.

Fig. 3. Pathogenicity of *Athelia rolfsii* on dichondra at 4 days post inoculation. (A) Healthy controls (upper part) and inoculated *D. donelliana* plants (lower part). Scale bar indicates 5 cm in length. (B) Healthy controls (upper part) and inoculated *D. micrantha* plants (lower part). Scale bars indicate 5 cm in length.

Molecular identification and phylogenetic analysis

The GenBank accession numbers of the ITS and LSU rDNA sequences of our fungal isolate BCRC FU30952 were MN380239 and MN368290, respectively (Table 1). Nucleotide BLAST search at GenBank with the sequences of the isolate revealed the highest identity with those of *Athelia rolfsii* (Curzi) C.C. Tu & Kimbr: only 1-2 base substitutions compared with the ITS sequences of Vietnamese *A. rolfsii* isolates (HQ895919 and HQ895962) [21] and *A. rolfsii* strain CBS191.62 (MH858139) [40] over a 461-bp alignment; and had 99.5% identity compared with the LSU sequence of *A. rolfsii* strain CBS745.84 over a 1,019-bp alignment. In the ML phylogenetic tree generated from the ITS sequences (Fig. 4), the fungus isolated from dichondra was placed within a clade comprising reference sequences of *A. rolfsii*.

The ITS, *rbcL*, and *matK* sequences of the plants in this study were listed in Table 2. The sequences of dichondra sp1 and dichondra sp2 shared close identities with dichondra sequences in Genbank. The obtained sequences of dichondra sp1 were highly identical to those of *D. donelliana* Tharp & M.C. Johnst. voucher JEPS118007 [37]: 100% for the ITS, 99.9% for the *rbcL*, and 100% for the *matK* sequences. Dichondra sp1 clustered with *D. donelliana* in the phylogenetic trees based on the three DNA regions (Fig. 5-7). On the other hand, sequences of dichondra sp2 were similar to those of *D. repens* J.R. Forst. & G. Forst. specimens from China (FJ980434) [6] and New Zealand (KT626692; voucher CHR 688766). Dichondra sp2 grouped with *D. repens* in the phylogenetic trees estimated from the ITS and *rbcL* sequences (Fig. 5-6). Although *matK* sequences of *D. repens* was currently not available in Genbank, the *matK* sequence of dichondra sp2 was distinct from those of other *Dichondra* species (Fig. 7).

Based on the morphological and molecular characteristics, the pathogen on dichondra was identified as *A. rolfsii* (synonymy: *Sclerotium rolfsii*). The two *Dichondra* species used in this study, namely dichondra sp1 and dichondra sp2, were identified as *D. donelliana* and *D. repens*, respectively.

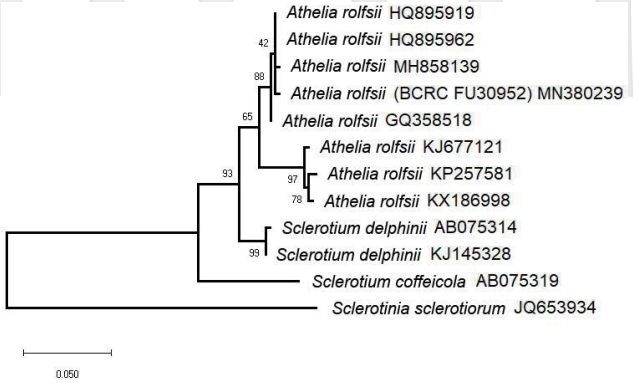

Fig. 4. Molecular phylogeny of *Athelia rolfsii* and close related species based on internal transcribed spacer sequences. The DNA sequences were aligned using MUSCLE strategy, and the phylogenetic tree was constructed using the Maximum likelihood method with 1,000 bootstrap replications. The bootstrap values are given at the internal branches.

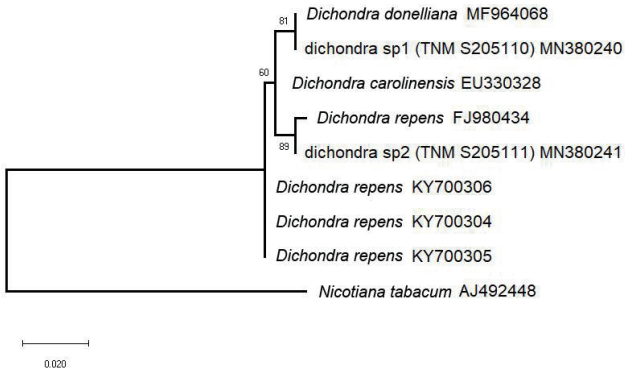

Fig. 5. Molecular phylogeny of *Dichondra* spp. based on internal transcribed spacer sequences. The DNA sequences were aligned using MUSCLE strategy, and the phylogenetic tree was constructed using the Maximum likelihood method with 1,000 bootstrap replications. The bootstrap values are given at the internal branches.

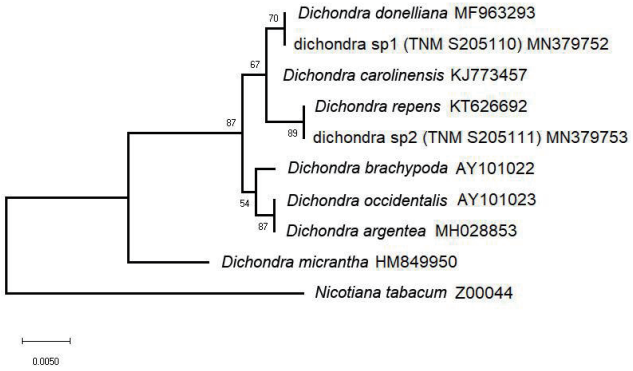

Fig. 6. Molecular phylogeny of *Dichondra* spp. based on *rbcL* sequences. The DNA sequences were aligned using MUSCLE strategy, and the phylogenetic tree was constructed using the Maximum likelihood method with 1,000 bootstrap replications. The bootstrap values are given at the internal branches.

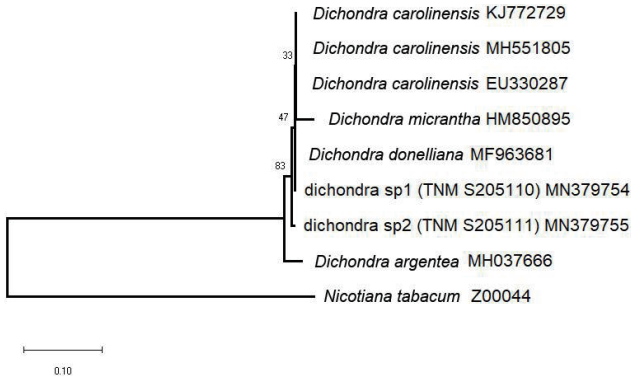

Fig. 7. Molecular phylogeny of *Dichondra* spp. based on *matK* sequences. The DNA sequences were aligned using MUSCLE strategy, and the phylogenetic tree was constructed using the Maximum likelihood method with 1,000 bootstrap replications. The bootstrap values are given at the internal branches.

Discussion

In this study, two morphologically distinct *Dichondra* species were used to prove the pathogenicity of the southern blight pathogen. We found that the identification of dichondra was hard because the flowers, an important character in the key to the species of *Dichondra*, was not observed in the field and only one species, *D. micrantha* Urb., was listed in Flora of Taiwan [33]. Dichondra sp1, which had larger leaves, resembled *D. donelliana* whereas dichondra sp2, which had smaller leaves, was morphologically similar to *D. micrantha* [36]. After integrating the molecular data, the identification of dichondra sp1 as *D. donelliana* was supported, but dichondra sp2 could not be placed under the name *D. micrantha* because the incongruence of the sequences between dichondra sp2 and *D. micrantha* in Genbank [28] (Fig. 6-7). Because dichondra sp2 grouped with *D. repens* in the molecular phylogeny and it also fit the morphological traits of *D. repens* in Tharp and Johnston [36], dichondra sp2 was then identified as *D. repens*. To the best of our knowledge, this is the first confirmed report of the existence of *D. donelliana* and *D. repens* in Taiwan based on the molecular data.

In 2018, southern blight disease was first found on *D. donelliana* in Dacun, Changhua, Taiwan. *Athelia rolfsii* was isolated from the diseased plants and then the pathogenicity of the fungus was demonstrated on *D. donelliana* and *D. repens*. It is possible that the inoculum was brought into the field through the introduction of diseased seedlings, or alternatively, the remains of the sclerotia and diseased host plants in the fields might be the source of infection [27]. In recent years, dichondra has been used as one of the ground covers in organic or sustainable agriculture [2, 8, 15]. The occurrence of

A. rolfsii on dichondra may pose a threat on other economic crops grown in the farmlands. To date, *Athelia rolfsii* is known to have a very wide host range with more than 1,200 host plants (https://nt.ars-grin.gov/fungaldatabases/). The pathogen has been reported to cause southern blight on *D. repens* in Italy, India [13], Australia, Brazil, and Hawaii and on *D. carolinensis* in the United States (https://nt.ars-grin.gov/fungaldatabases/). To our knowledge, this is the first report of *A. rolfsii* on *D. donelliana* in Taiwan and the fungus is able to infect *D. donelliana* and *D. repens* after inoculation. In addition, this is the first report of *D. donelliana* as a host of *A. rolfsii* in the world.

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摘 要

沈原民、林依佳、許嘉錦、黃冬青。2020。臺灣感染馬蹄金之白絹病菌。植物醫學62(1): 21-28。

2018年五月，在臺灣彰化縣大村的*Dichondra donelliana*馬蹄金植株上發現菌絲及菌核，分離得白絹病菌，藉形態與分子特徵鑑定該菌為*Athelia rolfsii*。病原性測試於*D. donelliana*、*D. repens*上進行，已由柯霍式法則完成病原性測試，且植物之鑑定由ITS、*rbcL*、*matK*序列支持。就我們所知，本研究為世界上首次紀錄*D. donelliana*為*A. rolfsii*之寄主植物。

關鍵詞：白絹病菌、馬蹄金、地被植物、分子鑑定、臺灣、園藝