玉川大学学術研究所紀要　第22号：29–35（2016）
Tamagawa University Research Review, 22, 29–35 (2016).

First Report of *Pestalotiopsis* sp. Causing Brown Leaf Spot Disease in the Lily

Shunsuke Nozawa[1)], Naoko Fujita[2)] and Kyoko Watanabe[1),3),*]

Tamagawa University Research Institute, Machida-shi, Tokyo, 194–8610 Japan.
Tamagawa University Research Review, 22, 29–35 (2016)

Abstract

In October 2008, brown leaf spot was first detected on the leaves of a rose red lily (*Lilium speciosum* var. *speciosum* L.) in Machida, Tokyo, Japan and ever since has been observed annually in August-October. The aim of this study was to clarify the pathogenic source of this disease. Typical symptoms observed were brown leaf spots surrounding the yellow leaf margins. Over time, the lesions enlarged but did not cover the entire leaf. The causative agent was a fungal species member of *Pestalotiopsis*, as determined via conidial morphology and molecular data. To our knowledge, this is the first report of *Pestalotiopsis* sp. causing brown leaf spot disease in lily in Japan. We propose to call this disease, brown leaf spot, "Kasshoku-hanyou-byo" in Japanese.

Keywords：brown leaf spot disease, fungal pathogen, *Pestalotiopsis*, novel disease, *Lilium speciosum* var. *speciosum* L.

Introduction

Lilies (*Lilium* spp.) are treasured ornamental plants, with the genus *Lilium* comprised of more than 100 species. In Japan, there are 10 native lily species, including the rose red lily (*Lilium speciosum* var. *speciosum* L.), which is a conservation-reliant species (Vulnerable) (Global red list of Japanese threatened Plants, http://www.kahaku.go.jp/english/research/db/botany/redlist/list/list 05_234_1.htm). Thus, protecting and sustaining this plant is absolutely crucial.

In early October 2008, brown leaf spots were observed in rose red lilies growing naturally in Machida, Tokyo, Japan and have been annually observed in August–October. Previously in Japan, there were not any reports of disease in the rose red lily, although in the US, fungal diseases of rose red lily caused by species *Fusarium oxysporum*, *Penicillium hirsutum*, and *Sclerotium rolfsii* are known (Miller et al. 1960).

The purpose of this study was to clarify the causative agent of this new disease in rose red lilies. Using Koch's postulates as the foundation for our investigations, we isolated and identified fungi in genus *Pestalotiopsis* as the fungal microbe responsible for disease, based on morphological and molecular data, and confirmed the pathogenicity in healthy lily plants. To our knowledge, this is the first report of brown leaf spot disease in lilies, caused by *Pestalotiopsis* sp.

1）Graduate School of Agriculture, Tamagawa University, Tamagawa-gakuen 6–1–1, Machida, Tokyo 194–8610, Japan
2）Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Saiwaicho 3–5–8, Fuchu, Tokyo 183–8509, Japan
3）Mycology & Metabolic Diversity Research Center, Tamagawa University Research Institute, Tamagawa-gakuen 6–1–1, Machida, Tokyo 194–8610, Japan
＊E-mail：wkyoko@agr.tamagawa.ac.jp

Materials and Methods

Isolation

To isolate the causative agent of brown leaf spot-like disease of rose red lilies in 2008, diseased leaves were collected and brown lesions were cut into 5mm^2 segments, sterilized with 10 % (v/v) NaCl for 1 min, and then placed on water agar plates (WA) to obtain monocultures. Emergent hyphae were transplanted to potato dextrose agar (PDA) plates for conidial formation. A monoculture was obtained from a conidium produced on the PDA plate. This strain was deposited to the Genebank Project NARO, Japan (MAFF) as MAFF 241683, and the culture collection of Tamagawa University (TAMA) was deposited as TAMA573.

Identification of pathogen

Morphological observation: The isolated strain was grown on leaf agar plates using *Hydrangea macrophylla* (Thun.) Ser. (Furukawa and Kishi 2002) to observe morphological characteristics. The sizes and characteristics of conidia (n = 30) were assessed under the light microscopy (BX51, Olympus, Tokyo, Japan).

Molecular analysis: MAFF 241683 DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. ITS, β-tubulin, and *tef1* gene regions were amplified using the following primer pairs: ITS5/ITS4 (White et al. 1990), Bt2a/Bt2b (Glass and Donaldson 1995), and pest_ef_f (Nozawa et al. 2017)/EF1−1567R (Rehner and Buckley 2005). PCR was performed in 10 µL reactions, which contained 7 µL of double distilled water, 1 µL of 10 × Ex Taq buffer with MgCl2, 0.8 µL of dNTPs (10 mM each), 0.1 µL of each primer (50 µM), 0.05 µL of Ex Taq DNA polymerase (5U µL^{-1}, Takara, Tokyo, Japan), and 1.0 µL of DNA template. Thermal cycling conditions were as follows. For ITS: initial denaturing step at 94℃ for 3 min, followed by 35 cycles at 95℃ for 1 min, 55℃ for 1 min, and 72℃ for 1 min, with a final extension step at 72℃ for 10 min. For β-tubulin: an initial denaturing step at 94℃ for 3 min, followed by 35 cycles at 94℃ for 1 min, 56℃ for 1 min, and 72℃ for 1 min, and then 10 min at 72℃. For *tef1*: an initial denaturing step at 94℃ for 3 min, followed by 35 cycles at 94℃ for 1 min, 57.5℃ for 1 min, and 72℃ for 1 min, and then 72℃ for 7 min. The PCR products were purified with the ExoSAP-IT reagent (GE Healthcare, Tokyo, Japan). The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) was used along with the same primers used in PCR. The sequencing products were purified using the ethanol precipitation method and then analyzed with an ABI Prism 3500 Sequencer (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. The sequences obtained by forward or reverse primers were processed with BioEdit (Hall 1999). These sequences were then deposited in the DNA Data Bank of Japan.

The sequence of ITS, β-tubulin, and *tef1* gene regions were aligned using Clustal W in BioEdit (Hall 1999). The combined dataset was performed with sequence data set obtained from database (Table 1) via three phylogenetic analyses [i.e., neighbor-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP)] using MEGA v. 5 (Tamura et al. 2011). Gaps were treated as missing data. The strengths of the internal branches in the trees obtained were tested by bootstrap analysis (Felsenstein 1985) using 1,000 replications. The ML method was performed based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). Initial trees for heuristic search were obtained automatically. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories; $+G$, parameter = 0.54).

Mycelial growth test: For the mycelial growth test, 7 d cultures of MAFF 241683 grown at 25℃ on PDA plates were used. A cork borer was applied to these cultures to make mycelial plugs (ϕ7 mm). These plugs were then put on a center of PDA plates. These plates were incubated at 4℃, 10℃, 15℃, 20℃, 25℃, or 30℃. After incubation for 4 d, mycelial growth per day was calculated. The average growth rate per day for each temperature was determined from five replicates.

Pathogenicity tests: Pathogenicity tests with MAFF 241683 were performed on rose red lily leaves in the field.

Table 1. Species and GenBank accession numbers.

Species	Culture No.	Location	Host	GeneBank accession		
				ITS	*β*-tubulin	*tef1*
Pestalotiopsis adusta	**ICMP 6088**	Fiji	On refrigerator door PVC gasket	JX399006	JX399037	JX399070
	MFLUCC 10–146	Thailand	*Syzygium* sp.	JX399007	JX399038	JX399071
P. anacardiacearum	**IFRDCC 2397**	China	*Mangifera indica*	KC247154	KC247155	KC247156
P. arceuthobii	**CBS 434.65**	USA	*Arceuthobium campylopodum*	KM199341	KM199427	KM199516
P. arengae	**CBS 331.92**	Singapore	*Arenga undulatifolia*	KM199340	KM199426	KM199515
P. australasiae	**CBS 114126**	New Zealand	*Knightia* sp.	KM199297	KM199409	KM199499
	CBS 114141	Australia	*Protea* sp.	KM199298	KM199410	KM199501
P. australis	CBS 111503	South Africa	*Protea neriifolia* × *susannae* cv. "Pink Ice"	KM199331	KM199382	KM199557
	CBS 114193	Australia	*Grevillea* sp.	KM199332	KM199383	KM199475
	CBS 114474	South Africa	*Protea neriifolia* × *susannae* cv. "Pink Ice"	KM199334	KM199385	KM199477
P. biciliata	**CBS 124463**	Slovakia	*Platanus* × *hispanica*	KM199308	KM199399	KM199505
	CBS 236.38	Italy	*Paeonia* sp.	KM199309	KM199401	KM199506
	CBS 790.68	Netherlands	*Taxus baccata*	KM199305	KM199400	KM199507
P. camelliae	CBS 443.62	Turkey	*Camellia sinensis*	KM199336	KM199424	KM199512
	MFLUCC 12-0277	China	*Camellia japonica*	JX399010	JX399041	JX399074
	MFLUCC 12–0278	China	*Camellia japonica*	JX399011	JX399042	JX399075
P. chamaeropis	CBS 113604	—	—	KM199323	KM199389	KM199471
	CBS 113607	—	—	KM199325	KM199390	KM199472
	CBS 186.71	Italy	*Chamaerops humilis*	KM199326	KM199391	KM199473
	CBS 237.38	Italy	—	KM199324	KM199392	KM199474
P. clavata	**MFLUCC 12-0268**	China	*Buxus* sp.	JX398990	JX399025	JX399056
P. colombiensis	**CBS 118553**	Colombia	*Eucalyptus eurograndis*	KM199307	KM199421	KM199488
P. diploclisiae	CBS 115449	Hong Kong	*Psychotria tutcheri*	KM199314	KM199416	KM199485
	CBS 115585	Hong Kong	*Diploclisia glaucescens*	KM199315	KM199417	KM199483
	CBS 115587	Hong Kong	*Diploclisia glaucescens*	KM199320	KM199419	KM199486
P. diversiseta	**MFLUCC 12-0287**	China	*Rhododendron* sp.	JX399009	JX399040	JX399073
P. ericacearum	**IFRDCC 2439**	China	*Rhododendron delavayi*	KC537807	KC537821	KC537814
P. furcata	**MFLUCC 12-0054**	Thailand	*Camellia sinensis*	JQ683724	JQ683708	JQ683740
P. gaultheria	**IFRD 411-014**	China	*Gaultheria forrestii*	KC537805	KC537819	KC537812
P. grevilleae	**CBS 114127**	Australia	*Grevillea* sp.	KM199300	KM199407	KM199504
P. hawaiiensis	**CBS 114491**	USA: Hawai	*Leucospermum* sp.	KM199339	KM199428	KM199514
P. hollandica	**CBS 265.33**	Netherlands	*Sciadopitys verticillata*	KM199328	KM199388	KM199481
P. humus	CBS 115450	Hong Kong	*Ilex cinerea*	KM199319	KM199418	KM199487
	CBS 336.97	Papua New Guinea	Soil	KM199317	KM199420	KM199484
P. inflexa	**MLFUCC 12-0270**	China	Unidentified tree	JX399008	JX399039	JX399072
P. intermedia	**MLFUCC 12-0259**	China	Unidentified tree	JX398993	JX399028	JX399059
P. kenyana	**CBS 442.67**	Kenya	*Coffea* sp.	KM199302	KM199395	KM199502
	CBS 911.96	—	Raw material from ager-agar	KM199303	KM199396	KM199503
P. knightiae	CBS 111963	New Zealand	*Knightia* sp.	KM199311	KM199406	KM199495
	CBS 114138	New Zealand	Knightia sp.	KM199310	KM199408	KM199497
P. linearis	**MFLUCC 12-0271**	China	*Trachelospermum* sp.	JX398992	JX399027	JX399058
P. malayana	**CBS 102220**	Malaysia	*Macaranga triloba*	KM199306	KM199411	KM199482
P. monochaeta	**CBS 144.97**	Netherlands	*Quercus robur*	KM199327	KM199386	KM199479
	CBS 440.83	Netherlands	*Taxus baccata*	KM199329	KM199387	KM199480
P. novae-hollandiae	**CBS 130973**	Australia	*Banksia grandis*	KM199337	KM199425	KM199511
P. oryzae	CBS 111522	USA: Hawai	*Telopea* sp.	KM199294	KM199394	KM199493
	CBS 171.26	Italy	—	KM199304	KM199397	KM199494
	CBS 353.69	Denmark	*Oryza sativa*	KM199299	KM199398	KM199496
P. papuana	**CBS 331.96**	Papua New Guinea	Coastal soil	KM199321	KM199413	KM199491
	CBS 887.96	Papua New Guinea	*Cocos nucifera*	KM199318	KM199415	KM199492
P. parva	**CBS 265.37**	—	*Delonix regia*	KM199312	KM199404	KM199508
	CBS 278.35	—	*Leucothoe fontanesiana*	KM199313	KM199405	KM199509
P. portugalica	**CBS 393.48**	Portugal	—	KM199335	KM199422	KM199510
P. rhododendri	**IFRDCC 2399**	China	*Rhododendron sinogrande*	KC537804	KC537818	KC537811
P. rosea	**MFLUCC 12-0258**	China	*Pinus* sp.	JX399005	JX399036	JX399069
P. scoparia	**CBS 176.25**	—	*Chamaecyparis* sp.	KM199330	KM199393	KM199478
P. spathulata	**CBS 356.86**	Chile	*Gevuina avellana*	KM199338	KM199423	KM199513
P. telopeae	CBS 113606	Australia	*Telopea* sp.	KM199301	KM199469	KM199559
	CBS 114161	Australia	*Telopea* sp.	KM199296	KM199403	KM199500
P. trachicarpicola	**OP068**	China	*Trachycarpus fortunei*	JQ845947	JQ845945	JQ845946
	MFLUCC 12–0263	China	Unidentified tree	JX399000	JX399031	JX399064
P. unicolor	MFLUCC 12–0275	China	Unidentified tree	JX398998	JX399029	JX399063
Pestalotiopsis sp. (this study)	MAFF 241683	Japan	*Lilium speciosum*	LC184194	LC151288	LC151289
Pseudopestalotiopsis theae	**MFLUCC 12-0055**	Thailand	Camellia sinensis	JQ683727	JQ683711	JQ683743

ex-type and type strains are printed in bold.

Mycelial plugs were placed on five leaves with or without wounds and covered with plastic bags to maintain high humidity. These were kept for 4 d, and then the plastic bags and agar plugs were removed. The treated plants were then maintained for 1.5 months.

Results and Discussion

Disease symptoms

In red rose lilies, symptoms were initially observed as pale brown leaf spots. Over time, the leaf spots expanded across the yellow leaf margins (usually 7–10 mm diameter), with some black dots on the leaves (Fig. 1). These symptoms did not spread across the entire plant and did not induce mortality. In addition, symptoms were observed during August–October.

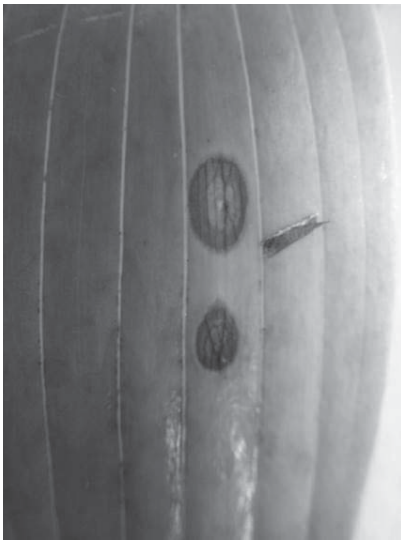

Figure 1: Brown leaf spot on rose red lily caused by *Pestalotiopsis* sp.

Identification of pathogen

Morphology: MAFF 241683 developed acervuli and conidia on leaf agar plates (Furukawa and Kishi 2002; Fig. 2). The conidia were comprised of five cells that were fusoid to ellipsoid in shape and 18.5–22.5 × 5.5–6.5 µm in size. In addition, MAFF 241683 was pale brown with concolorous median cells measuring 12.5–15 µm, and two or more of the cells usually had three apical appendages 8.5–16.5µm in length. These morphological features matched those of *Pestalotiopsis humus* and *P. papuana* (Table 2).

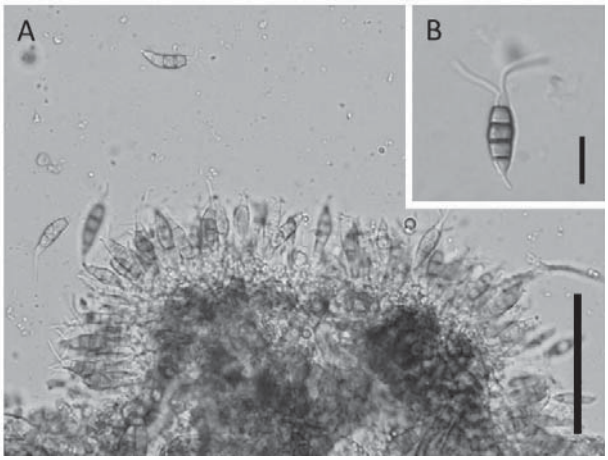

Figure 2: A. an acervulus on brown spot of a rose red lily leaf (bar = 50µm); B. conidium produced on a leaf agar (MAFF 241683) (bar = 10 µm)

Table 2. Comparison of conidial morphs between *Pestalotiopsis* sp. (from this study) and related species.

Species	Size (µm)	Median cells		Apical appendages	
		Length (µm)	Color	Length (µm)	Number
Pestalotiopsis sp. (this study)	18.5–22.5 × 5.5–6.5 (ave. 20.7 × 6.1)	12.5–15 (ave. 13.9)–	concolorous, pale brown	8.5–16.5 (ave. 12.6)	3
P. adusta[a)]	16–20 × 5–7 (ave. 18.7 × 6.2)	12.4–13.8 (ave. 13.2)	concolorous, olivaceous	7–15 (ave. 10)	2–3
P. humus[b)]	18.5–22 × 5–7 (ave. 20 × 6)	12–14 (ave. 12.8)	concolorous, brown	6.5–12 (ave. 9)	2–3
P. papuana[b)]	18–22 × 6–7.5 (ave. 20.5 × 6.7)	12–14 (ave. 12.8)	concolorous, brown	6.5–12 (ave. 9)	1–2

[a)] Maharachchikumbra et al. 2012.
[b)] Maharachchikumbra et al. 2014.

Molecular analysis: For molecular identification of MAFF 241683, phylogenetic analysis was performed based on the combined data sets of ITS (Accession no. LC184194), β-tubulin (Accession no. LC151288), and *tef1* (Accession no. LC151289) and those of 40 strains in database (Table 1). Total sequences comprised of 1,470 characters, including 556 characters of ITS sequences, 444 characters of β-tubulin sequences, and 470 characters of *tef1* sequences (including gaps). The ML, MP (35 trees: tree length = 1,268, consistency index = 0.512, retention index = 0.795, and composite index = 0.481), and NJ trees had similar topologies. The ML tree shown in Fig. 3 has bootstrap values greater than 70% at the nodes. The ML tree with the highest log-likelihood had a score of 8727.96. We revealed that MAFF 241683 belonged to *Pestalotiopsis*, which was supported by high bootstrap values (ML/MP/NJ: 100/99/100), and clearly separated from other species (Fig. 3).

Colony: Colonies grown for 6 days under light condition were on PDA were whitish mycelium on surface, and reverse of culture faint pink. Hypha grew at 4–30℃, with an optimum temperature of 25℃ (average 6.1 mm/day), on PDA plates (Figs. 4, 5). This colony was whitish to pale yellow, with dense, aerial mycelium on surface, with black, gregarious fruiting bodies; reverse of culture yellow to pale orange.

In summary, we identified MAFF 241683 as *Pestalotiopsis* sp., although it shared similarity with *P. humus* and *P. papuana* regarding conidial morphology. However, our phylogenetic analyses using ML, MP, and NJ trees distinguished MAFF 241683 from *P. humus* and *P. papuana* (Fig. 3).

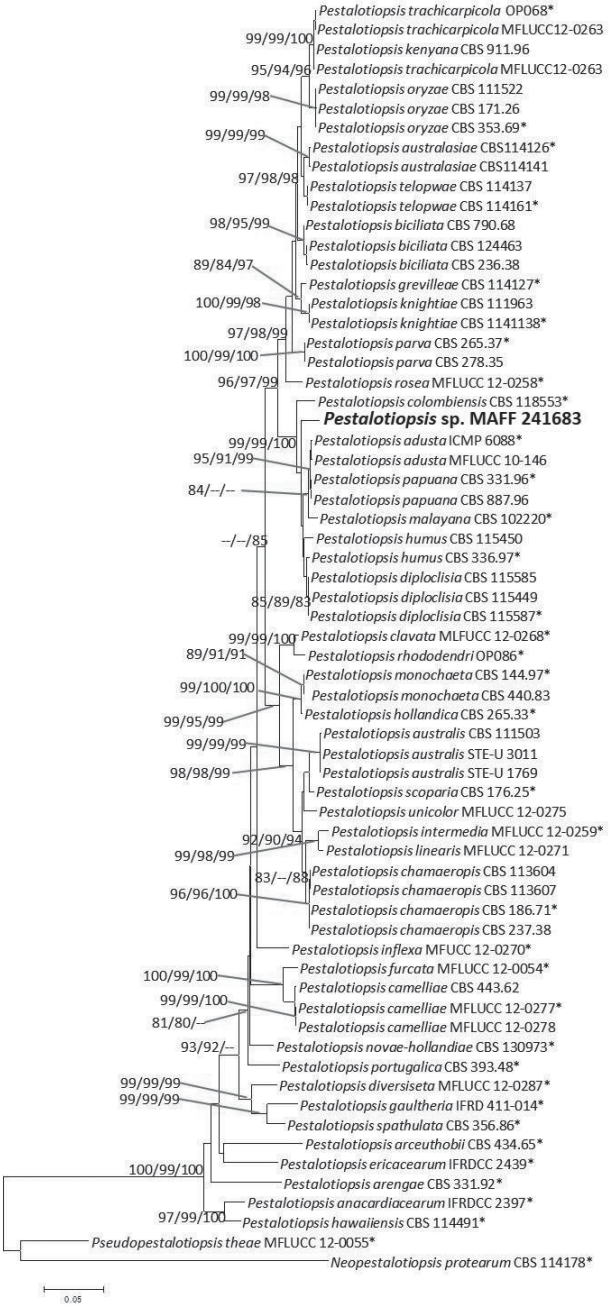

Figure 3: Phylogenetic tree of members of the genus *Pstalotiopsis* including *Neopestalotiopsis* and *Pseudopestalotiopsis* as out group, based on ML method using combine data set of ITS, β-tubulin and *tef1* sequences. BS values (1000 replications) are indicated at the nodes for ML/MP/NJ. A hyphen ('—') indicates bootstrap value is less than 70. An isolate in this study is shown in bold face. Asterisk "*" indicates ex-type or ex-epitype strains).

Pathogenicity test

Upon infection with MAFF 241683, symptoms appeared on all leaves (5/5) with lesions, albeit lessened (Fig. 6A). This strain was always isolated from these lesions. In control leaves with wound, the wounded parts turned brown but did not expand (Fig. 6B), and fungi were not

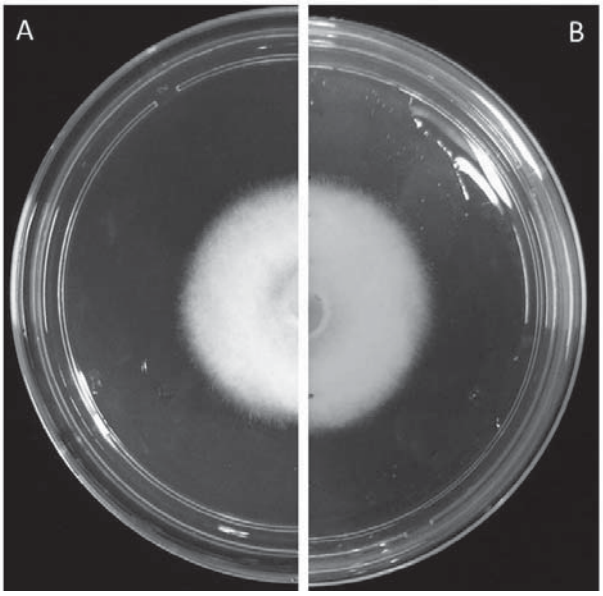

Figure 4: A colony of MAFF 241683 on PDA at 4 days after incubation at 25℃. A: from above, B: from below.

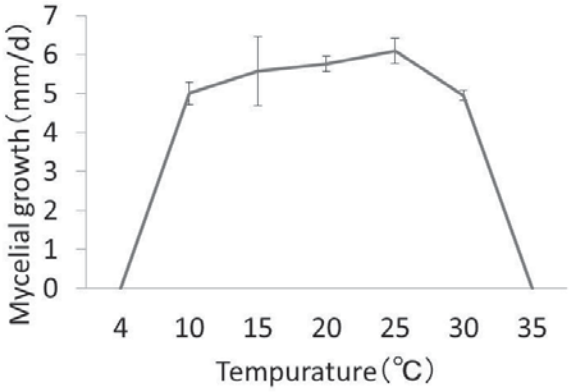

Figure 5: Growth rates at of colony various temperatures on PDA of MAFF 241683

isolated from these brown lesions. All leaves without wound remained healthy.

As aforementioned, molecular phylogenetic analyses determined that MAFF 241683 was distinct from *P. humus* and *P. papuana* (Fig. 3); however, we could not identify the species name and have reported MAFF 241683 as *Pestalotiopsis* sp. This strain had reduced pathogenicity as observed via milder symptoms in rose red lilies, and wounds were required to induce infection. MAFF 241683 had been reported as *P. adusta* which was isolated from

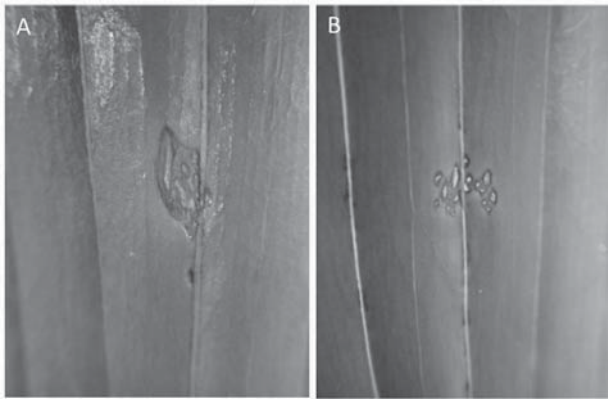

Figure 6: Pathogenicity test showing leaves inoculated with the isolate (A) and PDA as control (B)

lily (Watanabe et al. 2012), but the report does not include descriptions of the symptoms or pathogenicity tests following Koch's postulate. Furthermore, we show that this pathogen was misidentified in that study, because MAFF 241683 was clearly separated from *P. adusta* in phylogenetic tree (Fig. 3).

In conclusion, to our knowledge, this is a first report of brown leaf spot disease on a rose red lily (*Lilium speciosum* ver. *speciosum* L.) caused by *Pestalotiopsis* sp. formally. Furthermore, there is no record disease of Liliaceae caused by genus *Pestalotiopsis* in our knowledge. We propose to call this disease brown leaf spot, "Kasshoku-hanyou-byo" in Japanese.

References

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Furukawa T, Kishi K, 2002. Production of perithecia of various Ascomycotina on water agar medium emended with leaf pieces. *Journal of Phytopathology* **150**: 625–628.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied Environmental Microbiology* **61**: 1323–1330.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hasegawa M, Kishino H, Yano T, 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**: 160–174.

Maharachchikumbura SSN, Guo LD, Cai L, Chukeatirote E,

Wu WP, Sun X, Crous PW, Bhat DJ, McKenzie EHC, Bahkali AH, Hyde KD, 2012. A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species. *Fungal Diversity* **56**: 95–129.

Maharachchikumbura SSN, Hyde KD, Groenewald JZ, Xu J, Crous PW, 2014. *Pestalotiopsis* revisited. *Studies in Mycology* **79**: 121–186.

Miller PR, Weiss F, O'Brien MJ, 1960. Index of plant diseases in the United States. *U.S.D.A. Agriculture Handbook*, USA, Washington, D.C., pp 1–531.

Nozawa S, Yamaguchi K, Yen LTH, Hop DV, Phay N, Ando K, Watanabe K, 2017. Identification of two new species and a sexual morph from the genus *Pseudopestalotiopsis*. *Mycoscience*.

Rehner SA, Buckley E, 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1–a sequences. *Mycologia* **97**: 84–98.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731–2739.

Watanabe K, Nakazono T, Ono Y, 2012. Morphology evolution and molecular phylogeny of *Pestalotiopsis* (Coelomycetes) based on ITS2 secondary structure. *Mycoscience* **53**: 227–237.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols - A Guide to Methods and Applications*. San Diego, USA: Academic Press, pp 315–322.