RESEARCH NOTE

Unreported Post-harvest Disease of Apples Caused by *Plenodomus collinsoniae* in Korea

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ABSTRACT

This study was conducted to isolate and identify the fungal pathogen caused unreported post-harvest disease on apples (cv. Fuji) fruit in Korea. The disease symptoms on apples appeared as irregular, light to dark brown, slightly sunken spots. The three fungal strains were isolated from infected tissues of apple fruits and their cultural and morphological characteristics were completely consistent with those of *Plenodomus collinsoniae*. The phylogenetic analysis using the internal transcribed spacer (ITS) regions, beta-tubulin (*TUB*), and the second largest subunit of RNA polymerase II (*RPB2*) sequences revealed the closest relationship of the isolates with *Plenodomus collinsoniae* at the species level. The pathogenicity test showed the same dark brown spots on Fuji apple cultivar. Therefore, *P. collinsoniae* is a newly reported fungal agent causing post-harvest disease on apples in Korea.

Keywords: Apple, genus *Plenodomus,* Pathogenicity test, Phylogeny

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Apple (*Malus domestica*) is a commercially important and widely grown crop in the temperate regions of the world [1]. It is one of the world's leading fruits, with an estimated production of 89,329,179 tons [2]. Each country and region has local own cultivars and some cultivars are familiar all over the world [3]. However, post-harvest diseases of fruits cause heavy losses during storage resulting in considerable economic losses. To date, approximately 40 pathogens were identified as the causative agents of apple diseases [4], among them apple blotch, anthracnose, white rot, *Alternaria* leaf spot, and bacterial shoot blight have serious economic implication for apple cultivation [5]. Several causal agents of various diseases have been identified on apple fruits such as *Colletotrichum* spp. [6], *Botryosphaeria dothidea* [7], *Marssonina coronaria* [8], and *Fusarium decemcellulare* [9]. Recently, the abnormal fruit rot symptoms were observed during the screening of post-harvest diseases on apple fruits, which were collected from orchard located in Gunwi (36°16'27.1"N 128°28'17.6"E), Korea and stored under low-temperature conditions. In the present study, the isolated fungal strains are described and illustrated as a causal agent of newly recorded post-harvest disease on apples in Korea.

The symptoms of fruit rot disease were initially brown, later dark brown to dark red spots, irregular in shape (Fig. 1A-D), which were clearly differentiated from the typical symptoms of apple anthracnose, white rot, or *Fusarium* fruit rot. To isolate the causal agent from the abnormal spots, the surface of an apple was wiped with 70% ethanol and the diseased peel was removed using a sterilized blade. Then, the surface of the collected diseased tissues was sterilized for 30 seconds in 70% ethanol and 1% sodium hypochlorite and washed three times with sterilized double-distilled water. The surface-sterilized tissues were transferred onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates and maintained in an incubator at 25℃ [10]. As the result, the three strains were isolated from the diseased apples and then designated as KNU-20-A1, KNU-20-A2, and KNU-20-A3. To analyze the cultural characters, PDA, oatmeal agar (OA; Difco, Detroit, MI, USA) and autoclaved pine needle on 2% water agar (PNA) were prepared for the detail description of the isolated fungal strains [11]. After 10 days of incubation on PDA at 25℃, the colonies were 23-25 mm in diameter, gray to dark gray with white margin, circular to irregular with yellow pigmented halo around the colonies, reverse brown to dark brown with white margin (Fig. 1E and F). On OA, the colonies were 36-38 mm in diameter, white to gray in center, floccose, reverse brown with yellow margin (Fig. 1G and H). After 4 weeks of incubation, pycnidia-like conidiomata structures were appeared on OA and PNA, round to irregular, solitary to aggregate, and dark brown to black, and with the diam. of 600-750 μm (Fig. 1M and N). However, no conidial structures were observed. The strains also produced wide hyphae, branched mycelium, septate, smooth, brown, and light brown chlamydospores on PDA (Fig. 1O and P). The cultural and morphological characteristics were found to be similar with those of previously identified *Plenodomus collinosinae* (Table 1) [12]. The type strain of *Leptosphaeria collinsoniae* (=*Plenodomus collinsoniae*) has been reported as a sexual morph on the host called *Collinsonia canadensis*, and after that the strain was also combined with the strain *P. collinsoniae* CBS 120227 isolated from *Vitis coignetiae*, with the Perithecia; scattered, gradually blackening the stems, covered by the cuticle, finally bare, globose-conic, rugose, papillate, Asci; terete, short-stipitate, Sporidia; amber colored, biseriate, 5-8-septate, mostly 6-nucleate, and the asexual morph was not determined [13].

For molecular identification, the total genomic DNA was extracted from the three above-mentioned strains using a HiGene Genomic DNA prep kit (BIOFACT, Daejeon, Korea) following the manufacturer's instructions. The ITS regions, beta-tubulin (*TUB*), and the second largest subunit of RNA polymerase II (*RPB2*) genes were amplified using the primer sets ITS1F/ITS4 [14,15], Btub2Fd/Btub4Rd [16], and RPB2-5F2/fRPB2-7cR [17], respectively. Amplified PCR products were purified with EXOSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Macrogen Co., Ltd. (Daejeon, Korea). From the sequence analysis, sequences from the strains KNU-20-A1 (525, 344, and 1062 bp), KNU-20-A2 (567, 346, and 1046 bp), and KNU-20-A3 (553, 328, and 1035 bp) were obtained from the ITS regions, TUB, and RPB2 genes, accordingly. Comparative sequence analyses of the molecular markers of the three strains revealed high similarities of 99.8-100%, indicating their affiliation to the same species. A BLAST search of the NCBI database using sequences of ITS regions revealed the highest similarity of strains KNU-20-A1, KNU-20-A2, and KNU-20-A3 with *Plenodomus collinsoniae* CBS 120227 (99.6%). And the

closest species *P. influorescens* CBS 143.84 and *P. visci* CPC 35316 showed maximum 92.1-92.5% and 90.4%, respectively from the ITS regions. The partial sequences of *RPB2* gene of the three strains shared maximum 98.7% identity with that of *P. collinsoniae* CBS 120227, and only 89.4% and 90.7% similarities with the other closest relative, *P. influorescens* CBS 143.84 and *P. visci* CBS 122783, respectively. Based on the *TUB* gene sequence the three above-mentioned strains were close to *P. collinsoniae* CBS 120227 (97.9-98.2%), *P. influorescens* CBS 143.84 (85.4-86.1%), and *P. visci* CBS 122783 (86.8-87.5%). Using the three molecular markers, ITS regions, *RPB*2 and *TUB* genes, the closest neighbor of the three isolated strains were determined to be *P. collinsoniae* with the high values of the sequence similarities of 97.9-99.6% while no difference in sequences containing more or less base pairs between the markers from the isolated fungal strains. To confirm the relationship of the above-mentioned strains with *P. collinsoniae* at the species level, phylogenetic analysis using concatenated sequences of the ITS regions, *TUB* and *RPB*2 genes were performed. The sequences of allied species were retrieved from the National Center for Biotechnology Information (NCBI) (Table 2). Phylogenetic trees were constructed using neighbor-joining (NJ) [18], maximum-likelihood (ML) [19], and maximum-parsimony (MP) [20] methods, as implemented in MEGA7.0 [21]. The alignments were performed for each gene, and then the sequences were merged by using MEGA7.0 software program. The NJ analysis was performed using Kimura two-parameter distances [22] with gaps excluded from the analysis. A bootstrap analysis with 1000 replicate was performed to assess the support for clusters. In the phylogenetic tree (Fig. 2) all isolated strains occupied a position within the genus *Plenodomus* and clustered together with *P. collinsoniae*, indicating their closest relationship at the species level. Thus, strains KNU-20-A1, KNU-20-A2, and KNU-20-A3 were identified as *P. collinosinae* based on multi-locus phylogenetic analysis along with their cultural and morphological characteristics, which were completely consistent with those previously reported for this fungal species [12,13].

To confirm the pathogenicity of *P. collinosinae* isolated in this study, KNU-20-A1 was selected as the representative from the three strains and inoculated into healthy apples (cv. Fuji) fruits with three replications. The inoculum was prepared using strain KNU-20-A1 cultured for 4 weeks on PDA. The surface of a healthy apple was wiped with 70% EtOH and then air-dried. Two points of apple were wounded using a sterilized needle and colony agar blocks were attached and sealed using foil. Apple fruits inoculated with sterilized water were used as the control. All the inoculated fruits were incubated at 25℃, and after 3 days the colony agar blocks were removed. After 14 days, brown with slightly sunken spots were observed on apples which were identical to typical primary symptoms (Fig. 1I-L) while no symptoms were observed in unwounded fruits (data not shown). From each of the inoculated fruits *P. collinosinae* was re-isolated and the cultural and morphological characteristics were compared with those of the inoculated strains, and all characteristics were found to be the same (Table 1).

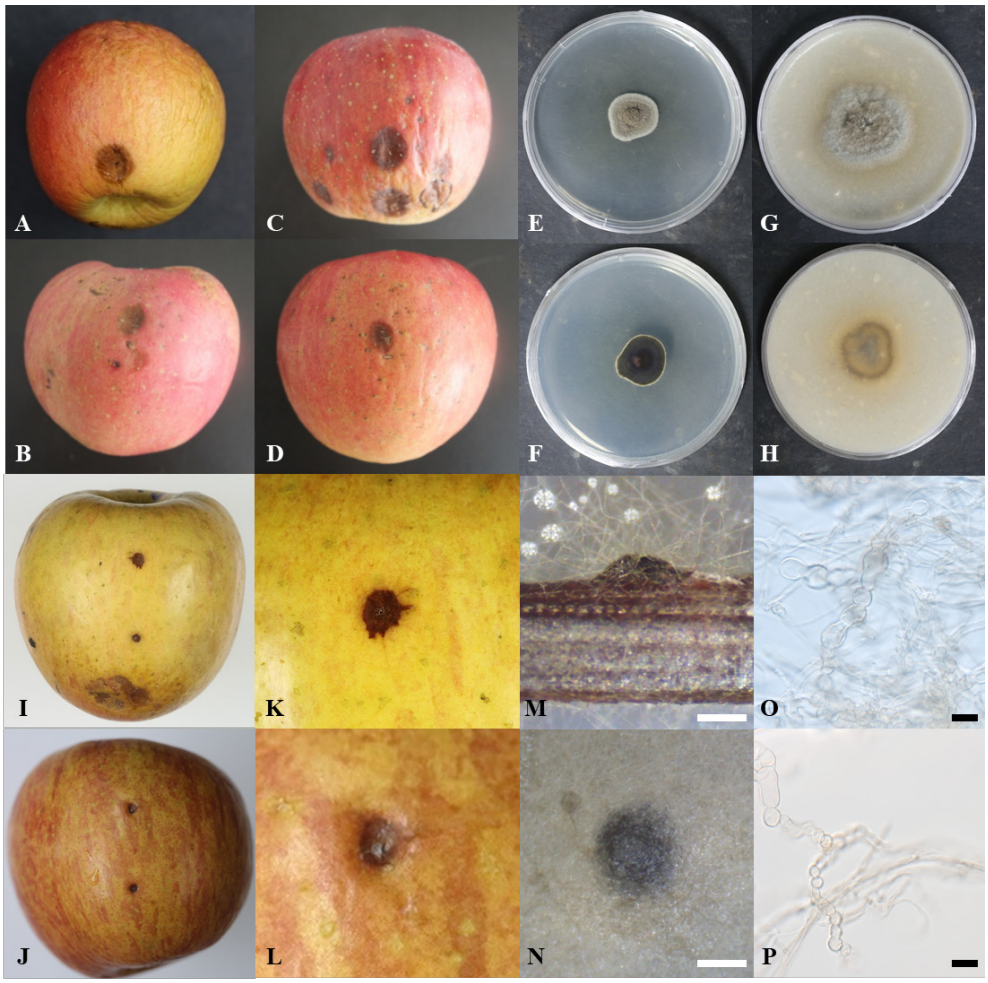

Fig. 1. Disease symptoms, cultural and morphological characteristics, and pathogenicity test on apples (cv. Fuji) with inoculation using colony agar blocks of *Plenodomus collinsoniae* KNU-20-A1. (A-D): Primary symptoms; (E, F): Colony of strain KNU-20-A1 on potato dextrose agar (PDA) after 10 days; (G,H): Colony of strain KNU-20-A1 on oatmeal agar (OA) after 10 days; (I, J): Inoculation after 14 days; (K, L): Enlarged picture; M: After 30 days, pycnidia-like structures on PNA (pine needle agar); (N): After 30 days, pycnidia-like structures on OA agar; (O, P): Hyphal structures of *P. collinsoniae* KNU-20-A1 (scale bars: M, N=500 μm; O, P=10 μm).

Table 1. Morphological characteristics of the strain KNU-20-A1 with reference to *P. collinsoniae*.

Characteristics	*P. collinsoniae* KNU-20-A1[a]	*P. collinsoniae* KNU-AP100C[b]
Colony	Gray to dark gray with white margin, circular to irregular with yellow pigmented halo around the colonies, reverse brown to dark brown with white margin on PDA. On OA, the colonies were white to gray in center, floccose.	White to light gray colonies in the center with a circular margin and reverse orange to light black color in the center with a wide white margin on PDA. On OA, the colonies were initially white to gray becoming brown in the center.
Hyphae	Produced wide hyphae, branched mycelium, septate, smooth, brown, and light brown chlamydospores on PDA.	Wide, branched mycelium, septate, smooth, brown, and light brown chlamydospores were found on PDA.
Conidiomata	Pycnidia-like conidiomata were appeared on OA and PNA after 4 weeks of incubation at 25°C.	Numerous conidiomata produced on PDA after 4 weeks of incubation at 25°C.
Conidia	No conidial structures were observed after 4 weeks of incubation at 25°C.	Conidial structures were not observed after 4 weeks of incubation at 25°C.

PDA: Potato dextrose agar; PNA: Pine needle agar; OA: Oatmeal agar.

[a] Fungal strain studied in this paper, [b] Source of the description [12].

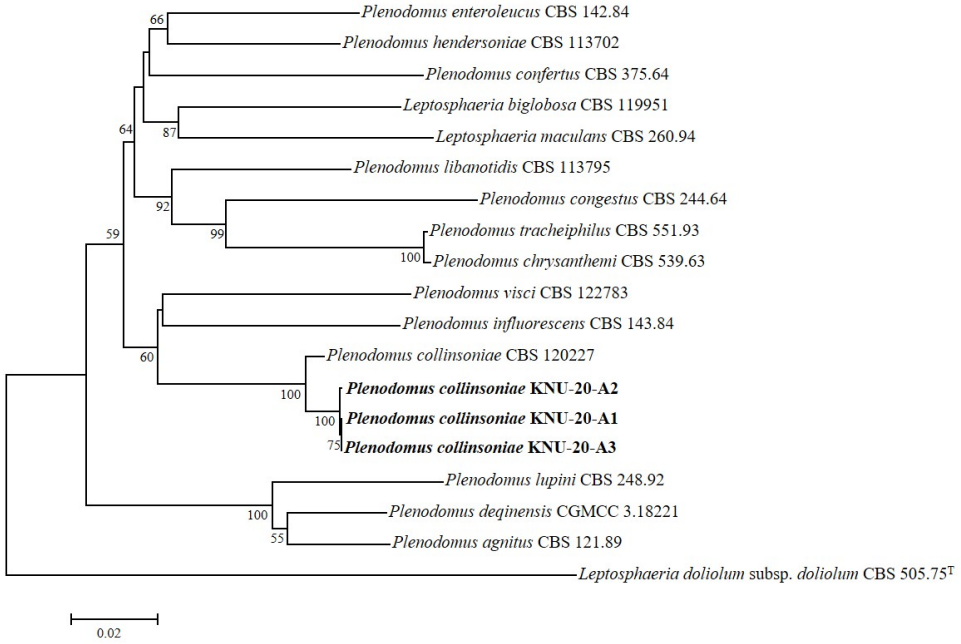

Fig. 2. Neighbor-joining phylogenetic tree based on the concatenated sequences of the internal transcribed spacer (ITS) regions, beta-tubulin (*TUB*), and RNA polymerase II (*RPB*2) genes showing the phylogenetic position of the three isolated strains (KNU-20-A1, KNU-20-A2, KNU-20-A3) among *Plenodomus* species and other closely related taxa. Bootstrap values (based on 1000 replications) greater than 50% are shown at branch points. The isolated strains are shown in bold. Bar, 0.02 substitutions per nucleotide position.

Table 2. List of species used in phylogenetic analyses with the GenBank accession numbers.

Species	Strain numbers	Hosts/habitats	GenBank accession numbers		
			ITS	*RPB*2	*TUB*
Plenodomus agnitus	CBS 121.89	*Eupatorium* sp.	JF740194	KY064036	KY064053
P. chrysanthemi	CBS 539.63	*Chrysanthemum* sp.	JF740253	KY064038	KY064055
P. collinsoniae	CBS 120227	*Vitis coignetiae*	JF740200	KY064039	KY064056
P. confertus	CBS 375.64	*Anacyclus radiatus*	AF439459	KY064040	KY064057
P. congestus	CBS 244.64	*Erigeron canadensis*	AF439460	KY064041	KY064058
P. deqinensis	CGMCC 3.18221	Soil	KY064027	KY064034	KY064052
P. enteroleucus	CBS 142.84	*Catalpa bignonioides*	JF740214	KY064042	KT266266
P. hendersoniae	CBS 113702	*Salix cinerea*	JF740225	KY064044	KT266271
P. influorescens	CBS 143.84	*Fraxinus excelsior*	JF740228	KY064045	KT266267
P. libanotidis	CBS 113795	*Seseli libanotis*	JF740231	KY064046	KY064059
P. lupini	CBS 248.92	*Lupinus mutabilis*	JF740236	KY064048	KY064061
P. tracheiphilus	CBS 551.93	*Citrus limonia*	JF740249	KY064049	KT266269
P. visci	CBS 122783	*Viscum album*	JF740256	KY064050	KY064063
Leptosphaeria biglobosa	CBS 119951	*Brassica rapa*	JF740198	KY064037	KY064054
L. doliolum subsp. *doliolum*	CBS 505.75T	*Urtica dioica*	NR155309	KY064035	JF740144
L. maculans	CBS 260.94	*Brassica oleracea*	MH862462	KY064047	KY064060
P. collinsoniae	**KNU-20-A1**	***Malus domestica***	**LC591836**	**LC591841**	**LC591846**
P. collinsoniae	**KNU-20-A2**	***Malus domestica***	**LC591837**	**LC591842**	**LC591847**
P. collinsoniae	**KNU-20-A3**	***Malus domestica***	**LC591838**	**LC591843**	**LC591848**

ITS: internal transcribed spacer; *RPB*2: RNA polymerase II; *TUB*: beta-tubulin.

The newly generated sequences indicated in bold.

In a previous study, the members of the *Plenodomus* seem to be cosmopolitan in distribution, since they have been recorded from both temperate and tropical countries (i.e. China, Greece, France, Japan, Netherlands, Peru, Spain, Taiwan) [23]. Until recent, 100 epithets of *Plenodomus* have been listed in the Index Fungorum database [24]. The host specificity of *Plenodomus* has not yet been clarified based on species from different plant families (Asteraceae, Lamiaceae, Liliaceae) [13]. *P. meliloti* was a low-temperature parasitic fungus found only in the provinces of Alberta and Saskatchewan in Canada [25], whereas, *P. morganjonesii* was obtained from partially degraded leaves from New Jersey [26]. *P. chrysanthemi* was isolated as an endophyte of *Chenopodium album* that represents a new host from Iran [27]. The causal agent of foot rot and storage tuber rot on sweet potato was identified as *P. destruens* from experimental fields in China [28]. A novel species, *P. sinensis* was introduced from *Tamarindus indica* L. (Fabaceae) and *Plukenetia volubilis* L. (Euphorbiaceae) in Yunnan Province, China [29]. Moreover, Brown root rot, caused by the fungal pathogen *Phoma sclerotioides* (=*Plenodomus meliloti*), was associated with winterkill, slow emergence from winter dormancy, and yield loss of alfalfa (*Medicago sativa* L.), and also was a problem with severe winters in Alaska and Alberta, Saskatchewan and Manitoba, Canada [30]. Although many fungal species belonging to the genus *Plenodomus* were isolated in several countries from diversified hosts, only *P. destruens* was reported as causing agent of storage tuber rot of sweet potato in Korea [31], but there are no studies of *P. collinsoniae* related to plant diseases in Korea. Recently, there are two species of *Plenodomus*, namely *P. sinensis* and *P. collinsoniae* that were isolated from a soil sample collected in abandoned apple orchard in Gyeongsangbuk-do, Korea [12]. Even, there is no enough information that the genus *Plenodomus* can cause diseases in apples, but there might be a relation or transfer of causal agents from soil to apples trees or fruits as well as causing disease. Furthermore, the genus *Plenodomus* includes several well-known important plant pathogens and is found as opportunistic fungi on several hosts. In this study, the strains were isolated from the infected disease apples (cv. Fuji) fruit and identified the fungal pathogen caused post-harvest disease on apples in Korea.

Furthermore, based on the results of pathogenicity test, the disease symptoms appeared very slowly on the inoculated apple fruits, so it is assumed that it can be observed in the long-term stored period in the low-temperature storage room condition. Still, there were no reported apple diseases caused by *Plenodomus* species in Korea. According to the results of the present study, *P. collinsoniae* can be a new fungal agent of the post-harvest disease of apple, and the ecology of *P. collinsoniae* should be further studied for the proper control. In conclusion, this is the first report of post-harvest disease on apple caused by *P. collinsoniae* in Korea.

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