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# Real-Time PCR Detection of Dogwood Anthracnose Fungus in Historical Herbarium Specimens from Asia

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# Abstract

*Cornus* species (dogwoods) are popular ornamental trees and important understory plants in natural forests of northern hemisphere. Dogwood anthracnose, one of the major diseases affecting the native North American *Cornus* species, such as *C. florida*, is caused by the fungal pathogen *Discula destructiva*. The origin of this fungus is not known, but it is hypothesized that it was imported to North America with its host plants from Asia. In this study, a TaqMan real-time PCR assay was used to detect *D. destructiva* in dried herbarium and fresh *Cornus* samples. Several herbarium specimens from Japan and China were detected positive for *D. destructiva*, some of which were collected before the first report of the dogwood anthracnose in North America. Our findings further support that *D. destructiva* was introduced to North America from Asia where the fungus likely does not cause severe disease.

# Introduction

In North America, several native *Cornus* species, especially *Cornus florida* (flowering dogwood) and *C. nuttallii* (Pacific dogwood), have been plagued by the dogwood anthracnose fungus *Discula destructiva* Redlin since the 1970's [1]. The disease threatens the ecological integrity of forest ecosystems and has caused massive economic losses for the nursery industry [2]. *Cornus florida and C. nuttallii* are both widely distributed understory trees in natural forests of the northern hemisphere, which provide food for birds and nutrient recycling through leaf litter [3,4,5]. These two species are members of the big bract morphological group [6] and are closely related to *Cornus* species native to Eastern Asia, such as *C. kousa* (Japanese dogwood), which appears to have resistance to the disease. *Cornus florida* and *C. kousa* are also valued ornamentals. *Cornus florida* is one of the most popular landscape trees in the United States with \$30,901,000 in total sales for 2007 [7].

Dogwood anthracnose was first noted in the west coast of the United States on *C. nuttallii* in 1979 [ $\underline{8}$ ], and was soon after reported on the east coast [ $\underline{9}$ ]. Redlin [ $\underline{1}$ ] concluded that isolates



**Competing Interests:** The authors have declared that no competing interests exist.

from both the east and west coast were morphologically indistinguishable and named the causal agent as a new fungal species, *Discula destructiva*. Several other studies reported that *D. destructiva* is distinct from any other North American *Discula* species [10, 11, 12,13]. The disease symptoms include bract necrosis, leaf spot, leaf blight, twig dieback, and trunk canker, which usually start to develop in the spring and early summer. Infected trees in forest settings, where inoculum levels are higher due to shade and moisture, can be killed in as little as one to three years [14]. Since the first reports in the 1970's, the disease has quickly spread through native North American dogwood populations, from British Columbia to northern California on the west coast and from Vermont to Georgia and Alabama in the east [15]. Within the United States, *D. destructiva* has resulted in mortality rates as high as 89% in some forests [16,17]. The disease had not been reported outside North America until 2002 when *D. destructiva* was detected on *C. florida* in Germany, and also in Italy in 2003 and Switzerland in 2009 [18,19].

The origin of *D. destructiva* is unknown but it is hypothesized to be an introduced species, similar to the chestnut blight pathogen [15, 20]. The sudden appearance of the disease near the USA ports, the low genetic variation within the pathogen population [21], and the natural resistance of the native Asian dogwood species suggest that *D. destructiva* was introduced, likely from Asia. It is hypothesized that *D. destructiva* is an endophyte or latent pathogen and does not typically cause disease on its native host plant when the hosts are not under other biotic or abiotic stresses. However, no previous work has been done on testing the presence of *D. destructiva* from dogwoods in Asia.

The identification of *D. destructiva* based on the fungal culture morphology and disease symptoms is problematic [22]. This fungus grows slowly on culture media and is often outgrown by the fast growing fungi inhabiting the same host plant tissue. Therefore, a negative detection of *D. destructiva* based on culturing is not reliable due to the high likelihood of false negative results. Furthermore, *D. destructiva* does not sporulate readily on the conventional media, making morphological identification challenging [14]. Disease symptoms caused by *D. destructiva* are similar to those caused by other pathogens such as *Colletotrichum acutatum* [1, 23], making it difficult to accurately detect the pathogen by symptoms alone.

To facilitate the study on the origin and distribution of *D. destructiva*, a real-time PCR assay has been developed for fast and accurate detection of this fungus, bypassing the need for culturing [22]. Rapid and reliable detection via real-time PCR is a valuable tool for diagnosing, monitoring, and narrowing down the location of the origin of the species. This assay was applied to test fresh dogwood samples but had not been used to survey dried herbarium specimens [22]. Historical herbarium samples can provide invaluable information about the presence or absence of the fungal species from a long range of time, which allows us to test the hypothesis on the origin of the disease. The objectives of this study were to: (i) test the presence of *Discula destructiva* in the historical herbarium *Cornus* specimens collected from Asia, Europe and North America using real-time PCR assay; and (ii) test the presence of *Discula destructiva* in recently collected fresh dogwood samples from Japan and USA using both culturing and realtime PCR assay.

# **Materials and Methods**

#### Study sites and sampling of fresh Cornus species

Samples were collected in late May to early June of 2010 and 2012 from temperate deciduous forests in the United States and Japan. The field studies did not involve endangered or protected species and no specific permissions were required for these locations. Mature leaf samples of wild *C. florida* were collected from five trees (13–30 cm diam.; 5–10 m. in height) each

year at the Hutchinson Memorial Forest in Somerset, New Jersey, USA (40°30′01″N 74° 34′02″W), within the native range of *C. florida*. The forest is a nature preserve, one of the few uncut forests in New Jersey [24, 25]. One branch each from the north, west and east-facing sides of the tree was collected from the top of the canopy [26]. Samples were kept at 4°C until DNA isolation. Three leaves from each branch were randomly selected for fungal isolation within three days of sample collection. Following the same sampling strategy, leaf samples of wild *C. kousa* were collected from a natural forest in the Ibaraki Prefecture in Japan (36° 14′60″N 140°5′20″E), with a similar climate to the United States sampling site. Five trees were sampled and analyzed in each site, each year giving a total of 20 tree samples.

# Herbarium specimens

Seventy herbarium specimens of a variety of *Cornus* species collected from Canada, Mexico, USA, China, Japan, Korea, Nepal, France, and Russia during 1909–2011 were obtained from the New York Botanical Garden and the Harvard Herbarium (<u>Table 1</u>). Required permissions for the specimen sampling were obtained from Dr. Stella Sylva of the New York Botanical Garden and Dr. Michaela Schumll from the Harvard Herbarium.

# Plant DNA extraction, real-time PCR and sequencing

For each sample, 50 mg leaf tissue was ground in liquid nitrogen. Genomic DNA was then isolated using Qiagen DNeasy Plant Mini kit (Qiagen, Germany) following the manufactures protocol (Tables 1 and 2). All real-time PCR reactions were performed on the StepOnePlus realtime PCR system (Applied Biosystems, CA, USA) following the procedures described in our previously published paper [22]. Primers used for the detection of *D. destructiva* were DdITS F1 and DdITS R1, along with the probe DdITS Probe1 [22]. The following conditions were used to carry out the real-time PCR reaction: 3 min of 95°C, followed by 45 cycles of 15 s at 95°C, and 40 s at 60°C. Each reaction consisted of 10 µl iTaq Supermix with ROX (Bio-Rad, CA, USA), 250 nM probe, 500 nM of each primer, and 4 µl template DNA for a total volume of 20 µl. A standard curve was also constructed using genomic DNA from D. destructiva isolate MD235, which is a type culture of this species [1]. A Ct value of less than 32 was counted as positive detection of *D. destructiva*. Each sample was tested in triplicate. The real-time PCR products were further subjected to purification using QIAquick PCR purification kit (Qiagen, CA), following the manufacturers protocol. The purified amplicons were sequenced by Gene-Wiz, Inc. (South Plainfield, NJ, USA) with primers DdITS\_F1 and DdITS\_R1 to confirm the identity of the amplified sequences.

# Fungal isolation and morphological identification

For the fresh *Cornus* leaf samples collected from USA and Japan, three leaves from each branch sampled from each tree were cut along various sections of the leaf (lamina tip, lamina margin to midrib, and lamina base) into multiple 0.5 cm segments that were surface-sterilized through sequential immersion in 0.5% sodium hypochlorite, 70% (v/v) ethanol, and rinsed three times in sterilized distilled water [27, 28]. Leaf segments were air dried and placed on Petri dishes containing 2% acidified MEA (AMEA). One liter AMEA contained 20 g malt extract (BD Bio-sciences, Sparks, MD), 20 g agar (BD Biosciences, Sparks, MD) and 1 ml of 85% lactic acid (Sigma-Aldrich, St. Louis, MO, USA). Five leaf segments from each sample were placed on a control AMEA plate for 30 seconds and then removed [27, 28, 29], which were used to monitor for epiphytic fungal growth. Petri dishes were incubated for six months under room temperature (22–24°C).



9C. alosJin Province, China197930 (0.86)138C. alosChina190934.0 (0.80)1342C. alosMontiss Kerlei, Flussia12936.0 (0.65)1426C. anorumCayuga Lake, NY194734.2 (0.65)147C. brachypociaNipen, Japan195131.2 (0.46)147C. brachypociaNipen, Japan195135.87 (1.02)158C. canodorsisProf. Varanashi, Japan1952-140C. canodorsisProf. Yamanshi, Japan1952-141C. canodorsisProf. Yamanshi, Japan194238.8 (0.20)142C. capitataVunan, China194238.1 (0.30)143C. capitataYunan, China194238.1 (0.30)144C. controversaJangi, China194238.1 (0.30)145C. controversaJangi, China194335.3 (1.70)148C. controversaJangi, China1953-149C. controversaJangi, China1953-149C. controversaJangi, China1953-150C. fordiaMunshi, Japan1953-161C. fordiaMuseisnu, USA19633.5 (0.6)17C. fordiaMunshi, Japan1963-184C. controversaMunshi, Japan1963-185C. fordiaMuseisnu, Japan1984-184C. fordiaMung'alan, China1984- <th>Sample</th> <th>Plant Species</th> <th>Location</th> <th>Year</th> <th>qPCR mean Ct value (Standard deviation)*</th>	Sample	Plant Species	Location	Year	qPCR mean Ct value (Standard deviation)*
H38C. akbaChina190334.0 (0.80)H42C. akbaMorise Kenniel, Fussia192936.0 (0.65)H26C. amomumCayuga Lako, NY194734.7 (0.65)H6C. brachypoodNippen, Japan195131.2 (0.49)H7C. brachypoodNippen, Japan195135.8 / (1.02)H8C. brachypoodNippon, Japan1952-H4C. canadensisProf. Winsu, Japan1952-H4C. caplataC. Napal, Nepal1972-L7C. caplataYunnan, China194238.0 (2.07)H2C. caplataYunnan, China194238.1 (3.03)H24C. caplataYunnan, China194238.1 (3.03)H24C. chinesisOmel-insin, China193835.3 (1.70)H18C. controversaMitobi, Japan1952-H19C. controversaJangu, China1953-H19C. controversaMitobi, Japan1953-H20C. controversaMitobi, Japan1953-H21C. controversaMitobi, Japan1953-H21C. controversaMitobi, Japan1953-H21C. controversaMitobi, Japan1953-H21C. controversaMitobi, Japan1953-H21C. controversaMitobi, Japan1963-H22C. floridaHuonis, Japan1964-H33C. flori	H9	C. alba	Jilin Province, China	1997	38.0 (0.86)
142C. atomMontes Kentes, Runsia192936.0 (0.65)146C. arnouruCayaga Lake, NY194734.7 (0.65)147C. brachypodaHondo, Japan195135.8 (1.62)148C. caradensisProv. Mutsu, Japan195135.8 (1.62)149C. caradensisProv. Mutsu, Japan1952-141C. caradensisProv. Mutsu, Japan1952-142C. caradensisYuman, China194638.2 (2.57)141C. capitataYuman, China194238.1 (0.30)142C. capitataYuman, China194836.3 (1.70)143C. cantoresaOmel-heine, China194836.3 (1.70)144C. controversaOmel-heine, China194836.1 (0.32)145C. controversaMutsu, Japan1953-147C. controversaMutsu, Japan1953-148C. controversaMutopik, Japan1953-149C. controversaMutopik, Japan1953-149C. controversaMutopik, Japan1953-141C. controversaMutopik, Japan1953-142C. controversaMutopik, Japan1953-143C. fordidHuang-shan, China1979-144C. controversaMutopik, Japan198436.0 (0.7)155C. fordidHuang-shan, China1984-156C. fordidHuang-shan, China <t< td=""><td>H38</td><td>C. alba</td><td>China</td><td>1909</td><td>34.0 (0.80)</td></t<>	H38	C. alba	China	1909	34.0 (0.80)
126     C. amachypoola     Nippen, Japan     1951     31.2 (0.46)       186     C. brachypoola     Nippen, Japan     1949     31.9 (0.62)       18     C. brachypoola     Nippen, Japan     1951     35.67 (1.02)       184     C. canadensis     Prov. Mulsu, Japan     1952     -       184     C. canadensis     Prov. Mulsu, Japan     1952     -       184     C. capitata     Yunan, China     1946     38.2 (2.25)       181     C. capitata     Yunan, China     1948     35.3 (1.70)       182     C. chinesis     Mt. Omei, China     1948     35.3 (1.70)       182     C. controversa     Mublik, Japan     1952     -       193     C. controversa     Japan, China     1953     -       1941     C. controversa     Japan     1953     -       1942     C. controversa     Misaisapi, USA     1953     -       1943     C. controversa     Misaisapi, USA     1953     -       1944     C. controversa     Misaisapi, USA     1953	H42	C. alba	Montes Kentei, Russia	1929	36.0 (0.65)
HetC. brachypodaNippen, Japan195131.2 (0.46)187C. brachypodaNippon, Japan195135.87 (1.02)18C. caradensisProv. Mutsu, Japan195236.07 (1.02)18C. caradensisProv. Mutsu, Japan195236.07 (1.02)18C. caradensisProv. Mutsu, Japan195236.02 (2.01)18C. caradensisProv. Mutsu, Japan195238.6 (2.34)18C. capitataVunan, China194638.6 (2.34)18C. capitataYunan, China194235.1 (3.70)18C. chinesisOmel-hein, China194235.1 (3.70)184C. chinesisOmel-hein, China194236.1 (3.70)184C. controversaOhahnizu, Japan1953-184C. controversaNanobici, Japan1953-184C. controversaNunobici, Japan1953-184C. controversaNunobici, Japan1953-185C. fordaMarasi, Japan1953-184C. controversaMissispi, USA197135.0 (0.54)185C. fordaMissispi, USA197135.0 (0.57)184C. fordaHuang-shan, China1979-185C. fordaNi Honshu, Japan1981-186C. fordaNi Honshu, Japan1981-187C. fordaNi Honshu, Japan1981-188C. fordaYamanashi Pref, Japan </td <td>H26</td> <td>C. amomum</td> <td>Cayuga Lake, NY</td> <td>1947</td> <td>34.7 (0.65)</td>	H26	C. amomum	Cayuga Lake, NY	1947	34.7 (0.65)
H7C. brachypodaHondo, Japan194931.9 (0.62)48C. brachypodaNippon, Japan1951.35.87 (1.02)43C. caradersisProf. Vannanshi, Japan1952.44C. caradersisProf. Vannanshi, Japan1959.80.0 (2.07)47C. cagibitaC. honpal, Nepal1972.48C. cagibitaYunnan, China1946.38.2 (2.25)411C. capibitaYunnan, China1942.38.1 (0.30)424C. chinesisOmet-hsien, China1942.38.1 (0.30)424C. chinesisMt. Ornei, China1952.418C. controversaJiangxi, China1952.419C. controversaJiangxi, China1953.420C. controversaAtma, Japan1953.421C. controversaMtmanit, Japan1953.422C. controversaMtmanit, Japan1955.436C. floridaMtseissippi, USA1955.457C. floridaNt, USA1951.458C. floridaNt, USA1951.459C. floridaNt, USA1951.450C. floridaNt, Horshu, Japan1984.451C. floridaNt, Horshu, Japan1984.452C. floridaNt, Horshu, Japan1984.453C. floridaNt, Horshu, Japan1984.454C. floridaN	H6	C. brachypoda	Nippen, Japan	1951	31.2 (0.46)
H8     C. banchypooda     Nippon, Japan     1951     35.87 (1.02)       H3     C. canadensis     Prot. Vatau, Japan     1952     .       H2     C. capitata     C. Napal, Nepsil     1972     .       H2     C. capitata     Yunnan, China     1946     38.2 (2.5)       H11     C. capitata     Yunnan, China     1942     38.6 (2.34)       H22     C. chinasis     Morei, China     1942     38.6 (2.34)       H23     C. chinasis     Morei, China     1942     38.6 (2.34)       H24     C. chinasis     Morei, China     1938     35.3 (1.70)       H16     C. controversa     Obshinizu, Japan     1952     .       H24     C. controversa     Minobik, Japan     1953     .       H24     C. controversa     Minobik, Japan     1953     .       H25     C. controversa     Minobik, Japan     1955     .       H26     C. controversa     Minobik, Japan     1955     .     .       H26     C. controversa     Minobik, Japan <t< td=""><td>H7</td><td>C. brachypoda</td><td>Hondo, Japan</td><td>1949</td><td>31.9 (0.62)</td></t<>	H7	C. brachypoda	Hondo, Japan	1949	31.9 (0.62)
H3     C. canaclerisis     Prov. Musu, Japan     1952     .       H4     C. canaclerisis     Pref. Yamanashi, Japan     1950     38.0 (2.07)       H4     C. capitata     C. Napal, Napal     1972     .       H10     C. capitata     Yunnan, China     1946     38.2 (2.25)       H23     C. chinesis     Omei-heien, China     1942     38.1 (0.30)       H24     C. chinesis     Mt. Omei, China     1942     38.1 (0.30)       H24     C. chinesis     Mt. Omei, China     1952     .       H18     C. controversa     Jalagxi, China     1952     .       C. controversa     Jalagxi, China     1953     .     .       H20     C. controversa     Mtmajapan     1953     .     .       H21     C. controversa     Mtmajapan     1955     .     .       H21     C. controversa     Mtmajapan     1955     .     .       H22     C. controversa     Mtomajapan     1951     .     .       H24     C. controversa	H8	C. brachypoda	Nippon, Japan	1951	35.87 (1.02)
44     C. canodensis     Pref. Yamanashi, Japan     1959     38.0 (2.07)       122     C. capitata     C. Napal, Nepal     1972     -       110     C. capitata     Yunnan, China     1946     38.2 (2.25)       111     C. capitata     Yunnan, China     1938     38.1 (0.30)       123     C. chinesis     Mt. Omel, China     1938     35.3 (1.70)       124     C. chinesis     Mt. Omel, China     1938     35.3 (1.70)       124     C. controversa     Jiangxi, China     1955     41.1 (0.32)       120     C. controversa     Minoki, Japan     1953     -       124     C. controversa     Minoki, Japan     1953     -       125     C. controversa     Minoki, Japan     1953     -       124     C. controversa     Minoki, Japan     1953     -       124     C. controversa     Minoki, Japan     1953     -       125     C. controversa     Minoki, Japan     1957     -       126     C. controversa     Minoki, Japan     1957 <td>H3</td> <td>C. canadensis</td> <td>Prov. Mutsu, Japan</td> <td>1952</td> <td>-</td>	H3	C. canadensis	Prov. Mutsu, Japan	1952	-
12     C. Rapitalia     C. Napala, Nepal     1972     -       110     C. capitala     Yunnan, China     1946     38.2 (2.25)       111     C. capitala     Yunnan, China     1942     38.1 (0.30)       123     C. chinesis     Omel-hsien, China     1942     35.3 (1.70)       124     C. controversa     Mich Omel, China     1983     35.3 (1.70)       124     C. controversa     Ohshimizu, Japan     1952     -       118     C. controversa     Jiangxi, China     1953     -       120     C. controversa     Mirna, Japan     1953     -       121     C. controversa     Mirna, Japan     1955     -       1424     C. controversa     Mira, Japan     1955     -       1425     C. fordra     Mississipi, USA     1971     2.6.4 (0.07)       156     C. fordra     Mississipi, USA     1975     -     -       1426     C. fordra     Mississipi, USA     1979     -     -       1430     C. fourda     NH (OSA)	H4	C. canadensis	Pref. Yamanashi, Japan	1959	38.0 (2.07)
H0     C. capitala     Yunnan, China     1946     38.2 (2.25)       H11     C. capitala     Yunnan, China     2000     38.6 (2.34)       H11     C. capitala     Yunnan, China     1932     38.1 (0.30)       H24     C. chinesis     Mt. Omel, China     1938     35.3 (1.70)       H24     C. chinesis     Mt. Omel, China     1938     35.3 (1.70)       H18     C. controversa     Ohshinizu, Japan     1952     -       H20     C. controversa     Munobiki, Japan     1953     -       H21     C. controversa     Munobiki, Japan     1955     -       H22     C. controversa     Munobiki, Japan     1955     -       H24     C. florida     Mt. Namai, Japan     1955     -     -       H24     C. florida     Msissispi, USA     1955     35.5 (0.63)     -       H25     C. florida     Juagsian, China     1971     -     -       H25     C. florida     Munoshi, Japan     1987     -     -       H26     C. flo	H2	C. capitata	C. Napal, Nepal	1972	-
H1     C. cpiltata     Yunnan, China     2000     38.6 (2.34)       H23     C. chinesis     Omei-hsien, China     1942     38.1 (0.30)       H24     C. chinesis     Mt. Omei, China     1943     38.3 (1.70)       H16     C. controversa     Ohshimizu, Japan     1956     31.6 (0.10)       H18     C. controversa     Jangxi, China     1975     41.1 (0.32)       H20     C. controversa     Nunobiki, Japan     1953     -       H21     C. controversa     Mina, Japan     1955     -       H22     C. controversa     Mi. Namari, Japan     1955     -       H48     C. florida     Mississippi, USA     1955     -       H48     C. florida     Louisiana, USA     1971     33.0 (0.34)       H7     C. florida     NH, USA     1985     38.0 (0.57)       H31     C. kousa     N. Honshu, Japan     1984     -       H32     C. kousa     Mingi Pref, Japan     1984     -       H33     C. kousa     Mingi Pref, Japan     1986	H10	C. capitata	Yunnan, China	1946	38.2 (2.25)
123     C. chinesis     Omei-hsien, China     1942     38.1 (0.30)       124     C. chinesis     Mt. Omei, China     1938     35.3 (1.70)       124     C. chinesis     Yubiso, Japan     1938     35.3 (1.70)       118     C. controversa     Ohshimizu, Japan     1952     -       120     C. controversa     Nunobiki, Japan     1953     -       121     C. controversa     Nunobiki, Japan     1953     -       122     C. controversa     Mt. Marai, Japan     1955     -       122     C. controversa     Mt. Sanai, USA     1955     35.5 (0.63)       124     C. forida     Mississippi, USA     1955     35.5 (0.63)       155     C. florida     Nt, Horshu, Japan     1971     33.0 (0.34)       156     C. florida     Nt, Horshu, Japan     1987     -       142     C. kousa     N. Honshu, Japan     1984     -       143     C. kousa     Yamanashi Pref., Japan     1984     -       143     C. kousa     Yamanashi Pref., Japan <t< td=""><td>H11</td><td>C. capitata</td><td>Yunnan, China</td><td>2000</td><td>38.6 (2.34)</td></t<>	H11	C. capitata	Yunnan, China	2000	38.6 (2.34)
H24     C. chrinesis     Mt. Omei, China     1938     35.3 (1.70)       H16     C. controversa     Ohshimizu, Japan     1956     31.6 (0.10)       H18     C. controversa     Jangxi, China     1975     41.1 (0.32)       H20     C. controversa     Jangxi, China     1975     41.1 (0.32)       H20     C. controversa     Mt. Mamari, Japan     1953     -       H21     C. controversa     Mt. Mamari, Japan     1955     -       H22     C. controversa     Mt. Mamari, Japan     1955     35.5 (0.63)       H22     C. controversa     Mt. Mamari, Japan     1985     32.8 (0.07)       H48     C. florida     Msissispi, USA     1985     32.8 (0.83)       H35     C. florida     Nu JSA     1985     32.8 (0.87)       H49     C. florida     Nu JSA     1986     33.8 (0.57)       H29     C. kousa     N. Honshu, Japan     1984     -       H31     C. kousa     Ming Pref, Japan     1984     -       H32     C. kousa     Ming Pref, Japan	H23	C. chinesis	Omei-hsien, China	1942	38.1 (0.30)
H16     C. controversa     Vuiso, Japan     1956     31.6 (0.10)       H18     C. controversa     Jangxi, China     1952     -       H20     C. controversa     Jangxi, China     1953     -       H21     C. controversa     Munobiki, Japan     1953     -       H21     C. controversa     Min Marri, Japan     1953     -       H22     C. controversa     Min Marri, Japan     1955     -       H48     C. forida     Missispipi, USA     1955     355 (0.63)       H46     C. florida     Nie, UsA     1957     32.8 (0.37)       H56     C. florida     Nie, UsA     1967     -       H27     C. florida     Nie, UsA     1979     -       H28     C. kousa     Nienshu, Japan     1984     -       H31     C. kousa     Minagi Pref., Japan     1984     -       H32     C. kousa     Minagi Pref., Japan     1982     -     -       H33     C. kousa     Moragi Pref., Japan     1985     -     -	H24	C. chinesis	Mt. Omei, China	1938	35.3 (1.70)
H18     C. controversa     Ohsimizu, Japan     1952     -       H19     C. controversa     Jiangxi, China     1975     41.1 (0.32)       H20     C. controversa     Nunobiki, Japan     1953     -       H21     C. controversa     Mt. Namari, Japan     1953     -       H22     C. controversa     Mt. Namari, Japan     1953     -       H22     C. controversa     Mt. Namari, Japan     1955     -       H24     C. forida     Mississippi, USA     1955     35.5 (0.63)       H48     C. florida     Louisana, USA     1971     33.0 (0.34)       T7     C. florida     N. Honshu, Japan     1985     38.0 (0.57)       H31     C. kousa     N. Honshu, Japan     1984     -       H32     C. kousa     Myaqi Pref, Japan     1984     -       H33     C. kousa     Myaqi Pref, Japan     1982     36.3 (1.13)       H34     C. kousa     Manashi Pref, Japan     1982     36.3 (1.13)       H35     C. kousa     Mino Pref, Japan     1985 <td>H16</td> <td>C. controversa</td> <td>Yubiso, Japan</td> <td>1956</td> <td>31.6 (0.10)</td>	H16	C. controversa	Yubiso, Japan	1956	31.6 (0.10)
H19     C. controversa     Jangxi, China     1975     41.1 (0.32)       420     C. controversa     Nunobiki, Japan     1953     -       421     C. controversa     Mina, Japan     1953     -       422     C. controversa     Mi. Namari, Japan     1955     -       422     C. controversa     Mi. Namari, Japan     1955     -       448     C. florida     Castens, NY     201     26.4 (0.07)       55     C. florida     Louisian, USA     1951     35.5 (0.63)       77     C. florida     Louisian, USA     1971     33.0 (0.34)       78     C. florida     Huagn-shan, China     1973     32.8 (0.37)       74     C. florida     Muagn-shan, China     1987     -       730     C. kousa     N. Honshu, Japan     1984     -     -       741     C. kousa     Minagni Pref., Japan     1984     -     -       743     C. kousa     Mara Pref., Japan     1984     -     -       7434     C. kousa     Mara Pref.,	H18	C. controversa	Ohshimizu, Japan	1952	-
120     C. controversa     Nunobiki, Japan     1953     -       121     C. controversa     Aira, Japan     1953     -       122     C. controversa     Mt. Namai, Japan     1955     -       148     C. foricka     Mt. Namai, Japan     1955     -       148     C. floricka     Mississippi, USA     1951     35.6 (0.63)       16     C. floricka     Muosississippi, USA     1971     33.0 (0.34)       17     C. floricka     Mug-shan, China     1979     -       129     C. kousa     N. Honshu, Japan     1988     38.0 (0.57)       130     C. kousa     N. Honshu, Japan     1984     -       132     C. kousa     Miyagi Pref., Japan     1984     -       133     C. kousa     Fluctshima Pref., Japan     1982     -       134     C. kousa     Honshu, Japan     1982     -       135     C. kousa     Nara Pref., Japan     1982     -       136     C. kousa     Mino Pref., Japan     1985     -	H19	C. controversa	Jiangxi, China	1975	41.1 (0.32)
121     C. controversa     Arima, Japan     1953     -       122     C. controversa     Mt. Namari, Japan     1950     -       1448     C. florida     Carstens, NY     2011     26.4 (0.07)       15     C. florida     Mississippi, USA     1955     35.5 (0.63)       166     C. florida     Louisiana, USA     1971     33.0 (0.34)       17     C. florida     Huang-shan, China     1979     -       156     C. japonica     Huang-shan, China     1987     -       1429     C. kousa     N. Honshu, Japan     1984     -       1330     C. kousa     N. Honshu, Japan     1984     -       134     C. kousa     Miyagi Pref, Japan     1984     -       135     C. kousa     Fukushim Pref, Japan     1982     36.5 (1.82)       136     C. kousa     Mina Pref, Japan     1982     36.3 (1.0)       137     C. kousa     Mino Pref, Japan     1982     36.3 (1.0)       138     C. kousa     Mino Pref, Japan     1982     36.6 (	H20	C. controversa	Nunobiki, Japan	1953	-
H22     C. controversa     Mt. Namari, Japan     1955     -       H48     C. forida     Carstens, NY     201     26.4 (0.07)       h5     C. florida     Mississipi, USA     1955     35.5 (0.63)       h6     C. florida     Louisiana, USA     1971     33.0 (0.34)       h7     C. florida     NH, USA     1985     32.8 (0.37)       156     C. japonica     Huang-shan, China     1979     -       129     C. kousa     N. Honshu, Japan     1987     -       130     C. kousa     N. Honshu, Japan     1984     -       132     C. kousa     Miyagi Pref, Japan     1984     -       133     C. kousa     Yamanashi Pref, Japan     1982     -       134     C. kousa     Yamanashi Pref, Japan     1982     -       135     C. kousa     Yamanashi Pref, Japan     1982     36.5 (1.82)       135     C. kousa     Nana Pref, Japan     1982     36.3 (1.13)       136     C. kousa     Mino Pref, Japan     1985     36.3 (1.13)<	H21	C. controversa	Arima, Japan	1953	-
H48     C. florida     Carstens, NY     2011     26.4 (0.07)       15     C. florida     Mississipi, USA     1955     35.5 (0.63)       166     C. florida     Louisian, USA     1987     33.0 (0.34)       166     C. florida     Hu, USA     1987     32.8 (0.37)       155     C. japonica     Huang-shan, China     1979     -       129     C. kousa     N. Honshu, Japan     1987     -       130     C. kousa     Namashi Pref, Japan     1984     -       132     C. kousa     Miyagi Pref, Japan     1984     -       133     C. kousa     Yamanashi Pref, Japan     1984     -       134     C. kousa     Miyagi Pref, Japan     1982     -       135     C. kousa     Mara Pref, Japan     1982     -       136     C. kousa     Mino Pref, Japan     1985     36.3 (1.13)       137     C. kousa     Mino Pref, Japan     1985     36.3 (1.075)       143     C. kousa     Mino Pref, Japan     1985     36.1 (0.75)	H22	C. controversa	Mt. Namari, Japan	1955	-
h5     C. florida     Mississippi, USA     1955     35.5 (0.63)       h6     C. florida     Louisiana, USA     1971     33.0 (0.34)       h7     C. florida     Huang-shan, China     1979     -       H29     C. kousa     N. Honshu, Japan     1987     -       H30     C. kousa     N. Honshu, Japan     1988     38.0 (0.57)       H31     C. kousa     Yamanashi Pref., Japan     1984     -       H32     C. kousa     Miyagi Pref., Japan     1984     -       H33     C. kousa     Yamanashi Pref., Japan     1982     -       H34     C. kousa     Yamanashi Pref., Japan     1982     -       H35     C. kousa     Yamanashi Pref., Japan     1982     -       H35     C. kousa     Mara Pref., Japan     1982     -       H36     C. kousa     Mikolu, Japan     1985     36.3 (1.13)       H37     C. kousa     Mino Pref., Japan     1985     -       H40     C. kousa     Mino Pref., Japan     1986     -	H48	C. florida	Carstens, NY	2011	26.4 (0.07)
h6     C. florida     Louisiana, USA     1971     33.0 (0.34)       17     C. florida     NH, USA     1985     32.8 (0.37)       156     C. florida     Huang-shan, China     1979     -       149     C. kousa     N. Honshu, Japan     1987     -       130     C. kousa     N. Honshu, Japan     1984     -       131     C. kousa     Miyagi Pref., Japan     1984     -       132     C. kousa     Miyagi Pref., Japan     1984     -       133     C. kousa     Yamanashi Pref., Japan     1984     -       134     C. kousa     Yamanashi Pref., Japan     1982     -       135     C. kousa     Yamanashi Pref., Japan     1982     -       136     C. kousa     Mara Pref., Japan     1982     36.3 (1.13)       139     C. kousa     Mino Pref., Japan     1985     36.3 (1.0.75)       140     C. kousa     Mino Pref., Japan     1988     36.1 (0.75)       141     C. kousa     Montes Kentei, Japan     1988     36.1 (0.75	n5	C. florida	Mississippi, USA	1955	35.5 (0.63)
h7   C. florida   NH, USA   1985   32.8 (0.37)     156   C. japonica   Huang-shan, China   1979   -     129   C. kousa   N. Honshu, Japan   1987   -     130   C. kousa   N. Honshu, Japan   1988   38.0 (0.57)     131   C. kousa   Yamanashi Pref, Japan   1984   -     132   C. kousa   Miyagi Pref, Japan   1984   -     133   C. kousa   Yamanashi Pref, Japan   1984   -     134   C. kousa   Yamanashi Pref, Japan   1982   -     135   C. kousa   Yamanashi Pref, Japan   1982   -     136   C. kousa   Nara Pref, Japan   1982   -     137   C. kousa   Nara Pref, Japan   1982   -     138   C. kousa   Mino Pref, Japan   1988   36.1 (0.75)     149   C. kousa   Mino Pref, Japan   1988   36.1 (0.75)     141   C. kousa   Montes Kentei, Japan   1988   36.1 (0.75)     141   C. kousa   Montes Kentei, Japan   1978   -	n6	C. florida	Louisiana, USA	1971	33.0 (0.34)
H56     C. japonica     Huang-shan, China     1979     -       H29     C. kousa     N. Honshu, Japan     1987     -       H30     C. kousa     N. Honshu, Japan     1988     38.0 (0.57)       H31     C. kousa     Yamanashi Pref, Japan     1984     -       H32     C. kousa     Miyagi Pref, Japan     1984     -       H33     C. kousa     Miyagi Pref, Japan     1984     -       H34     C. kousa     Honshu, Japan     1982     -       H35     C. kousa     Honshu, Japan     1982     38.5 (1.82)       H36     C. kousa     Honshu, Japan     1982     36.3 (1.13)       H37     C. kousa     Mino Pref, Japan     1985     -       H40     C. kousa     Mino Pref, Japan     1985     36.1 (0.75)       H41     C. kousa     Mino Pref, Japan     1988     36.1 (0.75)       H41     C. kousa     Mino Pref, Japan     1968     36.7 (0.03)       H43     C. kousa     Montes Kentei, Japan     1968     36.1 (0.75)	n7	C. florida	NH, USA	1985	32.8 (0.37)
129   C. kousa   N. Honshu, Japan   1987   -     130   C. kousa   N. Honshu, Japan   1988   38.0 (0.57)     131   C. kousa   Yamanashi Pref., Japan   1984   -     132   C. kousa   Miyagi Pref., Japan   1984   -     133   C. kousa   Fukushima Pref., Japan   1984   -     134   C. kousa   Yamanashi Pref., Japan   1982   -     135   C. kousa   Yamanashi Pref., Japan   1982   -     134   C. kousa   Yamanashi Pref., Japan   1982   38.5 (1.82)     135   C. kousa   Nara Pref., Japan   1982   -     136   C. kousa   Nara Pref., Japan   1985   36.3 (1.13)     137   C. kousa   Mino Pref., Japan   1985   36.3 (1.075)     143   C. kousa   Mino Pref., Japan   1988   36.1 (0.75)     144   C. kousa   Montes Kentei, Japan   1988   36.1 (0.75)     144   C. kousa   Montes Kentei, Japan   1978   -     144   C. kousa   Montes Kentei, Japan	H56	C. japonica	Huang-shan, China	1979	-
H30   C. kousa   N. Honshu, Japan   1988   38.0 (0.57)     H31   C. kousa   Yamanashi Pref., Japan   1984   -     H32   C. kousa   Miyagi Pref., Japan   1984   -     H33   C. kousa   Fukushima Pref., Japan   1984   -     H34   C. kousa   Yamanashi Pref., Japan   1982   -     H35   C. kousa   Honshu, Japan   1982   -     H36   C. kousa   Honshu, Japan   1982   -     H37   C. kousa   Honshu, Japan   1982   -     H37   C. kousa   Nikoku, Japan   1982   -     H37   C. kousa   Mino Pref., Japan   1985   36.3 (1.13)     H39   C. kousa   Mino Pref., Japan   1985   36.3 (1.075)     H40   C. kousa   Mino Pref., Japan   1988   36.1 (0.75)     H41   C. kousa   Montes Kentei, Japan   1968   36.7 (0.03)     H43   C. kousa   Montes Kentei, Japan   1978   35.6 (1.11)     H44   C. kousa   Motya, Japan   1977   41.3 (2.9	H29	C. kousa	N. Honshu, Japan	1987	-
H31   C. kousa   Yamanashi Pref., Japan   1984   -     H32   C. kousa   Miyagi Pref., Japan   1984   -     H33   C. kousa   Fukushima Pref., Japan   1984   36.0 (0.12)     H34   C. kousa   Yamanashi Pref., Japan   1982   -     H35   C. kousa   Honshu, Japan   1982   -     H36   C. kousa   Nara Pref., Japan   1982   -     H37   C. kousa   Honshu, Japan   1982   -     H37   C. kousa   Nara Pref., Japan   1978   -     H37   C. kousa   Mino Pref., Japan   1985   36.3 (1.13)     H39   C. kousa   Mino Pref., Japan   1985   -     H40   C. kousa   Mino Pref., Japan   1985   -     H41   C. kousa   Mino Pref., Japan   1988   36.1 (0.75)     H43   C. kousa   Montes Kentei, Japan   1965   -     H44   C. kousa   Tokyo, Japan   1978   -     H44   C. kousa   Mit Fuji, Japan   1977   41.3 (2.98)  H	H30	C. kousa	N. Honshu, Japan	1988	38.0 (0.57)
H32   C. kousa   Miyagi Pref., Japan   1984   -     H33   C. kousa   Fukushima Pref., Japan   1984   36.0 (0.12)     H34   C. kousa   Yamanashi Pref., Japan   1982   -     H35   C. kousa   Honshu, Japan   1982   38.5 (1.82)     H36   C. kousa   Nara Pref., Japan   1978   -     H37   C. kousa   Shikoku, Japan   1985   36.3 (1.13)     H39   C. kousa   Mino Pref., Japan   1985   -     H40   C. kousa   Mino Pref., Japan   1988   36.1 (0.75)     H41   C. kousa   Miyagi Pref., Japan   1988   36.1 (0.75)     H41   C. kousa   Monto Pref., Japan   1988   36.1 (0.75)     H41   C. kousa   Montos Kentei, Japan   1968   36.1 (0.75)     H44   C. kousa   Tokyo, Japan   1978   -   -     H44   C. kousa   Tokyo, Japan   1978   -   -     H44   C. kousa   Mt. Fuji, Japan   1977   41.3 (2.98)     H47   C. kousa   M. Hotta, J	H31	C. kousa	Yamanashi Pref., Japan	1984	-
H33     C. kousa     Fukushima Pref., Japan     1984     36.0 (0.12)       H34     C. kousa     Yamanashi Pref., Japan     1982     -       H35     C. kousa     Honshu, Japan     1982     38.5 (1.82)       H36     C. kousa     Nara Pref., Japan     1978     -       H37     C. kousa     Shikoku, Japan     1985     36.3 (1.13)       H39     C. kousa     Mino Pref., Japan     1985     36.3 (1.07)       H40     C. kousa     Mino Pref., Japan     1985     36.1 (0.75)       H41     C. kousa     Mino Pref., Japan     1988     36.1 (0.75)       H41     C. kousa     Mt. Ohyama, Japan     1968     36.7 (0.03)       H43     C. kousa     Mto Ohyama, Japan     1965     -       H44     C. kousa     Tokyo, Japan     1978     -       H45     C. kousa     Mt. Fuji, Japan     1978     -       H46     C. kousa     Mt. Huji, Japan     1976     38.8 (1.67)       H47     C. kousa     M. Hotta, Japan     1976     3	H32	C. kousa	Miyagi Pref., Japan	1984	-
H34   C. kousa   Yamanashi Pref., Japan   1982   -     H35   C. kousa   Honshu, Japan   1982   38.5 (1.82)     H36   C. kousa   Nara Pref., Japan   1978   -     H37   C. kousa   Shikoku, Japan   1985   36.3 (1.13)     H39   C. kousa   Mino Pref., Japan   1935   -     H40   C. kousa   Miyagi Pref., Japan   1988   36.1 (0.75)     H41   C. kousa   Miyagi Pref., Japan   1968   36.7 (0.03)     H43   C. kousa   Montes Kentei, Japan   1965   -     H44   C. kousa   Tokyo, Japan   1978   35.6 (1.11)     H45   C. kousa   Tokyo, Japan   1978   -     H46   C. kousa   Mt. Fuji, Japan   1977   41.3 (2.98)     H47   C. kousa   Mt. Hotta, Japan   1965   34.1 (0.47)     H49   C. kousa   J. Murata, Japan   1976   38.8 (1.67)     H51   C. kousa   J. Murata, Japan   1987   35.6 (1.01)     H52   C. kousa   J. Murata, Japan   1987	H33	C. kousa	Fukushima Pref., Japan	1984	36.0 (0.12)
H35C. kousaHonshu, Japan198238.5 (1.82)H36C. kousaNara Pref., Japan1978-H37C. kousaShikoku, Japan198536.3 (1.13)H39C. kousaMino Pref., Japan1935-H40C. kousaMiyagi Pref., Japan198836.1 (0.75)H41C. kousaMontes Kentei, Japan196836.7 (0.03)H43C. kousaMontes Kentei, Japan1965-H44C. kousaMontes Kentei, Japan197835.6 (1.11)H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H54C. kousaIupei-Szechuan, China196036.3 (0.50)	H34	C. kousa	Yamanashi Pref., Japan	1982	-
H36C. kousaNara Pref., Japan1978-H37C. kousaShikoku, Japan198536.3 (1.13)H39C. kousaMino Pref., Japan1935-H40C. kousaMiyagi Pref., Japan198836.1 (0.75)H41C. kousaMt. Ohyama, Japan196836.7 (0.03)H43C. kousaMontes Kentei, Japan1965-H44C. kousaTokyo, Japan197835.6 (1.11)H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H35	C. kousa	Honshu, Japan	1982	38.5 (1.82)
H37C. kousaShikoku, Japan198536.3 (1.13)H39C. kousaMino Pref., Japan1935-H40C. kousaMiyagi Pref., Japan198836.1 (0.75)H41C. kousaMt. Ohyama, Japan196836.7 (0.03)H43C. kousaMontes Kentei, Japan1965-H44C. kousaTokyo, Japan197835.6 (1.11)H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H36	C. kousa	Nara Pref., Japan	1978	-
H39C. kousaMino Pref., Japan1935-H40C. kousaMiyagi Pref., Japan198836.1 (0.75)H41C. kousaMt. Ohyama, Japan196836.7 (0.03)H43C. kousaMontes Kentei, Japan1965-H44C. kousaTokyo, Japan197835.6 (1.11)H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H37	C. kousa	Shikoku, Japan	1985	36.3 (1.13)
H40C. kousaMiyagi Pref., Japan198836.1 (0.75)H41C. kousaMt. Ohyama, Japan196836.7 (0.03)H43C. kousaMontes Kentei, Japan1965-H44C. kousaTokyo, Japan197835.6 (1.11)H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H39	C. kousa	Mino Pref., Japan	1935	-
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H43C. kousaMontes Kentei, Japan1965-H44C. kousaTokyo, Japan197835.6 (1.11)H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H41	C. kousa	Mt. Ohyama, Japan	1968	36.7 (0.03)
H44C. kousaTokyo, Japan197835.6 (1.11)H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H43	C. kousa	Montes Kentei, Japan	1965	-
H45C. kousaTokyo, Japan1978-H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H44	C. kousa	Tokyo, Japan	1978	35.6 (1.11)
H46C. kousaMt. Fuji, Japan197741.3 (2.98)H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H45	C. kousa	Tokyo, Japan	1978	-
H47C. kousaM. Hotta, Japan196534.1 (0.47)H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H46	C. kousa	Mt. Fuji, Japan	1977	41.3 (2.98)
H49C. kousaJ. Murata, Japan197638.8 (1.67)H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H47	C. kousa	M. Hotta, Japan	1965	34.1 (0.47)
H51C. kousaKukhansan, Korea198735.6 (1.01)H52C. kousaYakushima, Japan196136.3 (0.50)H53C. kousaHupei-Szechuan, China196036.3 (0.50)H54C. kousaIsl. Tsushima, Japan196836.7 (0.82)	H49	C. kousa	J. Murata, Japan	1976	38.8 (1.67)
H52   C. kousa   Yakushima, Japan   1961   36.3 (0.50)     H53   C. kousa   Hupei-Szechuan, China   1960   36.3 (0.50)     H54   C. kousa   Isl. Tsushima, Japan   1968   36.7 (0.82)	H51	C. kousa	Kukhansan, Korea	1987	35.6 (1.01)
H53   C. kousa   Hupei-Szechuan, China   1960   36.3 (0.50)     H54   C. kousa   Isl. Tsushima, Japan   1968   36.7 (0.82)	H52	C. kousa	Yakushima, Japan	1961	36.3 (0.50)
H54     C. kousa     Isl. Tsushima, Japan     1968     36.7 (0.82)	H53	C. kousa	Hupei-Szechuan, China	1960	36.3 (0.50)
	H54	C. kousa	Isl. Tsushima, Japan	1968	36.7 (0.82)

#### Table 1. Real-time PCR results of Cornus herbarium samples with species name, location, and year of collection.

(Continued)

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#### Table 1. (Continued)

Sample	Plant Species	Location	Year	qPCR mean Ct value (Standard deviation)*
H55	C. kousa	Yamanashi Pref., Japan	1982	35.0 (0.74)
H57	C. kousa	Hupei-Szechuan, China	1960	36.3 (1.45)
H59	C. kousa	Kyushu, Japan	1983	35.7 (0.13)
H60	C. kousa	Honshu, Japan	1973	32.2 (0.42)
H61	C. kousa	Yamanashi Pref., Japan	1976	
H65	C. kousa	Shennongjia Forest, China	1980	34.67 (0.87)
H66	C. kousa	Kiangsi, China	1975	33.5 (0.06)
H27	C. macrophylla	Metasequoia area, China	1960	-
n1	C. nuttallii	Oregon, USA	1987	35.4 (1.22)
n2	C. nuttallii	BC, Canada	1963	-
n3	C. nuttallii	California, USA	1976	-
n4	C. nuttallii	Oregon, USA	1975	-
n8	C. nuttallii	California, USA	1988	33.7 (0.63)
n9	C. nuttallii	California, USA	1968	33.0 (0.24)
H12	C. paucinervis	Hupei-Szechuan, China	1974	40.2 (2.50)
H28	C. paucinervis	Hubei Prov., China	1980	36.2 (1.11)
H25	C. sanguinea	Nancy, France	1959	34.2 (0.46)
H58	C. stolonifera	Mt. Potosi, Mexico	1968	-
H64	C. stolonifera	Nuevo Leon, Mexico	1960	34.35 (0.74)
H62	C. stolonifiera	Chihuahua, Mexico	1972	36.3 (1.44)
H63	C. stolonifiera	Chihuahua, Mexico	1977	33.5 (0.38)
H13	C. walteri	Sichuan Prov., China	2007	31.2 (0.28)
H14	C. walteri	Hupeh:Shenlungkai, China	1976	31.1 (0.16)

\*Highlighted in bold are the samples with positive detection of *Discula destructiva*, with Ct value < 32.0, and confirmed with DNA sequencing. -: no amplification signals.

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Emerging colonies were sub-cultured to obtain pure fungal isolates. After a week, subcultures growing in AMEA were grouped into morphotaxa [28, 30, 31] based on spore morphology (if present), as well as colony characteristics such as shape, color, texture, aerial hyphae, and margin. If more than three isolates were present in a morphotaxon, three representative isolates were selected for sequencing. If there were fewer than three isolates in a morphotaxon, all isolates were sequenced.

# Fungal DNA extraction, PCR, sequencing and identification

Fungal isolates were grown on AMEA at room temperature for four days to two weeks depending on growth rate. Genomic DNA was extracted from mycelium with Qiagen DNeasy Plant Mini kit (Qiagen, Germany) following the manufacturer's protocol. The internal transcribed spacer (ITS) of the rRNA genes was amplified with ITS1 and ITS4 primers [32]. ITS1F was used with ITS4 if no PCR product was found with ITS1 and ITS4. PCR reaction mixture (25  $\mu$ l) consisted of 5  $\mu$ l of 5X GoTaq Flexi Buffer (Promega, WI, USA), 1.5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 2  $\mu$ l of 10 mM dNTPs mix, 1  $\mu$ l of 10 mM forward primer and 1  $\mu$ l of 10 mM reverse primer, 0.125  $\mu$ l (5U/ $\mu$ l) of GoTaq DNA polymerase (Promega, WI, USA), and a maximum of 25 ng/ $\mu$ l of genomic DNA. The PCR cycling conditions were as follows: 94°C for 5 minutes, followed by 32 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and primer extension at 72°C for 1.5 minutes, followed by a final extension at 72°C for 5 minutes. PCR products



Sample	Plant Species	Location	Year	qPCR mean Ct value (Standard deviation)*
US10-1	C. florida	USA	2010	
US10-2	C. florida	USA	2010	-
US10-3	C. florida	USA	2010	30.6 (1.90)**
US10-4	C. florida	USA	2010	34.9 (0.60)
US10-5	C. florida	USA	2010	17.01 (0.43)**
US12-1	C. florida	USA	2012	-
US12-2	C. florida	USA	2012	-
US12-3	C. florida	USA	2012	-
US12-4	C. florida	USA	2012	-
US12-5	C. florida	USA	2012	-
JAP10-1	C. kousa	Japan	2010	23.9 (0.42)
JAP10-2	C. kousa	Japan	2010	-
JAP10-3	C. kousa	Japan	2010	-
JAP10-4	C. kousa	Japan	2010	-
JAP10-5	C. kousa	Japan	2010	38.3 (0.46)
JAP12-1	C. kousa	Japan	2012	38.2 (1.43)
JAP12-2	C. kousa	Japan	2012	34.8 (0.76)
JAP12-3	C. kousa	Japan	2012	35.0 (0.59)
JAP12-4	C. kousa	Japan	2012	34.1 (0.91)
JAP12-5	C. kousa	Japan	2012	34.8 (0.76)

Table 2. Real-time PCR detection results of fresh Cornus leaf samples collected from New Jersey, USA and Ibaraki Prefecture, Japan in 2010 and 2012.

\*Highlighted in bold are the samples with positive detection of *Discula destructiva*, with Ct value < 32.0, and confirmed with DNA sequencing. -: no amplification signals.

\*\* Cultures of Discula destructiva were also obtained from these samples.

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were verified using gel electrophoresis and purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) following the manufacturer's instructions. Purified PCR products were sequenced by GeneWiz, Inc. (South Plainfield, NJ, USA) using primers ITS1, ITS4 and ITS1F [32].

Fungal isolates were identified based on morphology and Mega BLASTn for each of the ITS sequences against GenBank. The ITS sequences (ca. 500 bp) were compared against a curated GenBank database using their Mega BLAST program on a local server. Top sequences that matched >97% similarity were considered belonging to the same operational taxonomic unit (OTU). DNA sequences obtained in this study are deposited in GenBank under accession numbers KJ921855-KJ921969.

# Results

## **Real-time PCR results**

Six herbarium samples (H6, H7, H13, H14, H16 and H48) (<u>Table 1</u>) had Ct values below 32, the cutoff Ct value, which was decided based on the positive and negative control real-time PCR readings, and the sequencing results of the real-time PCR amplicons. Samples H6, H7, H13, H14, and H16 were from various *Cornus* species located in Japan and China collected during 1949–2007. H48 was collected in USA in 2011. The real-time PCR assay also detected *D. destructiva* from three recently collected fresh samples from USA and Japan (<u>Table 2</u>). All samples with positive real-time PCR detection results were verified to have a 191 bp amplicon, the sequence of which matched to the ITS sequence of *D. destructiva* (100% identity).

## Cornus endophyte analysis

A total of 371 fungal culture isolates were obtained from 1825 leaf segments from the 20 fresh *Cornus* samples collected from USA and Japan in 2010 and 2012, and 121 representative isolates were sequenced. A total of 48 OTUs were identified that belong to 26 genera (<u>S1 Table</u>). There was no fungal growth present on the control plates after surface sterilization of the leaf samples. *Discula destructiva* was isolated in culture from the USA samples but not from the samples from Japan.

## Discussion

The origin of the dogwood anthracnose pathogen had been a mystery [15]. The current hypothesis is that the fungus was introduced from Asia to North America in the 1970's [15, 20]. It has later spread into Europe due to trade [22]. However, there had been no report on the presence of this fungal species in Asia. This study is the first time report of positive detection of *D. destructiva* from dogwood samples in Asia. The fact that some of the *D. destructiva*-positive herbarium samples were collected in Asia before the first disease outbreak in North America provides evidence for the introduction hypothesis.

A major challenge in searching for the dogwood anthracnose fungus and other slow-growing microscopic species is a lack of accurate and sensitive detection methods. The real-time PCR assay allowed for sensitive and reliable testing for both fresh [22] and herbarium samples. In this study, we successfully detected the dogwood anthracnose fungus from dried specimens collected up to 66 years ago. The results here present an early attempt to utilize this molecular method to detect fungal endophytes or pathogens from historical herbarium samples.

A live culture of *D. destructiva* has not been obtained from Asia, likely due to its slow growth rate in culture and the small number of fresh samples included in this study. The positive molecular detection from Asia indicates that further sampling efforts in Japan and other areas of Asia likely will yield *D. destructiva* cultures, which will provide long-awaited materials for future studies, in order to better understand the origin, dispersal and evolution of this *Cornus*-associated fungus.

# **Supporting Information**

**S1** Table. List of identified fungal OTUs and their abundance by year and location. (DOCX)

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# **Author Contributions**

Conceived and designed the experiments: NZ SM. Performed the experiments: SM EW. Analyzed the data: SM NZ. Contributed reagents/materials/analysis tools: NZ HM JZ. Wrote the paper: SM NZ HM JZ.

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