Pathogenicity and Distribution of *Fusarium solani* Isolates Associated with *Erythrina* Decline in Japan

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Abstract

Erythrina spp. trees have been declining since the 2000s worldwide, and fungi belonging to *Fusarium solani* species complex (FSSC) have been suggested to be a causal factor of decline and mortality of *Erythrina variegata* trees in Okinawa Island, Japan. In addition to the FSSC isolate grouped as “it-1” based on ITS sequence data (previously called strain A), we conducted an inoculation experiment with two isolates grouped as “it-2” (previously strain B), which is genetically close to it-1. Two it-2 isolates originating from two islands showed pathogenicity to *E. variegata* with the same symptoms as those caused by it-1 isolate. We also found the isolates of it-1 and it-2 were widely distributed, including on Ishigaki Island, ~400 km south of Okinawa Island across the ocean.

All isolates of it-1 and it-2 belong to the ambrosia *Fusarium* clade of the FSSC, a group of symbionts of ambrosia beetles, including the pathogens of *Fusarium* dieback in avocados and teas. The detection of ambrosia beetles *Euwallacea* spp. from our specimens provided information on the vectors of the pathogens. Our present results suggest the fungi of the FSSC could be responsible for the *Erythrina* decline in other areas with damage.

Keywords: *Erythrina variegata*, wilt disease, *Quadrastichus erythrinae*, *Fusarium solani* species complex, ambrosia *Fusarium*, ambrosia beetle

The decline and mortality of coral trees (*Erythrina* spp.) have been increasing since the 2000s in a wide area of tropical and subtropical regions of Asia, Hawaii, and a few regions on the American continent (Uechi et al. 2007). *Erythrina* trees are designated in agroforestry as shade trees for pepper, cacao, and coffee and as ornamental trees in parks (Kumar et al. 2010). *Erythrina variegata*, the Indian coral tree, which is widely planted in the southern islands of Japan, has also shown decline and mortality since the 2000s. Although an infestation by the gall wasp *Quadrastichus erythrinae* has been believed to be the cause of the decline, based on the observation that many galls are found on the leaves of declining trees (Kim et al. 2004; Rubinoff et al. 2010; Uechi et al. 2007), there was no scientific data on the causal relationship between the gall wasp infestation and the decline and mortality of *Erythrina* trees. It is well known that the injection of insecticides in the trunks of *Erythrina* trees does not always effectively prevent decline. In Hawaii, biological control with a parasitic wasp from Africa was recently conducted to protect an indigenous *Erythrina* species (Messing et al. 2008; Reimar 2008). However, Yalemar et al. (2009) reported that this method did not always have positive effects.

For this reason, the authors thought that the real causal factor of the decline and mortality should be found in order to protect *Erythrina* trees, and we focused on the pathological incidences in trees at various stages of decline before death. Kuroda et al. (2017) found that specific fungi are distributed consistently in the discolored xylem of declining trees. In addition, anatomical observation proved that

those trees died of water deficit due to the blockage of water conduction in the discolored xylem in which *Fusarium* spp. were distributed. These results suggested that the occurrences might be results of wilt disease caused by fungi. From the DNA sequencing of the rDNA-ITS gene repeat, the fungi most frequently detected in declining trees were revealed to belong to the *Fusarium solani* species complex (FSSC) (recently reported as *Neocosmospora* [Lombard et al. 2015]). The detected isolates were grouped on the basis of the similarity in the base sequences, and two groups of them, which include many isolates from various specimens, were named strain A and strain B in the previous report (Kuroda et al. 2017). Those groupings were renamed as it-1 (strain A) and it-2 (strain B) in the present report. The artificial inoculation of healthy seedlings with an isolate of it-1 was conducted to check the pathogenicity, and it showed the same symptoms as naturally declining trees of *E. variegata* on Okinawa Island, that is, an abnormal defoliation associated with decreased water uptake, necrosis of the cambium and phloem with an odor, and, finally, the death of the whole tree. By the successful re-isolation of the inoculated fungus from dead seedlings, Kuroda et al. (2017) concluded that an isolate—P2-f11 of it-1 obtained from Okinawa Island—is pathogenic to *E. variegata* and that *E. variegata* is dying from a new disease caused by a fungus in the it-1 group belonging to the FSSC.

The next steps were to check the pathogenicity of other isolates belonging to the group it-2 of the FSSC. In addition, surveillance was necessary on the geographical distributions of the FSSC pathogenic to *E. variegata* to find areas affected by the pathogens and to reveal the routes of infection from diseased to healthy trees. Therefore, the aims of this research are (i) to add fundamental information of geographic distribution of FSSC associated with declining *Erythrina* trees and (ii) to check the pathogenicity of the isolate group it-2 in addition to it-1 (Kuroda et al. 2017). In the preceding investigation, isolates phylogenetically the same as it-1 and it-2 were both detected at two sites 17 km apart on Okinawa Island (Kuroda et al. 2017). This suggested that pathogenic fungi might be distributed in a wide area, even beyond Okinawa Island or Japan. Confirmation of the universal detection of the pathogenic fungus in all declining *E. variegata* is essential to find strategies to protect this tree species. For that purpose, geographical surveillance of the FSSC associated with *Erythrina*

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decline was expanded to Ishigaki Island, 400 km from Okinawa Island. In addition, geographical differences in pathogenicity were compared by an artificial inoculation experiment using two it-2 isolates that were detected at different sites. If *Erythrina* mortality in tropical Asian countries, the American continent, and Hawaii is also caused by similar fungi or closely related to our pathogenic isolates of the FSSC, the present information will be helpful in those areas.

Materials and Methods

Surveying of declining trees and sampling. *E. variegata* trees planted as ornamental or shade trees were surveyed on Ishigaki and Okinawa Islands, Okinawa Prefecture, Japan, in 2017. Specimens were taken from declining trees, as indicated in Table 1. The level of decline in trees was evaluated based on the method used in Kuroda et al. (2017) as follows: level 1 trees are extensively damaged with sparse leaves; level 2 trees are moderately damaged but still have abundant leaves; and level 3 trees appear healthy, although they have infestations of *Q. erythrinae*.

Two declining trees, Is1 and Is2, were selected from a private garden in Ibaruma, Ishigaki City, Ishigaki Island. Tree Is1 had abundant leaves, although it was abnormally defoliated and had galls of *Q. erythrinae* (level 2, moderate damage). Tree Is2 had few leaves and was judged to be close to death (level 1, extensive damage). On Okinawa Island, two roadside trees, On1 and On2, with sparse leaves (level 1) were selected at Nakama, Onna-son Village, Kunigami-gun. In addition, one tree, R10, with abundant leaves with galls of *Q. erythrinae* (level 2) was selected at the University of the Ryukyus, Nishihara-cho, Nakagami-gun. From the selected trees, declining branches were cut or wood blocks with necrotic tissue were cut from the stems and were processed for further observation and the detection of fungi in the laboratory. In the preceding investigation (Kuroda et al. 2017), we found that it is difficult to judge whether *Erythrina* trees are infected or healthy because most of the *Erythrina* trees in Okinawa Prefecture have been infested by *Q. erythrinae* and subsequently have lost some leaves or young shoots. In addition, owners, including the local government, did not permit us to cut down apparently healthy ornamental or roadside trees. Therefore, we used tissues without necrosis and discoloration from slightly affected trees as a kind of control for comparing fungal distribution.

Sampling and identification of beetles. Trunks and branches of declining trees were checked for pinholes and galleries of ambrosia beetles on the surface, or, after harvest, galleries in the xylem of trunks and branches were checked for galleries by splitting specimens with a chisel. When adult beetles were found inside the tissue, they were collected and identified under a binocular microscope based on their morphology.

Macroscopic observation and the procedure of fungal isolation. Harvested branches or stem blocks were cut into smaller pieces and observed with a stereoscopic microscope (SMZ1500, Nikon, Tokyo, Japan), and the cut surfaces were photographed with a digital camera. First, the macroscopic conditions of the phloem and xylem of slightly damaged trees (level 2) were compared with those of severely damaged trees (level 1), and abnormal areas, including discolored xylem and necrotic phloem, were processed for the detection of fungi as follows.

Each specimen (Table 1, Is1 to R10) was dissected into small blocks (5 × 5 × 5 mm) with disposable knife blades. These blocks were washed with 70% ethanol, and the surfaces were sterilized with aqueous sodium hypochlorite (effective chlorine concentration 0.5%). The blocks were placed on potato dextrose agar (PDA) in 9-cm Petri dishes and incubated at 25°C. Another block (2 × 3 × 3 cm) of each sample block was kept in a sterilized empty Petri dish. After 5 days, hyphae emerging from the blocks were directly picked with a lancet and incubated on PDA as described above.

Isolate identification and phylogenetic analysis. For the phylogenetic analysis of isolates, DNA sequencings of the rDNA-ITS gene repeat and the translation elongation factor (*EF-1α*) gene were conducted as follows. Mycelial cultures grown on PDA were suspended in 100 μl of Tris-EDTA buffer, incubated at 95°C for 10 min, and used as a PCR template. The rDNA-ITS was amplified with primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), and *EF-1α* was amplified with primers EF-1H (5'-ATGGGTAAGGAAGCAAGAC-3') and EF-2T (5'-GGAAGTACCAGTGATCATGTT-3') (O'Donnell 2000) in a 20-μl reaction of KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan).

DNA sequencing was conducted using ABI PRISM 3100 and 3130xl Genetic Analyzer (Thermo Fisher Scientific, U.S.A.) at the Biosignal Research Center, Kobe University, including all of the

Table 1. Condition of sampled specimens from declining *Erythrina variegata*

Sampling date	Tree no.	Sampling site	Tree height (m)	Leaf quantity level ^a	Condition of sampled parts
June 21, 2017	Is1	A private garden Ibaruma, Ishigaki City	8	2	Non-discolored xylem
	Is2		10	1	Base of a branch with discolored xylem and necrotic phloem
June 22, 2017	On1	Roadside at Nakama, Onna-son Village, Kunigami-gun	5	1	Base of two branches with discolored xylem and necrotic phloem
	On2		4	1	Base of the trunk with discolored xylem and necrotic phloem
June 23, 2017	R10	Univ. of the Ryukyus Nishihara-cho, Nakagami-gun	7	2	Base of branch with discolored xylem and necrotic phloem

^a Level 1 = small quantity of leaves; level 2 = abundant leaves; and level 3 = healthy condition.

Table 2. Inoculation of healthy *Erythrina variegata* seedlings with two isolates of it-2 of the *Fusarium solani* species complex from two sites on Okinawa and Ishigaki Islands

Treatment	Specimen no.	Number of seedlings	Inoculum (it-2)		Height (cm)	Diameter (mm)	Number of leaves
			Isolate name	Detected site			
Inoculation	Ru-ino 1–24	24	R1-BC6 ^a	Univ. of the Ryukyus, Okinawa Island	23–51	7.6–14.9	18–37
	Is-ino 1–24	24	Is2-f4 ^b	Private garden, Ishigaki Island	24–52	7.4–15.9	13–39
Control	WCt 1–6	6	– ^c		25–52	7.1–13.0	12–29
	NCt 1–6	6	– ^c		21–33	6.5–10.6	20–26

^a Detected from the specimen R1 sampled on May 20, 2014 (Kuroda et al. 2017).

^b Detected from the specimen Is2 sampled on June 21, 2017.

^c Without fungus inoculation on the wounded control (WCt 1–6) and nonwounded control (NCt 1–6).

isolates for comparison with the previously obtained data of strains A and B, it-1 and it-2 (Kuroda et al. 2017). Nucleotide sequences were aligned with the program ClustalW and manually optimized using the program MEGA version 7 (Kumar et al. 2016). Isolates not belonging to the FSSC were identified to the genera level by using the ITS gene sequence as a BLAST query. DNA sequencing of the *EF-1 α* was conducted for the phylogenetic analysis of selected isolates that showed sequence data that were the same as or close to those of it-1 and it-2. We used the sequence data of *F. staphyleae* and 31 FSSC isolates reported by Aoki et al. (2018), Costa et al. (2016), Kasson et al. (2013), O'Donnell et al. (2008, 2015), and Zhang et al. (2006), which were obtained from GenBank for multiple sequence alignment using ClustalW and analysis by MEGA version 7. The phylogenetic relationship was inferred using the maximum likelihood method based on the Jukes–Cantor model (Jukes and Cantor 1969). All positions containing gaps and missing data were eliminated. Nodal supports were assessed via 1,000 bootstrap replicates. *F. staphyleae* was used as an outgroup species in the dendrogram.

Microscopic observation of declining trees. Specimens were processed by light microscopy to observe the distribution of fungal hyphae and the effects of infection. Specimens were cut into small blocks (1 × 1 × 2 to 2 × 2 × 3 cm) and fixed in formalin, acetic acid, and 50% ethyl alcohol (FAA, 5:5:90, v/v) for 1 week. They were subsequently washed for 1 day under tap water. With a sliding microtome (Ritratome REM-710, Yamato Koki Co., Asaka, Saitama, Japan), sections (transverse, tangential, and radial) 20 to 30 μ m thick were cut from the tissue blocks. Some of the sections were stained with periodic acid-Schiff to stain polysaccharides and toluidine blue O to stain fungal hyphae (Feder and O'Brien 1968). A part of each section was mounted onto a glass slide without staining to observe the natural colors and cytological changes. Microscopic observation (ECLIPSE 80i, ECLIPSE Ni-U, Nikon) was focused on the distribution of fungal hyphae and was compared with those of the previous study by Kuroda et al. (2017).

Inoculation experiment. Sixty 1-year-old *E. variegata* seedlings (height, 21 to 52 cm; diameter, 6.5 to 15.9 mm; number of leaves, 12 to 39) were provided by Ryukyu Sankei Co. in Okinawa Prefecture and were used in the inoculation experiment (Table 2). Two isolates—R1-BC6, detected on Okinawa Island in 2014 (Kuroda et al. 2017), and Is2-f4, detected on Ishigaki Island in 2017—were used as inocula. These isolates both belong to the FSSC and, based on

the coincidence of the rDNA-ITS data with primers ITS5 and ITS4, were categorized as it-2. The inocula were cultured on toothpicks placed on PDA in 9-cm Petri dishes at 25°C for 2 weeks. Inoculation was conducted on the lower stems of 48 seedlings on July 31,

Fig. 2. Development of external symptoms in *Erythrina variegata* seedlings for 49 days after the inoculation date. Seedlings numbered 1 to 24 in the sections labeled Ru-ino and Is-ino were those inoculated with the isolate R1-BC6 detected on Okinawa Island or Is2-f4 detected on Ishigaki Island, respectively (Table 2). WCt = control specimens inoculated with sterilized toothpicks; NCt = no-wound control specimens; S = sampling date; O = leaf drooping; ⊙ = leaf wilting; ● = complete defoliation; △ = fluid exudation; ■ = necrosis of cortex and phloem; and × = death.

Fig. 1. Inoculation of healthy *Erythrina variegata* seedlings with toothpicks with mycelia of the it-2 isolates. Four toothpicks were used to pierce the stem 1.5 cm above the ground and at 1-cm intervals upward, shifting 45° between each hole.

Fig. 3. Sampling sites of *Erythrina variegata* specimens and distances between sites.

2017, following the method of Morita et al. (2016) and Sumida et al. (2016). Before the inoculation, four holes were drilled, piercing each stem of *E. variegata* seedlings at 1.5 cm above the ground and at 1-cm intervals upward from the lowest hole, shifting 45° horizontally between each hole (Fig. 1). Then, toothpicks with mycelia were inserted into the four holes. The inoculated sites were covered with Parafilm. Twenty-four seedlings were inoculated with R1-BC6, and the others were inoculated with Is2-f4. As a control without fungus, sterilized toothpicks were inserted into six seedlings. Healthy seedlings were also used as no-wound control specimens (Table 2). The inoculated and control seedlings were placed on the two adjacent balconies under the roof at a 1-m distance separated by a mesh partition at Kobe University. Control specimens were put ~5 m from inoculated seedlings. All seedlings were watered once a day. During this experiment period, from July 31 to September 18, 2017, the temperature was as high as 34.1°C and as low as 22.0°C.

After the inoculation, external symptoms, such as leaf drooping and defoliation, were monitored every day. The death of specimens was determined by complete defoliation and the death of cambial cells and apical meristems. Seedlings were harvested from each inoculated group on the fourth day and at 7-day intervals thereafter (Table 2, Fig. 2). Two control seedlings were harvested on

Fig. 4. A heavily damaged *Erythrina variegata* tree harvested at Ibaruma, Ishigaki Island, on June 21, 2017. **A**, Tree Is2 with dead branches; arrow points to the sampled part. **B**, Xylem discoloration (Dis), phloem, and cambium necrosis (Nec) occurring in the trunk of Is2.

Fig. 5. Galleries of ambrosia beetle *Euwallacea* spp. found in a declining *Erythrina variegata* tree at University of the Ryukyus. **A**, Galleries (arrows) at the exfoliated bark of a dead branch. **B**, Discolored xylem around a gallery (G) in a dead branch. **C**, A cross-sectional microphoto along a beetle gallery (G). Hyphae (arrow h) are elongating from the gallery (G) through vessels (v) and ray tissue (r) and invading into axial parenchyma cells (ap) adjacent to vessels. Stained with toluidine blue O.

Fig. 6. Lateral views of **A**, *Euwallacea funereus*, **B**, *E. fomicatus*, and **C**, *E. piceus* detected in declining *Erythrina variegata* trees in Ishigaki (A) and Okinawa (B, C) Islands.

inoculation day and 49 days later. Internal symptoms were then observed using anatomical techniques. The harvested stems were cut at heights of 3, 13, and 23 cm from the base. The cut surfaces were observed with a stereoscopic microscope. Specimen blocks (1 × 1 × 2 cm) were then taken from the adjacent areas, fixed in FAA, and processed for light microscopy, as in the case of naturally infected trees. The distribution of hyphae in the tissues and the response of host cells to the fungal infection were examined by light microscopy. Tissues of control specimens were compared with those of fungus-inoculated seedlings. Reisolation of the inoculated fungi was conducted with the same method of fungal isolation from naturally infected trees. Tissue blocks (5 × 5 × 5 mm) were cut from harvested seedlings and incubated in PDA. DNA sequencing of the rDNA-ITS gene repeat was conducted to check whether reisolated fungi have the same nucleotide sequences as the FSSC it-2 isolates, R1-BC6 and Is2-f4 of it-2.

Results

Macroscopic and microscopic characteristics of declining trees. The decline and mortality of *E. variegata* were also serious on Ishigaki Island ~400 km south of Okinawa Island across the ocean

(Fig. 3). In Ibaruma, northern Ishigaki Island, galls of *Q. erythrinae* were frequently found on leaves and petioles. Leaves were lost resulting in defoliation of many *E. variegata* trees. In the Onna-son Village, central Okinawa Island, gall formation was also abundant, and the leaves were abnormally reduced on many *E. variegata* trees planted along the road. The conditions of the trees harvested from Ishigaki and Okinawa Islands in 2017 are shown in Table 1. In the trunks or branches of heavily damaged and defoliated trees harvested from Ibaruma, Ishigaki Island (specimen Is2) and from Onna-son, Okinawa Island (specimens On1 and On2) (level 1, Table 1; Fig. 4A), pale gray discolored xylem was observed with the naked eye (Fig. 4B, Dis). Although specimen R10 from the University of the Ryukyus had abundant leaves (level 2, Table 1), a thick branch was dead, and grayish discoloration was found on the crosscut surface of the branch. The bark of the trunks and the branches surrounding the discolored xylem was soft and oozed liquid when pressed with the fingertips. Necrosis of the phloem and cambium (Fig. 4B, Nec) had occurred in the wide area adjacent to the discolored xylem. A putrid smell was detected from the necrotic and softened tissue. The bark of such necrotic parts was exfoliated easily from the trunk surface. In specimen Is1 from Ishigaki Island, which had abundant

Table 3. Tissue condition in declining *Erythrina variegata* trees and dominantly detected fungi

Tree no.	Sampled part	Cultured part	Number of cultured pieces	Number of isolates	Isolate names ^a	FSSC ^b isolate named by rDNA-ITS allele	Other isolated fungi ^c
Is1	Trunk	Non-discolored xylem	10	4	Is1-b5 Is1-b1	it-2 it-3	<i>Scedosporium</i> sp. <i>Curvularia</i> sp.
Is2	Base of a branch	Discolored xylem	28	25	Is2-f2, Is2-g6, Is2-g8 Is2-f4, Is2-f6, Is2-g2, Is2-g7, Is2-g9 Is2-g10, Is2-h3 Is2-g11 Is2-g3, Is2-g5, Is2-g13, Is2-g14 Ig2-g15, Is2-h2	it-1 it-2 it-3 it-4	<i>Scedosporium</i> sp. <i>Peniophora</i> sp. <i>Arthrotrichum</i> sp. <i>Peniophora</i> sp. <i>Geotrichum</i> sp.
		Discolored xylem	25	13	Is2-a2, Is2-b3 Is2-e1, Is2-e3, Is2-e6, Is2-e7 Is2-f8	it-3 it-4	<i>Scedosporium</i> sp. <i>Pythiogeton</i> sp. <i>Arthrimum</i> sp. <i>Hypocrea</i> sp. <i>Ceriporia</i> sp.
		Exfoliated bark	7	4	Is2-c2, Is2-c3	it-1	<i>Botryosphaeria</i> sp. <i>Penicillium</i> sp. <i>Paracremonium</i> sp.
On1	Top of a branch	Discolored xylem	21	20	On1-c1, On1-c2, On1-c3, On1-c4 On1-c8, On1-c9, On1-c11, On1-c13 On1-c18 On1-c5, On1-c6, On1-c7, On1-c10 On1-c12, On1-c14, On1-c15, On1-c16 On1-c19, On1-c20	it-1 it-3	
	Base of a branch	Discolored xylem	51	43	On1-a1, On1-a8, On1-b2, On1-b9 On1-b1, On1-b5, On1-b7, On1-b8 On1-b11, On1-b13, On1-b14, On1-b15 On1-b16, On1-b20, On1-b21, On1-b22 On1-b23, On1-b24, On1-b25 On1-a2, On1-a3, On1-a4, On1-a5 On1-a6, On1-a7, On1-a10, On1-a12 On1-a13, On1-a14, On1-a15, On1-a16 On1-a17, On1-a20, On1-a21, On1-a22 On1-a23, On1-a24, On1-a25, On1-a26	it-1 it-2 it-3	<i>Ceriporia</i> sp. <i>Lasiodiplodia</i> sp.
On2	Basal trunk	Discolored xylem	20	17	On2-a5, On2-a6, On2-b2, On2-b3 On2-a3, On2-a4, On2-a7, On2-c2 On2-c3 On2-a1, On2-a2	it-1 it-2 it-3	<i>Lasiodiplodia</i> sp. <i>Pichia</i> sp. <i>Paracremonium</i> sp.
R10	Base of a branch	Discolored xylem	15	12	R10-a12 R10-a1, R10-a2, R10-a7, R10-a9 R10-a11	it-1 it-2	<i>Nectriaceae</i> sp.

^a The isolates named by authors.

^b FSSC = *Fusarium solani* species complex.

^c Genera identified by ITS sequence and BLAST analysis on GenBank.

leaves and galls (level 2, Table 1), discolored xylem and necrotic tissues were not found.

In the dead branches of specimen R10 and the main stem of Is2, galleries of ambrosia beetles were densely formed in the discolored xylem (Fig. 5A and B), whereas galleries were not found in specimens Is1, On1, and On2. Living ambrosia beetles were found in galleries formed in the xylem of specimens R10 and Is2. Beetles from R10 were identified as *Euwallacea fornicatus* and *E. piceus*, and those from Is2 were *E. funereus* (Fig. 6).

Under the light microscope, pale yellow to brown substances were observed in some vessels and axial parenchyma cells in the discolored xylem of specimens Is2, On1, On2, and R10, as in the case of the previous study (Kuroda et al. 2017). Fungal hyphae were observed in this discolored xylem area. Hyphae had penetrated into the lumen of vessels and wood fibers from ray tissue. In the tissue surrounding the beetle galleries of specimens Is2 and R10, hyphae were abundant in ray tissues and axial parenchyma cells adjacent to the galleries (Fig. 5C).

Table 4. *Fusarium* isolates used and their allele types

Isolate	Host tree	Locality	Island	Allele ^a		Genotype
				rDNA-ITS	<i>EF-1α</i>	
On1-a1	On1	Onna-son	Okinawa Island	it-1	ef-4 (LC490894)	1-4
R10-a12	R10	Univ. of the Ryukyus	Okinawa Island	it-1	ef-5 (LC490895)	1-5
On2-b3	On2	Onna-son	Okinawa Island	it-1	ef-3 (LC490893)	1-3
P2-f11 ^b	P2	Peace Memorial Park	Okinawa Island	it-1 (LC198903)	ef-1 (LC198905)	1-1
On1-b9	On1	Onna-son	Okinawa Island	it-1	ef-1	1-1
On1-c1	On1	Onna-son	Okinawa Island	it-1	ef-1	1-1
On2-a6	On2	Onna-son	Okinawa Island	it-1	ef-1	1-1
R1-BC6 ^b	R1	Univ. of the Ryukyus	Okinawa Island	it-2	ef-1	2-1
On2-a4	On2	Onna-son	Okinawa Island	it-2	ef-1	2-1
On2-c2	On2	Onna-son	Okinawa Island	it-2	ef-1	2-1
On1-a3	On1	Onna-son	Okinawa Island	it-3	ef-1	3-1
Is2-f2	Is2	Ibaruma	Ishigaki Island	it-1	ef-2 (LC490892)	1-2
R10-a11	R10	Univ. of the Ryukyus	Okinawa Island	it-2	ef-8 (LC490898)	2-8
Is2-c2	Is2	Ibaruma	Ishigaki Island	it-1	ef-6	1-6
Is2-c3	Is2	Ibaruma	Ishigaki Island	it-1	ef-6	1-6
Is2-g6	Is2	Ibaruma	Ishigaki Island	it-1	ef-6	1-6
Is2-g8	Is2	Ibaruma	Ishigaki Island	it-1	ef-6	1-6
Is2-f4	Is2	Ibaruma	Ishigaki Island	it-2 (LC476551)	ef-6 (LC490896)	2-6
Is2-f6	Is2	Ibaruma	Ishigaki Island	it-2	ef-6	2-6
Is2-g2	Is2	Ibaruma	Ishigaki Island	it-2	ef-6	2-6
Is2-h3	Is2	Ibaruma	Ishigaki Island	it-2	ef-6	2-6
On1-b1	On1	Onna-son	Okinawa Island	it-2	ef-6	2-6
Is1-b5	Is1	Ibaruma	Ishigaki Island	it-2	ef-7 (LC490897)	2-7
Is2-g7	Is2	Ibaruma	Ishigaki Island	it-2	ef-9 (LC490899)	2-9
Is2-g9	Is2	Ibaruma	Ishigaki Island	it-2	ef-9	2-9
Is2-g10	Is2	Ibaruma	Ishigaki Island	it-2	ef-9	2-9
On1-c5	On1	Onna-son	Okinawa Island	it-3	ef-10	3-10
On1-b12	On1	Onna-son	Okinawa Island	it-3 (LC476552)	ef-10 (LC490900)	3-10
Is1-b1	Is1	Ibaruma	Ishigaki Island	it-3	ef-11 (LC490901)	3-11
Is2-a2	Is2	Ibaruma	Ishigaki Island	it-3	ef-11	3-11
Is2-b3	Is2	Ibaruma	Ishigaki Island	it-3	ef-11	3-11
Is2-g11	Is2	Ibaruma	Ishigaki Island	it-3	ef-11	3-11
On2-a1	On2	Onna-son	Okinawa Island	it-3	ef-11	3-11
Is2-g5	Is2	Ibaruma	Ishigaki Island	it-4 (LC476553)	ef-12 (LC490902)	4-12
Is2-g3	Is2	Ibaruma	Ishigaki Island	it-4	ef-12	4-12
Is2-e1	Is2	Ibaruma	Ishigaki Island	it-4	ef-12	4-12
On1-a8	On1	Onna-son	Okinawa Island	it-1	—	—
On1-b2	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c2	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c3	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c4	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c8	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c9	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c11	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c13	On1	Onna-son	Okinawa Island	it-1	—	—
On1-c18	On1	Onna-son	Okinawa Island	it-1	—	—
On2-a6	On2	Onna-son	Okinawa Island	it-1	—	—
On2-b2	On2	Onna-son	Okinawa Island	it-1	—	—
On1-b5	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b7	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b8	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b11	On1	Onna-son	Okinawa Island	it-2	—	—

(Continued on next page)

^a Accession numbers in GenBank are shown in parentheses.

^b Referred to Kuroda et al. (2017).

Geographical distribution and identification of fungi belonging to the FSSC. Table 3 shows a list of fungi detected from all specimens from three sampling sites (Table 1; Fig. 3). Twenty-three isolates having the same nucleotide sequences of the rDNA-ITS gene that were assigned to the pathogenic isolate it-1 of the FSSC were detected from specimens Is2, On1, On2, and R10. Thirty-three isolates having the same nucleotide sequences of the rDNA-ITS gene as it-2 were detected from all of the harvested trees. Isolates of it-2 have rDNA-ITS genes with only one nucleotide substitution compared

with those of it-1. Fungi grouped as it-1 and it-2 of the FSSC were distributed on both Ishigaki and Okinawa Islands.

DNA sequencing revealed four (it-1 to it-4) and 12 (ef-1 to ef-12) alleles at rDNA-ITS and *EF-1 α* , respectively, among *Fusarium* isolates tested (Table 4). By combining these alleles at the two gene loci, the 36 *Fusarium* isolates were classified into 15 unique multilocus genotypes. These genotypes were designated using abbreviations of each contributing allele. For example, the isolate that shows it-2 and ef-1 alleles was designated as having a 2-1 genotype. A

Table 4. (Continued from previous page)

Isolate	Host tree	Locality	Island	Allele ^a		Genotype
				rDNA-ITS	<i>EF-1α</i>	
On1-b13	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b14	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b15	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b16	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b20	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b21	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b22	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b23	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b24	On1	Onna-son	Okinawa Island	it-2	—	—
On1-b25	On1	Onna-son	Okinawa Island	it-2	—	—
On2-a3	On2	Onna-son	Okinawa Island	it-2	—	—
On2-a7	On2	Onna-son	Okinawa Island	it-2	—	—
On2-c3	On2	Onna-son	Okinawa Island	it-2	—	—
R10-a1	R10	Univ. of the Ryukyus	Okinawa Island	it-2	—	—
R10-a2	R10	Univ. of the Ryukyus	Okinawa Island	it-2	—	—
R10-a7	R10	Univ. of the Ryukyus	Okinawa Island	it-2	—	—
R10-a9	R10	Univ. of the Ryukyus	Okinawa Island	it-2	—	—
On1-a2	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a4	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a5	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a6	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a7	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a10	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a12	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a13	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a14	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a15	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a16	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a17	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a20	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a21	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a22	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a23	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a24	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a25	On1	Onna-son	Okinawa Island	it-3	—	—
On1-a26	On1	Onna-son	Okinawa Island	it-3	—	—
On1-b10	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c6	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c7	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c10	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c12	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c14	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c15	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c16	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c19	On1	Onna-son	Okinawa Island	it-3	—	—
On1-c20	On1	Onna-son	Okinawa Island	it-3	—	—
On2-a2	On2	Onna-son	Okinawa Island	it-3	—	—
Is2-e3	Is2	Ibaruma	Ishigaki Island	it-4	—	—
Is2-e6	Is2	Ibaruma	Ishigaki Island	it-4	—	—
Is2-e7	Is2	Ibaruma	Ishigaki Island	it-4	—	—
Is2-g13	Is2	Ibaruma	Ishigaki Island	it-4	—	—
Is2-g14	Is2	Ibaruma	Ishigaki Island	it-4	—	—
Is2-g15	Is2	Ibaruma	Ishigaki Island	it-4	—	—
Is2-g16	Is2	Ibaruma	Ishigaki Island	it-4	—	—
Is2-h2	Is2	Ibaruma	Ishigaki Island	it-4	—	—

Fig. 7. Maximum likelihood phylogenetic tree based on *EF-1 α* nucleotide sequences. Numbers at nodes represent bootstrap support >50% from 1,000 replicates. Three clades in the *Fusarium solani* species complex and the ambrosia *Fusarium* clade are indicated by bold internodes. Accession numbers and NRRL (the ARS Culture Collection) or FRC (*Fusarium* Research Center) numbers are shown. Isolates are denoted by the isolate name with the genotype in parentheses as shown in Table 4.

Nucleotide sequence analysis of the ITS and the *EF-1 α* indicated that it-2, it-3, and it-4 belong to clade 3 of the FSSC as defined by O'Donnell et al. (2008), which is the same as it-1 (Kuroda et al. 2017, P2-f11; Table 4). Figure 7 shows the phylogenetic tree inferred from the nucleotide sequences of the *EF-1 α* using 31 *Fusarium* isolates from GenBank and 36 representative *Fusarium* isolates that were detected at all sampling sites. Each isolate shows the isolate name with its genotype on the phylogenetic tree. Specifically, all of our isolates belong to the ambrosia *Fusarium* clade (AFC). Fungi isolated at a lower frequency were not identified because they were less likely to be pathogens.

Development of external symptoms after inoculation with two isolates of it-2. Representative characteristics of external symptoms are shown in Figures 8 and 9. All 48 seedlings inoculated with isolates of either R1-BC6 or Is2-f4 showed external symptoms. Twelve of these seedlings finally died, but some of the inoculated seedlings survived (Fig. 2). Leaf drooping was observed starting 3 or 4 days after inoculation in 58.3 and 66.7% of seedlings inoculated with isolates from Okinawa Island and Ishigaki Island, respectively (Fig. 2, open circle and double circle). Defoliation then occurred rapidly in 75 and 95.8% of the seedlings inoculated with each isolate within a few days of leaf drooping, or even without symptoms on leaves (Figs. 8B, 8C, and 9). Complete defoliation occurred on 50 and 58.3% of the seedlings inoculated with each isolate beginning 4 days after inoculation (Fig. 2, filled circle; Fig. 8D). Red to brown fluid began to exude from the lower stems of seedlings with the symptom of defoliation (Fig. 2, open triangle; Fig. 8E). Necrosis of the cortex and phloem with an odor occurred starting 21 days after inoculation (Fig. 2, filled rectangle). Symptoms did not develop synchronously among the fungus-inoculated seedlings. Similarly, death also occurred independently; for instance, five seedlings with R1-BC6 died between 8 and 35 days after inoculation, whereas seven seedlings with Is2-f4 died between days 14 and 49. Symptom development ceased in a few seedlings with partial defoliation, and one or two shoots sprouted at the inoculation sites (Fig. 9). Isolate Is2-f4 from Ishigaki Island demonstrated rapid symptom development compared with R1-BC6 from Okinawa Island. All control seedlings showed no external symptoms.

In the reisolation experiment, the two isolates either R1-BC6 or Is2-f4 of it-2 were reisolated from tissues adjacent to the inoculation sites of fungus-inoculated seedlings. In contrast, fungi used for the inoculation experiment were not detected in any of the 12 control seedlings.

Development of internal symptoms after inoculation with two isolates of it-2. Internal symptoms observed in the tissue around the inoculation sites are shown in Figure 10. Xylem discoloration detectable with the naked eye became more widespread in the later period, especially 14 days after inoculation. In seedlings without external symptoms, xylem discoloration had not spread. In the completely defoliated seedlings, the area of xylem discoloration covered a large proportion of the stem's cross section. In dead specimens around 21 days after inoculation, the xylem was completely discolored and had been desiccated. In some inoculated seedlings, cambial activity continued partially as defoliation progressed. Tissues of control seedlings without wounds had no abnormalities, and those with just a wound had formed narrow dark-colored discoloration around the drilled wound.

Under a light microscope, pale yellow to brown substances were observed in parenchyma cells in the above-mentioned discolored xylem. The colored substances had plugged the lumen of some vessels (Fig. 11). Fungal hyphae were densely distributed in vessels and axial parenchyma cells around the inoculation sites. In all of the control seedlings, no hyphae were present in the stem tissues. In the controls with wounds, a small amount of a pale-brown substance had accumulated in some vessels just around the wound.

Discussion

In the present study, 25% of seedlings inoculated with isolates characterized as it-2 (R1-BC6 from Okinawa Island or Is2-f4 from Ishigaki Island) were killed after the development of symptoms, such

as abnormal defoliation and necrosis of phloem with odor, similarly to naturally declining trees. Compared with the case of it-1 (P2-f11), which killed one-third of the inoculated seedlings (Kuroda et al. 2017), the mortality rate and internal and external symptoms caused by the inoculation of it-2 isolates were the same. By the successful reisolation of it-2 FSSC fungi from dead seedlings, we judged that the two it-2 isolates belonging to the FSSC (R1-BC6 and Is2-f4) are pathogenic to *E. variegata*.

In the artificial inoculation with isolates of it-2, internal symptoms—(i) gray to brownish xylem discoloration associated with the stoppage of xylem sap movement and (ii) necrosis of cambium and phloem in the area of fungal distribution—were the same as those of naturally dying trees. They were also same as seedlings inoculated with an isolate of it-1 (Kuroda et al. 2017). Xylem discoloration is known to occur as a defense mechanism to protect trees from microbial invasion by secondary metabolites, and the discolored area is called “wound heartwood” or “pathological heartwood” (Hillis 1987). Water conduction stops in wound heartwood; therefore, the xylem sap flow decreases as the discolored xylem area enlarges in tree stems (Kuroda and Yamada 1996). The mechanism of tree wilt has been well explained in preceding reports on Japanese oak wilt caused by *Raffaelea quercivora* (Kuroda 2001) and fig canker caused by *Ceratocystis fusicola* (Kajii et al. 2013). During experiments in which fig seedlings were inoculated with pathogenic *C. fusicola* (Morita et al. 2016; Sumida et al. 2016) and *Pittosporum tobira* seedlings with pathogenic *F. solani* (Yamaoka et al. 2015), severe and wide discoloration occurred in the xylem, and the vessels became dysfunctional due to the production of secondary metabolites. Due to the similarities in the internal symptoms of these tree diseases, the decline and mortality of *E. variegata* trees infected with the FSSC will also be categorized as a wilt disease. The survival of inoculated seedlings was due to a small amount of water conduction maintained in the xylem that was newly formed by the partial survival of the cambium after infection. Although the cause of abnormal defoliation of *E. variegata* has been believed to be due to infestation by the gall wasp, *Q. erythrinae*, the present results and the preceding report by Kuroda et al. (2017) suggest the possibility that defoliation may be accelerated by the water deficit in upper tree bodies infected with pathogenic fungi.

The decline and mortality of *E. variegata* trees also occurred on Ishigaki Island, located 400 km south of Okinawa Island, with the same symptoms. From the fact that the nucleotide sequences of the ITS of 11 isolates obtained from Ishigaki Island were in accordance with the isolates of it-1 and it-2 found on Okinawa Island (Kuroda et al. 2017), *Erythrina* decline is caused by the specific fungi of the FSSC widely including Ishigaki Island. Fungi belonging to it-1 and it-2 were also widely distributed on Okinawa Island, judging from their detection in the Onna-son Village, located 70 km north of Peace Memorial Park. However, the history of the wide distribution of these fungi over hundreds of kilometers is unknown. The route of pathogen spread from dead trees to healthy trees is unknown.

F. solani f. sp. *mori*, a pathogen of *Pittosporum tobira* and a member of the FSSC, also has a wide distribution ~1,650 km inside Japan (Masuya et al. 2012), and the distribution history is similarly unknown.

The route of pathogen spread at close range was hypothesized to be as follows. *Fusarium* spp. mainly inhabit soil, and the infection of adjacent trees occurs frequently via root contact underground (Aoki et al. 2014). The present and preceding investigations (Kuroda et al. 2017) found that adjacent *Erythrina* trees planted along roads often die off sequentially. This suggests that infection at close range occurs via soil and root contact; therefore, we are currently investigating the fungi inhabiting soil under declining trees. The process of disease spread in a wide region over the sea is still unknown. In Taiwan, located ~320 km from Ishigaki Island, *E. variegata* is also severely declining, and infestation by the gall wasp *Q. erythrinae* was first reported in 2003 (Yang et al. 2004). In addition, we have information that *Fusarium solani* has been found in declining *E. variegata* trees there. The fungi are possibly the same or closely related to isolates of it-1 and it-2. We may need to consider some contribution of human activities for the long-distance spread of pathogens.

Naturally declining trees with thick trunks, commonly over 30 cm in diameter, demonstrate slower symptom development, and it takes more than several years from the decrease of leaves to phloem necrosis and complete death. If trees take many years from infection to mortality, the declining thick trees will have a long history after infection. In such cases, investigating infection routes and the contribution of vectors is difficult because we cannot obtain newly infected trees.

Fig. 9. Defoliation of *Erythrina variegata* seedlings inoculated with the it-2 isolate (Is2-f4). Each line shows the number of leaves of each specimen until harvesting.

Fig. 8. External symptoms observed in *Erythrina variegata* seedlings inoculated with the it-2 isolate (R1-BC6): examples of specimen Ru-no 8 (A to D) and Ru-no 12 (E) are shown. A, Healthy seedling on the day of inoculation. B, Leaf drooping 3 days after the inoculation. C, Defoliation 4 days after inoculation. D, Death 7 days after inoculation. E, Fluid exudation (arrow) from the lower stem 4 days after inoculation.

By phylogenetic analysis, all isolates that were grouped into it-1 to it-4 based on the analysis of ITS were revealed to be members of the AFC. AFC members are symbionts of ambrosia beetles (Kasson et al. 2013). *F. pseudensiforme*, known as a pathogen of Fusarium dieback of avocados (Eskalen et al. 2012; Kasson et al. 2013), is phylogenetically very close to some isolates from *E. variegata*, including the pathogenic ones, P2-f11, R1-BC6, and Is2-f4. Another two isolates (On1-b12 and On1-c5) that have the 3-10 genotype are very close to AF17338 NRRL 22354, which has not been named, in the phylogenetic tree. Three isolates (Is2-g5, Is2-g3, and Is2-e1) that have the 4-12 genotype might be *F. oligoseptatum*, known as a pathogen that causes the decline of *Ailanthus altissima* (tree of heaven) (Aoki et al. 2018). Two pathogenic FSSCs—*F. euwallacea*, which is another pathogen of avocados, and *F. ambrosium*, which is a pathogen of tea trees (O'Donnell et al. 2015)—are also close to some isolates detected in this study. Almost all isolates were genetically close to each other, even though they were distributed in different places of Ishigaki and Okinawa Islands. From the above-mentioned conditions of the phylogenetic data, it seems difficult to name the fungi pathogenic to *E. variegata* as a new species because there are a number of unknown fungi close to our isolates. Phylogenetic research should be continued so the pathogenic fungi can be named.

Among ambrosia beetles, the genus *Euwallacea* is known as a symbiont of *Fusarium* spp. in the AFC (Freeman et al. 2013; O'Donnell et al. 2015). We found *Euwallacea fornicatus* (the tea shot-hole borer), which is reportedly a symbiont of *F. ambrosium* and *F. euwallacea*, in declining *E. variegata* trees at the University of the Ryukyus. This species has a wide host range of more than 100 species of plants and trees, including avocado and tea in Asia (Eskalen et al. 2013; Freeman et al. 2013; O'Donnell et al. 2015; Ploetz et al. 2013) and *E. variegata* in China (Li et al. 2016). This ambrosia beetle is known as an insect pest of mango trees in Japan (Yamaguchi et al. 2006). Among other two beetle species, *E. piceus* is widely distributed in Oceania, Africa, and Asia (Wood and Bright 1992), and *Erythrina subumbrans* (December tree) is known as one of the host plants. This species was first

discovered by us in living trees in Japan. The last one, *E. funereus*, is found in Oceania and Southeast Asia, and the hosts trees are *Sterculia alata* and *Ficus infectoria* (Beaver and Liu 2010). This species was also first described by us in Japan. Judging from the data of *Euwallacea interjectus* as a vector of the pathogen *C. ficicola* (Kajii et al. 2013; Morita et al. 2016; Sumida et al. 2016), detailed field research will be necessary to examine the relationship between ambrosia beetles and the distribution of pathogens of *E. variegata* trees.

In conclusion, the present study revealed that fungi belonging to it-1 and it-2 are pathogenic to *E. variegata*, categorized in the AFC of the FSSC, and are responsible for the mortality of *E. variegata* trees in a geographically wide area of Okinawa Prefecture, Japan. Besides pathogenic isolates of it-1 and it-2, such as P2-f11 (Kuroda et al.

Fig. 11. Accumulation of pale yellow to brown substances (cs) observed in the vessels (v) and axial parenchyma cells (ap) as a defense reaction. Cross section of the specimen Ru-ino 12 without staining.

Fig. 10. Macroscopic view of internal symptoms observed on the cross and radial sections of seedlings inoculated with the it-2 isolates. No symptoms (14 days): discoloration progressed in the xylem, phloem, and cortex. Partial defoliation (21 days): wide discoloration in the xylem, but for the regeneration of cambium and additional xylem formation, water conduction was partly maintained. Complete defoliation and death (14 and 21 days): complete xylem discoloration and necrosis of the cambium. Control (49 days): no discoloration in the xylem but only around the inoculation sites. Dis = discolored xylem; NDi = non-discolored xylem; Ino = inoculation site; NX = newly formed xylem; Fn = functional xylem; and Nec = necrotic cambium and phloem.

2017), Is2-f4, and R1-BC6, isolates genetically close to them were found and grouped as it-3 and it-4 in Okinawa Prefecture. Detailed research should be conducted on the pathogenicity and the phylogenetic relationship between them. Judging from data that *F. solani* was found in declining *Erythrina* spp. in Pakistan and India (Faizal et al. 2006; Javaid et al. 2005), genetically close species in the FSSC may be widely distributed in tropical and subtropical Asia and may be pathogenic to other *Erythrina* spp. or other tree species, such as tea or mango. Unfortunately, there is insufficient information on the FSSC found in Pakistan and India because these fungi were just optically identified without DNA analysis. We cannot tell whether their fungi are phylogenetically the same or close to the pathogenic isolates. Investigations on the distribution of the AFC species will be necessary across a wide area of tropical Asia, Hawaii, and other regions of *Erythrina* decline and mortality.

Two important subjects remain to be investigated. First, the effects of *Q. erythrinae* and the FSSC should be analyzed separately. *Q. erythrinae* is responsible for decreased flowering by gall formation, but the wasp cannot cause tree mortality. The effect of *Q. erythrinae* on the physiological condition of *Erythrina* trees is still unclear. One possibility is that the wasp infestation might add some stress to the host and promote the pathogen's activity. The second possibility is that infection with the pathogenic FSSC may decrease the protective reaction of hosts and facilitate the wasp infestation. The third is that the two organisms do not cooperate at all in the decline of *E. variegata* trees. Judging from the fact that *E. variegata* had been spontaneously dying in Okinawa Prefecture before the discovery of *Q. erythrinae* infestation in that area (Kameyama 2018), the pathogen seems to kill trees without the wasp's contribution. Further detailed investigations are necessary on the physiological aspects of host trees.

The second subject is how to control this wilt disease. As a protective procedure in Okinawa Prefecture, insecticide has been injected to kill the larvae of *Q. erythrinae* in galls. Although *Q. erythrinae* is not the cause of *Erythrina* mortality, Okinawa Prefecture is starting to biologically control *Q. erythrinae* with *Eurytoma erythrinae*, a wasp indigenous to Africa, following the strategy demonstrated in Hawaii (Bell et al. 2013). This method has not proved to be effective in preventing decline in Hawaii (Yalemar et al. 2009), and the induction of foreign species for pest control should be reconsidered because we cannot predict the impact of the induced organisms on domestic species. Based on the results of our investigations, we recommend fungicide injections to prevent the colonization of pathogenic fungi in the trunk. To keep *E. variegata* flowering and decrease mortality, pathogenic fungi should be controlled. Otherwise, planting *Erythrina* spp. that are native to Africa and known to be resistant to *Q. erythrinae* infestation (Messing et al. 2008) will be effective at maintaining flowering and as another procedure to prevent infection with the pathogenic *Fusarium* spp.

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