

ORIGINAL ARTICLE

Field survey of Fusarium stem rot of lisianthus (*Eustoma grandiflorum*) cultivated in Okinawa, Japan

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Abstract

Lisianthus (*Eustoma grandiflorum*) has become a major flowering plant in Okinawa, the southernmost prefecture of Japan. Its cultivation area has increased steadily with each passing year for two decades. Simultaneously, many types of lisianthus diseases related to damping-off symptoms have also increased dramatically. To create a strategy for preventing the disease, disease symptoms and pathogenic organisms of primary problematic disease with seasonal variation in the emergence were investigated. The symptoms were diagnosed as Fusarium stem rot (Kukigusare-byo) and the pathogen of the disease was identified as *Fusarium avenaceum* based on multigene sequences analyses. Indeed, the PCR result of the isolated strain in this study was the same as that isolated from lisianthus plants with Fusarium stem rot in Hokkaido Prefecture. Furthermore, the pathogen is clustered separately from the other *F. avenaceum* strains isolated from lisianthus in the USA. Diseased lisianthus plants spread throughout greenhouses even though several fungicides were applied. Additionally, they appeared from November to January and increased to 0.3% of the total number. Fusarium stem rot was found in 43.8% of the total number of farms from 2020–2021 in Okinawa Main Island.

Keywords: conidia, Fusarium stem rot, Kukigusare-byo, PCR detection, phylogenetic analysis, sporodochia

Introduction

In Okinawa, lisianthus (*Eustoma grandiflorum*) has become a major flowering plant. Okinawa's subtropical climate enables lisianthus shipping at a high price to other prefectures in Japan during winter and spring (from December to May) when other prefectures find it relatively challenging to grow them for commercial purposes. Although this flowering plant has been cultivated for only about two decades in Okinawa, its cultivation area has increased steadily with each passing year. In 2019, according to the official figures of the Okinawa prefectural government, the following data were recorded: (1) cultivation area – 1,221a; (2) the number of shipments – 2.46 million; and (3) the shipment value – 364 million yen. In 2019, the amount of production was recorded as the 10th highest in Japan.

From 2013–2015, there was a dramatic improvement in its cultivation with associated factors, such as cultivation area increased by 4.8 times, the number of shipments increased by 3.2 times, and the number of farmers increased by 5.3 times. However, simultaneously, many types of lisianthus diseases also increased dramatically. Moreover, various symptoms appearing in production sites are associated with damping-off, accordingly reflecting the variety of pathogenic organisms. Hence, a detailed examination should be conducted to identify lisianthus diseases related to damping-off to create a strategy for preventing these diseases. Recently, we identified strains showing diseases similar to Fusarium stem rot in greenhouses of farmers in Okinawa Main Island. Fusarium stem rot,

an extremely problematic disease of lisianthus, was first found to be caused by *Fusarium avenaceum* in each of the regions in Hokkaido Prefecture, Japan around 1986 (Iwata 1991; Horita and Kodama 1995). Consequently, the disease has spread all over Japan, such as to the Ibaraki and Kumamoto prefectures (Tomita *et al.* 2005). Additionally, *F. avenaceum* is isolated from lisianthus in many states of the United States, including Florida, California, and Connecticut and in many other countries (Nalim *et al.* 2009). In this study, disease symptoms and pathogenic organisms of *Fusarium* stem rot, with seasonal variation in the emergence of the disease in Okinawa Main Island were investigated.

Materials and Methods

Lisianthus sample

Lisianthus strains were planted in four rows in a furrow and resulted in about 950 plants in one furrow in greenhouses A and B of a farmer in Okinawa City (Supplementary Fig. 1). “Engage Boy” and “Moana Light Pink” were each planted in two furrows alternately in greenhouse A on October 16th, 2020. “Celeb Christal” and “Celeb Grape” were planted in two furrows alternately in greenhouse B on November 6th, 2020. Greenhouses A and B were separate but stood side by side. All plants of all varieties were investigated and the plants that had *Fusarium* stem rot-like symptoms were sampled each day. Furthermore, lisianthus plants that exhibited *Fusarium* stem rot-like symptoms from each of the regions in the Okinawa Main Island were sampled from a total of 32 farmers from various regions: seven from Nakijin village, one from Motobu town, and seven from Nago city in the northern area; one each from Okinawa City (the same as above) and Uruma city in the middle area; and six from Yaese town, three from Nanjo city, and six from Itoman city in the southern area.

Fungal isolation

Small pieces of infected stems were cut from the edge of significant lesions using a sterile blade. The pieces were soaked in 70% ethanol for 30 s and 1.0% hypochlorous acid for 60 s, then soaked twice in sterile distilled water for a few seconds. After the pieces were dried on sterile paper, they were placed on an agar plate. The plates were incubated at 25°C and examined to determine if hyphal grew from the pieces 3–4 days later. When hyphal grew from a piece, the hyphal tip was cut with agar and moved to a potato dextrose agar (PDA, Difco Laboratories) plate. To confirm the appearance

of fungi, the PDA plate was incubated at 25°C for a week until the hyphae almost covered the entire plate.

Koch’s postulates

For the Koch’s postulates test to determine the causal agent of the disease, a young lisianthus seedling (“Celeb Pink”) was used. Mycelia of the fungal isolates, R2-30 cultured on a PDA plate, were placed on the stem of the seedling alongside a piece of PDA. Three seedlings were placed in sealed boxes with damp paper under greenhouse conditions at ~25°C. Fungi were reisolated from the diseased parts of the seedlings onto an agar plate and the hyphal tip was moved to a PDA plate. Subsequently, the morphologies of the fungi were examined to fulfill the last stage of Koch’s postulates (Bradbury 1970).

PCR detection

Fungal genomic DNA of R2-30 was extracted using a DNeasy Plant Mini Kit following the manufacturer’s protocols (QIAGEN). PCR amplification was conducted in a 25- μ l reaction mixture in a GeneAmp PCR system 9700 (Applied Biosystems Inc.). The reaction mixture was comprised of 12.5- μ l Sapphire Amp Fast PCR Master Mix, 1- μ l JIAf (10-pmol/ μ l; 5’-GCTAATTCTTAACTTACTAGGGGCC-3’) and JIAr (10 pmol/ μ l; 5’-CTGTAATAGGTTATT-TACATGGGCG-3’) primers (Turner *et al.* 1998), 1- μ l fungal genomic DNA template (approximately 30-ng), and 9.5- μ l sterile distilled water. Amplification was conducted using the following cycling conditions: primary denaturation for 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, and a final extension step for 5 min at 72°C (Turner *et al.* 1998). The PCR product of about 300 bp was confirmed using gel electrophoresis.

Sequencing and phylogenetic analysis

Genomic DNA from fungal mycelium of R2-30 grown on potato dextrose agar (PDA; Becton Dickinson, USA) was extracted via physical disruption using beads (Nippon Gene Co., Ltd.). Four loci, i.e., nuclear ribosomal internal transcribed spacer (ITS) regions, including ITS1, 5.8S, and ITS2, partial translation elongation factor (*tef1*), partial RNA polymerase largest subunit (*rpb1*), and partial RNA polymerase second largest subunit (*rpb2*) gene regions, were amplified and sequenced, respectively. The primers used in this study are listed in Supplementary Table 1. Polymerase chain reactions (PCR) were performed with BIOTAQ DNA Polymerase (Bioline, UK) for ITS, *tef1*, and *rpb2*. Amplification was performed with the

following cycling conditions: primary denaturation for 5 min at 94°C, 40 cycles of 30 s at 94°C, 40 cycles of 30 s at 52°C, 40 cycles of 30 s at 72°C and a final extension step for 7 min at 72°C. For *rpb1*, PCR was performed with TaKARA Ex taq (Takara Bio, Japan). Amplification was performed using the following cycling conditions: 40 cycles of 10 s at 98°C, 40 cycles of 30 s at 55°C, 40 cycles of 1 min at 72°C and a final extension step for 7 min at 72°C. The sequences were assembled with ChromasPro 2.1.10 (Technelysium Pty, Ltd., South Brisbane QLD, Australia). Multiple alignments were performed with CLUSTALW (Thompson *et al.* 1994), and the final alignments were manually introduced. Ambiguous positions and alignment gaps were excluded from the analysis. The neighbor-joining (Saitou and Nei 1987) phylogenetic tree with the Kimura two-parameter model (Kimura 1980) was constructed using the MEGA ver. 7.0 (Kumar *et al.* 2016). A bootstrap test with 1000 iterations was used to assess the reliability of the branches (Felsenstein 1985). The positions with gaps and the regions of uncertain nucleotide alignment were excluded from the phylogenetic

analyses. Neighbor-joining trees were constructed on the basis of each gene or concatenated sequence.

Results

Diagnosis of *Fusarium* stem rot

Symptoms of *Fusarium* stem rot on lisianthus plants were observed in greenhouses A and B in Okinawa City. Diseased lisianthus plants exhibited brown discoloration in the stem from the lowest part near the root (Fig. 1A and B). Furthermore, leaves became discolored, turning brown from the side of the stem, which kept spreading. Moreover, the entire plant wilted. Furthermore, there were cases in which orange sporodochia were formed on the lowest part of the stem when the disease progressed (Fig. 1B and C). The conidia was crescent shaped (Fig. 1D). The fungi which appeared to be *Fusarium*, R2-30 was isolated primarily from the infected part of lisianthus plants, “Moana Light Pink”. Mycelia of the fungal isolates were



Fig. 1. Symptoms of *Fusarium* stem rot and sporodochia on lisianthus plants: A – the plant of variety “Celeb Grape” with disease symptoms (April 15, 2021); (B–C) – orange sporodochia formed on “Celeb Christal” (January 4, 2021); (D) crescent shaped conidia observed through a light microscope

white or pale yellow on a PDA plate and colored a PDA plate dark red or pale red (Fig. 2A). *Fusarium avenaceum* specific primers, JIAf/r amplified a single PCR product of the expected size (about 300 bp; Harukuni and Fujio 2016) from all tested *F. avenaceum* isolates but not from an *F. oxysporum* isolate causing Fusarium wilt (Fig. 2B). The symptoms of Fusarium stem rot reported in Hokkaido (The Phytopathological Society of Japan 2022; Harukuni and Fujio 2016) were the same as those in this study. As a result, these symptoms were diagnosed as Fusarium stem rot (Kukigusare-byo).

Koch's postulates

Koch's postulates showed that mycelia of the fungi were extended on the stem ~7 days following inoculation, and it was confirmed that the stem was infected by the fungi. Control seedlings were asymptomatic. Reisolated fungi grew on PDA plates after 7 days of incubation and grown fungi exhibited the same appearance.

Phylogenetic analysis of *Fusarium avenaceum* isolate, R2-30

ITS sequence-based NJ phylogeny (Supplementary Fig. 2) showed that R2-30 (MAFF247602) and *F. avenaceum*, including one strain from *Eustoma grandiflorum* (lisianthus) in Poland, and related *F. tricinctum* species complex (FTSC) species formed a monophyletic lineage with moderate bootstrap support (92%). The bootstrapped NJ phylogeny inferred from *tef1*, *rpb1*, and *rpb2* genes also demonstrated a monophyletic lineage with a *F. avenaceum* in the FTSC clade (Supplementary Figs 3–5). Furthermore, the concatenated NJ phylogeny generated from *tef1*, *rpb1*, and *rpb2* genes sequences showed that R2-30 and *F. avenaceum* formed a monophyletic cluster in the FTSC clade with moderate bootstrap support (100%) (Fig. 3). Therefore, R2-30 was identified as *F. avenaceum* based

on multigene sequence analyses. The bootstrapped NJ phylogeny inferred from *tef1* gene demonstrated divergence of R2-30 and *F. avenaceum*, including 15 strains isolated from lisianthus in the USA as referred from Nalim *et al.* (2009), in the clade of FTSC (Fig. 4), and R2-30 is clustered separately from the other *F. avenaceum* strains from lisianthus.

The occurrence of Fusarium stem rot

Lisianthus strains diseased with Fusarium stem rot spread throughout the two greenhouses, regardless of the variety (Fig. 5A). Furthermore, the diseased plants appeared at the early growth stage to the late growth stage, from November to January (Fig. 5B). The rate of increase was high from November to January in greenhouse A, whereas it was high in January and April in greenhouse B. In January Fusarium stem rot occurred frequently in both houses. Eventually, the accumulated numbers of diseased plants in greenhouses A and B were 26 and 18, which were 0.3% and 0.2% of the total number, respectively. No differences in the degree of disease between “Engage Boy,” “Moana Light Pink,” “Celeb Christal,” and “Celeb Grape” were observed morphologically. Furthermore, as for the occurrence rate on the investigated farms, Fusarium stem rot occurred in 43.8% of the 32 farms in each of the regions in Okinawa Main Island (Fig. 6).

Discussion

Fusarium avenaceum is predominantly soil-borne and common in temperate regions throughout the world, but it is also reported in warmer or subtropical regions (Leslie and Summerell 2006). This species is also known to be a phytopathogenic fungus which can cause stem and root disease in various plant hosts,

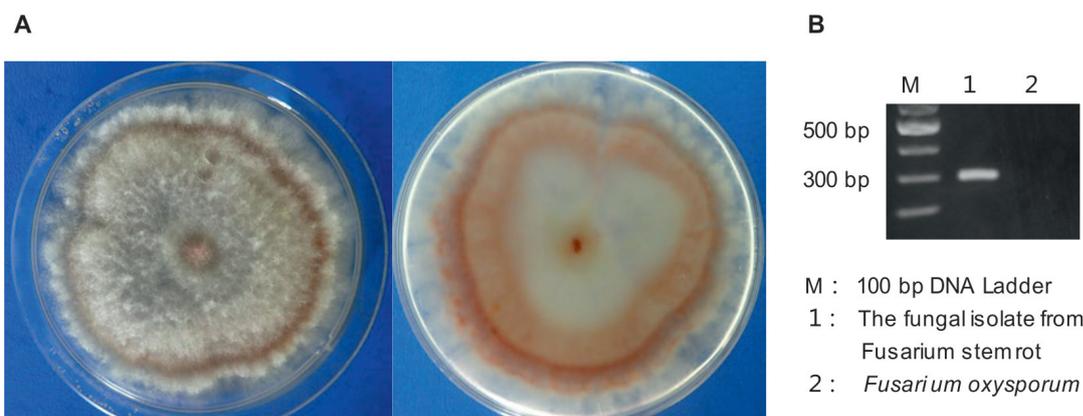


Fig. 2. The isolate grown on a potato dextrose agar (PDA) plate (A) and PCR detection using JIAf/r primers (B)

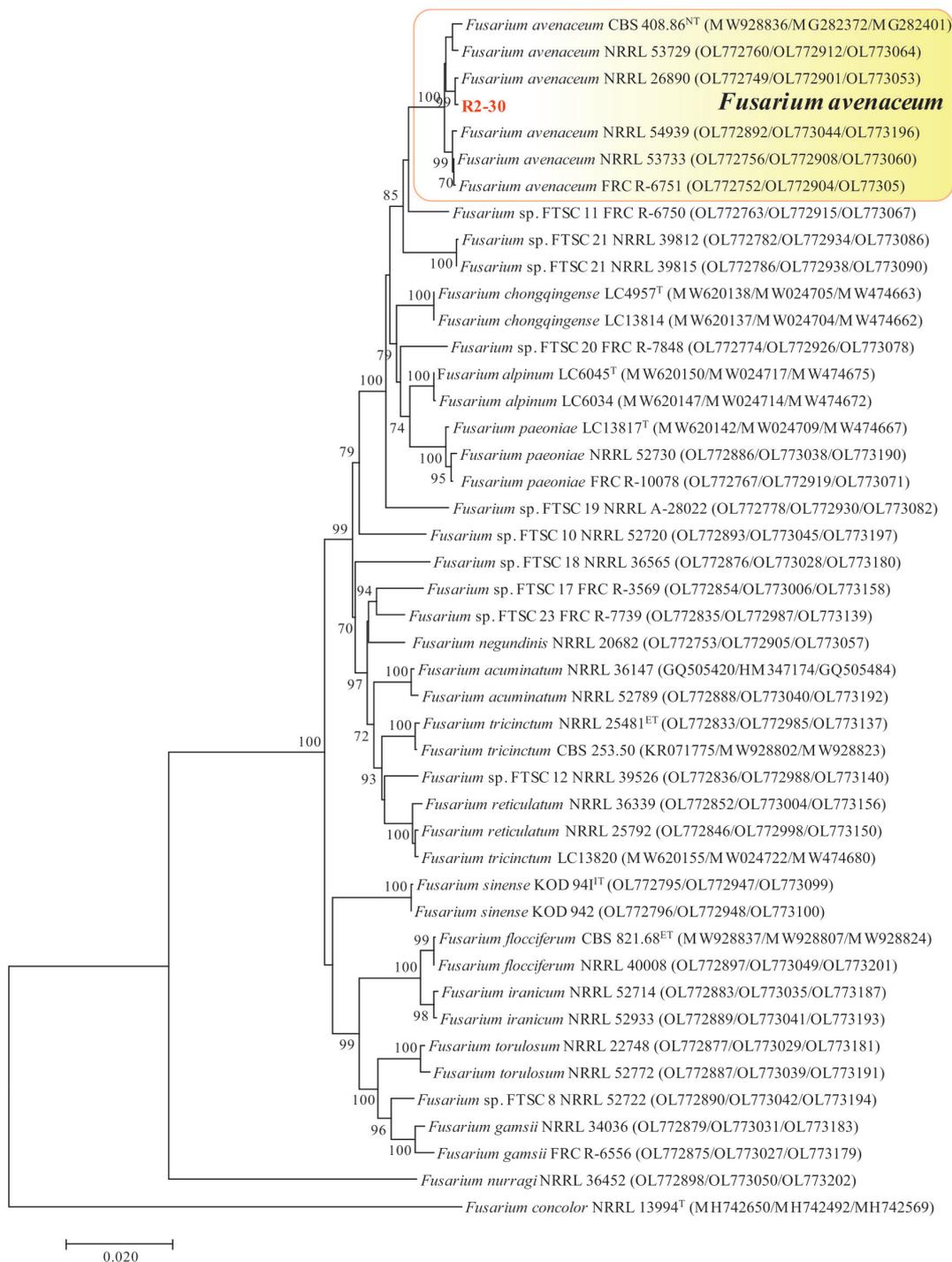


Fig. 3. Neighbor-Joining (NJ) phylogeny inferred from combined *tef1*, *rpb1*, and *rpb2* sequences of R2-30 and species of *Fusarium tricinctum* species complex. Numbers on the nodes are NJ Bootstrap values above 70%. Ex-type, ex-epitype, isotype, and neotype strains are indicated with ^T, ^{ET}, ^{IT} and ^{NT}, respectively

and head blight in wheat. This species is not associated with any human or animal toxicoses/pathogens, however, it produces mycotoxins such as beauvericin, fusarin C, and moniliformins (Leslie and Summerell 2006). *Fusarium avenaceum* from lisianthus were scattered within the clade of FTSC, and did not form distinct groups based on host species or locality (Nalim et al. 2009; Laraba et al. 2022; Yli-Mattila et al. 2022).

In our molecular phylogenetic analysis of *tef1* gene, our isolate, R2-30, and *F. avenaceum* from lisianthus demonstrated divergence of phylogenetic position (Fig. 4). This suggests that *F. avenaceum* strains from lisianthus may contain a number of cryptic species.

In the Database of Plant Diseases in Japan (https://www.gene.affrc.go.jp/databases-micro_pl_diseases.php) and Common Names of Plant Diseases in

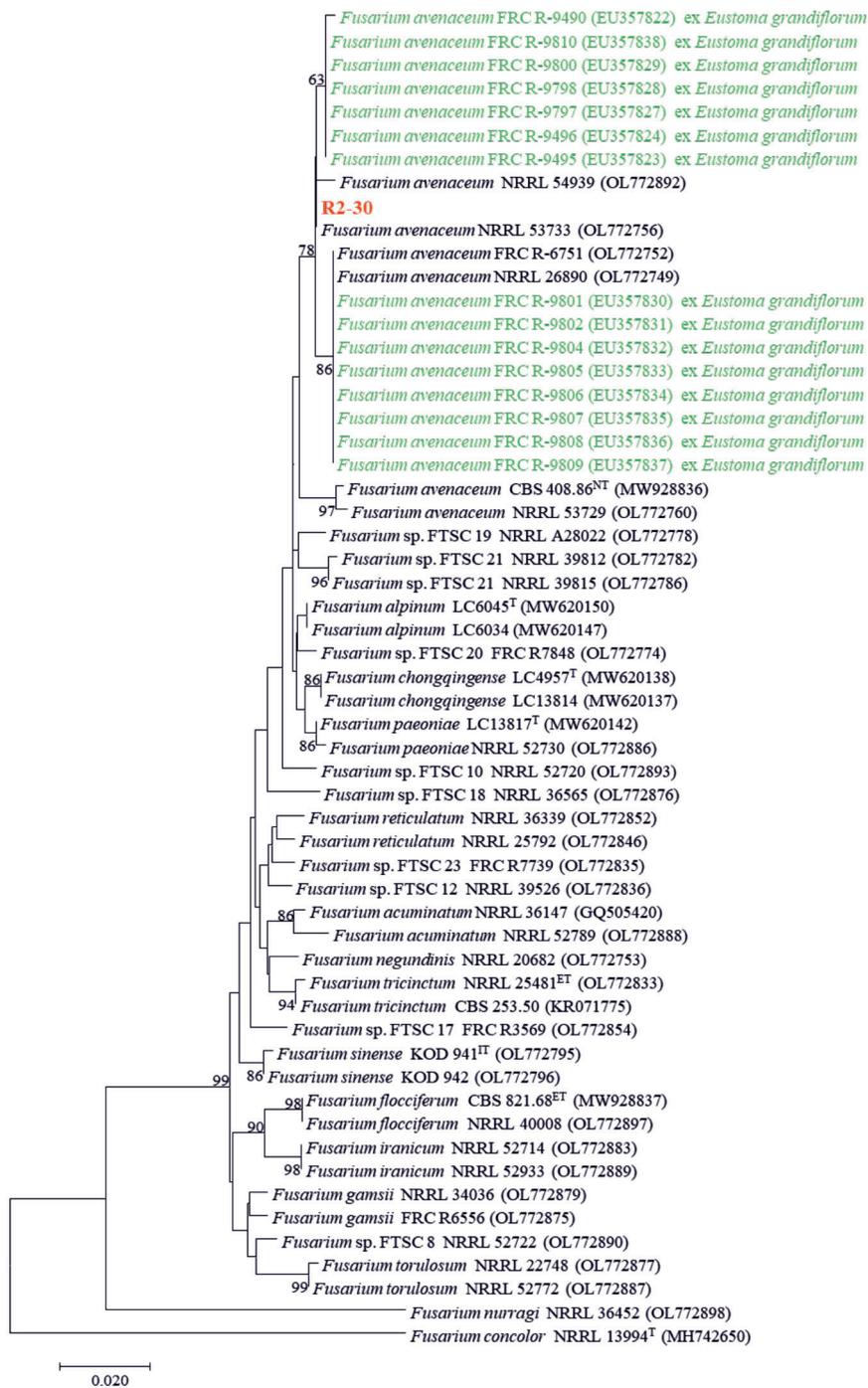


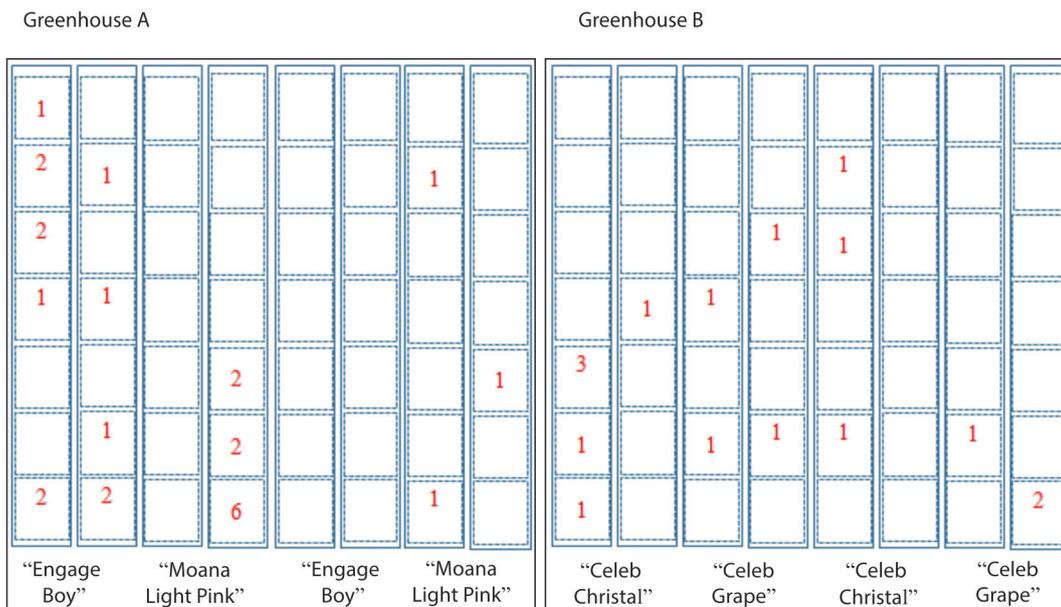
Fig. 4. Neighbor-Joining (NJ) phylogeny inferred from *tef1* sequences of R2-30, 15 strains from lisianthus, and species of *Fusarium tricinatum* species complex. Numbers on the nodes are NJ Bootstrap values above 70%. Ex-type, ex-epitype, isotype, and neotype strains are indicated with ^T, ^{ET}, ^{IT} and ^{NT}, respectively

Japan (The Phytopathological Society of Japan 2022), *F. avenaceum* is recorded as a plant-pathogen for stem rot of lisianthus in Hokkaido Prefecture. This *F. avenaceum* four strains ex *E. grandiflorum* are preserved in NARO Genebank, but these strains could not be included in molecular phylogenetic analyses, because sequence data of these strains are not released to the public. However, the PCR result in this study was the same as one from lisianthus plants with *Fusarium* stem rot in Hokkaido Prefecture (Fig. 2). Actually, the ITS

region sequences of the pathogens isolated in each area in Okinawa Main Island were different (data not shown). Further phylogenetic analysis of these pathogens should be carried out next.

In greenhouses, as preventive measures against fungal diseases, the farmer of greenhouses A and B applied soil disinfectant and many types of fungicides before as well as after planting the lisianthus plants (Table 1). First, Basamide, including dazomet and methyl isothiocyanate, was sprayed on soil 20 days before

A



Each rectangle on the solid line represents each furrow with four rows planted. Values in each section indicate the number of lisianthus plants with Fusarium stem rot. Blank indicates no disease. In each section, there are about 140 lisianthus plants cultivated

B

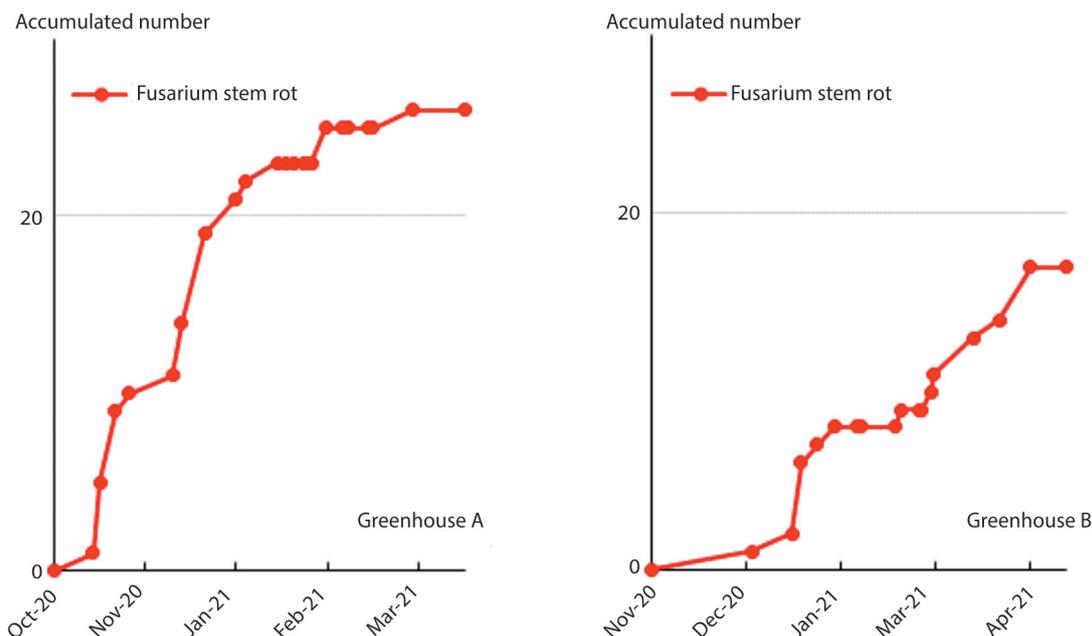


Fig. 5. The occurrence of Fusarium stem rot: A – the location of the disease occurrence; B – the accumulated number of the disease

planting and Benrate wettable powder was perfused a day before planting in these greenhouses. Basamide is registered for Pythium rot. There were no diseased lisianthus plants with Pythium rot in these greenhouses; thus, it is possible to conclude that Basamide may prevent Pythium rot. The number of diseased plants with Fusarium stem rot was ten for the first month since lisianthus were planted in greenhouse A. In

contrast, there were only one for the first month and two for the first two months since they were planted in greenhouse B. It is not obvious that this perfused fungicide, Benrate wettable powder prevented Fusarium stem rot, but the diseased plants increased to 0.3% of the total number in greenhouse A and 0.2% of the total number in greenhouse B. However, Fusarium root rot did not occur in these greenhouses and the fungicide

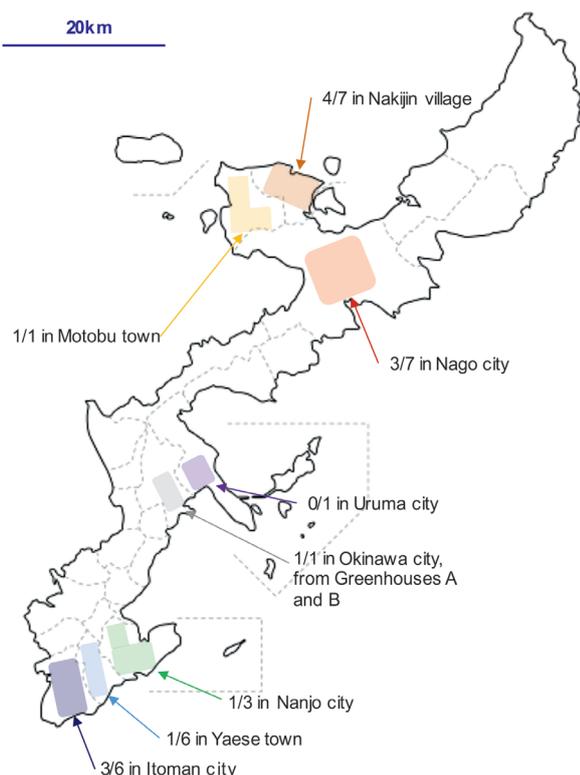


Fig. 6. Map of Okinawa, indicating the occurrence rate of Fusarium stem rot in 32 farms in each of the regions

may contribute to preventing the disease. In fact, the pesticides registered for Fusarium stem rot were not sprayed in these greenhouses. Actually, only Orthocide wettable powder 80 is registered as a spraying type pesticide for Fusarium stem rot. Captan in Orthocide was more effective against Fusarium wilt of chickpea than thiophanate-methyl (Arshi and Shabbir 2020), which was sprayed for sclerotial rot in the greenhouses (Table 1) in this study. Also, captan inhibited mycelial growth of *F. graminearum* associated with corn and soybean

seed more effectively than azoxystrobin and trifloxystrobin (Broders *et al.* 2007). Spraying Orthocide had the possibility of preventing the disease more and verifying its effect is the next step. Actually, the disease incidence in Okinawa was relatively high and was distributed in the southern, middle, and northern areas of Okinawa Main Island (Fig. 6). Thus, the disease incidence in Okinawa Main Island from 2020–2021 was 43.8%. In fact, other Fusarium diseases also occurred in the Okinawa Main Island, which we hope to report in the next study.

In conclusion, Fusarium stem rot appeared frequently despite spraying several fungicides though irregularly. Choosing appropriate soil disinfectants and fungicides for the environment and spraying them at appropriate intervals are the keys to preventing these diseases. Further studies are required to search for effective methods to prevent these diseases, such as choosing fungicides and soil disinfestation for inhibiting these pathogens. Further, evaluating resistant varieties against Fusarium stem rot are warranted for the next study.

Date availability statement

The sequence is available from the Bioinformatics and DDBJ Center database DDBJ at www.ddbj.nig.ac.jp with accession numbers LC724043 (ITS), LC724044 (tef1), LC724045 (rpb1), and LC724046 (rpb2).

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Table 1. The fungicides applied to lisianthus plants in greenhouses A and B

The fungicide name	Compound	Registration for lisianthus	The date of application
Basamide	dazomet	Pythium rot	September 24 (greenhouses A and B)
Benrate wettable powder	benomyl	Fusarium root rot	October 15 (greenhouse A)
Zimandasen wettable powder	mancozeb	Anthrax	November 5 (greenhouse B)
Polyoxin AL water solvent	polyoxin complex	Powdery mildew Gray mold	November 16 (greenhouse A)
Topgin M wettable powder	thiophanate-methyl	Sclerotial rot	January 9 (greenhouses A and B)
Savior flowable 20	fludioxonil	Gray mold	January 25 (greenhouses A and B)
			February 18 (greenhouses A and B)

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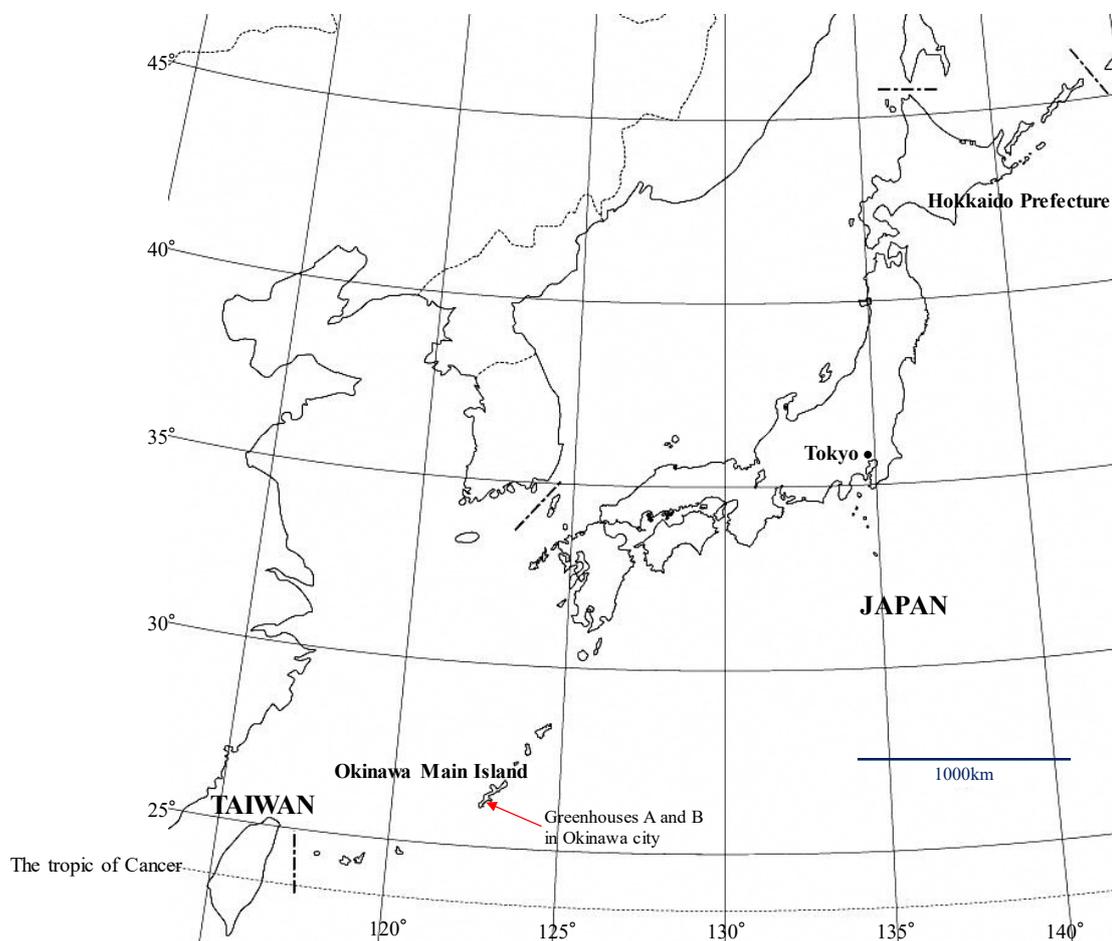
SUPPLEMENTARY MATERIAL

The authors are fully responsible for both the content and the formal aspects of the supplementary material. No editorial adjustments were made.

Supplementary Table 1. PCR Primers information of PCR amplification of the four loci

Locus	Primers	Sequence of primer (5'→3')	Direction	References
ITS	ITS5	GGAAGTAAAAGTCGTAACAAG	forward	White <i>et al.</i> (1990)
	ITS4	TCCTCCGCTTATTGATATGC	reverse	
	NL4 ^a	GGTCCGTGTTTCAAGACGG	reverse	O'Donnell (1993)
<i>tef1</i>	EF1	ATGGGTAAGGARGACAAGAC	forward	O'Donnell <i>et al.</i> (1998)
	EF2	GGARGTACCAGTSATCATGTT	reverse	
<i>rpb1</i>	Fa	CAYAARGARTCYATGATGGGWC	forward	O'Donnell <i>et al.</i> (2010)
	R8	CAATGAGACCTTCTCGACCAGC	reverse	
	F7	CRACACAGAAGAGTTTGAAGG	forward	
	R9	TCARGCCCATGCGAGAGTTGTC	reverse	
	F6 ^a	CTGCTGGTGTATCATTACG	forward	
	F8 ^a	TTCTTCCACGCCATGGCTGGTCG	forward	
	G2R ^a	GTCATYTGDDGDGCDGGYTCDC	reverse	
	Amp3f	GAYTACATCTTCAAYCGTCAGCC	forward	
Amp3r	GTTCTTGGAHGACACACCRGCG	reverse		
<i>rpb2</i>	RPB2-5f2	GGGGWGWAYCAGAAGAAGGC	forward	Reeb <i>et al.</i> (2004)
	fRPB2-7cr	CCCATRGCTTGYTRCCCAT	reverse	Liu <i>et al.</i> (1999)
	fRPB2-7cf	ATGGGYAARCAAGCYATGGG	forward	
	RPB2-11ar	GCRTGGATCTTRCRTCACC	reverse	

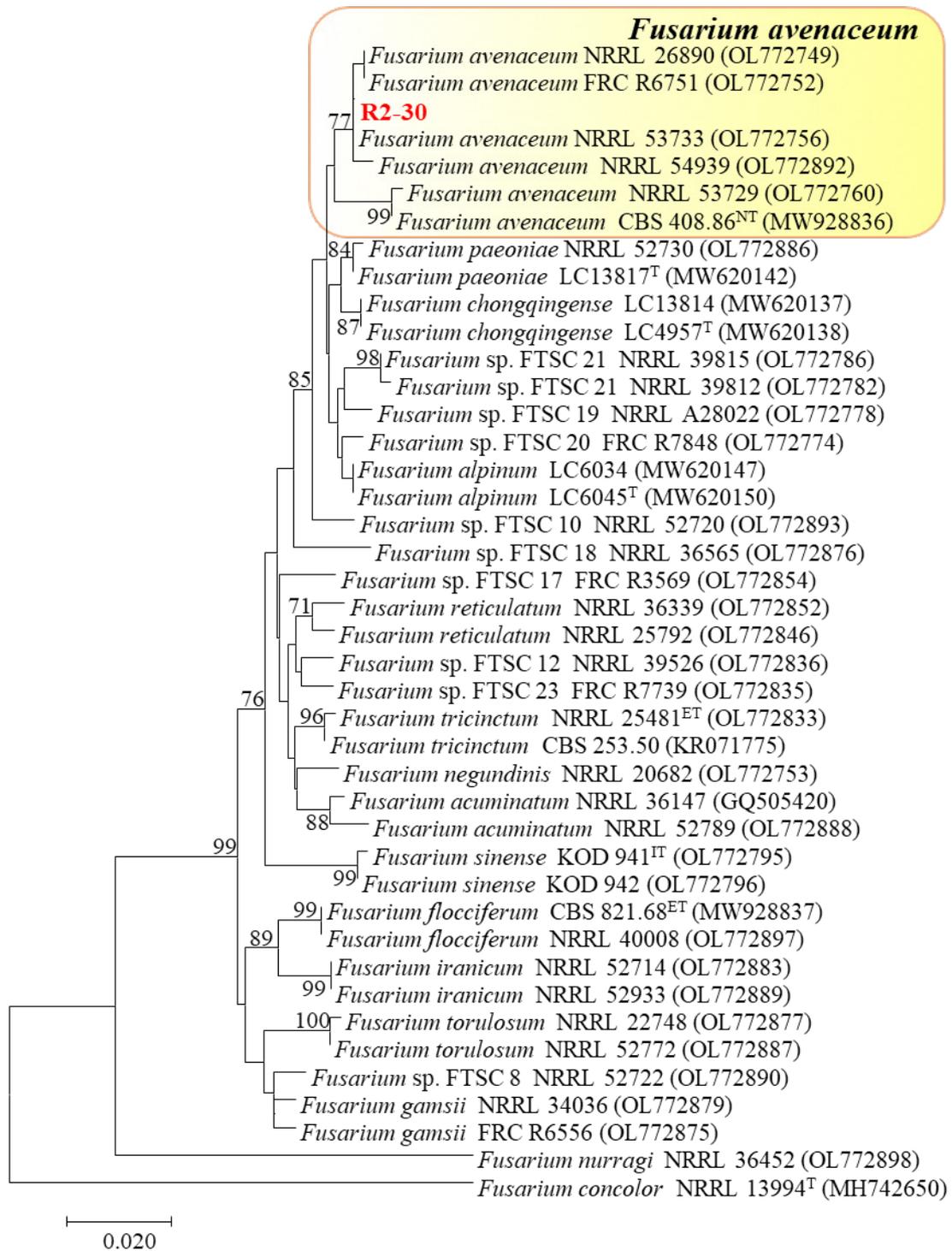
^aused only for sequencing reactions



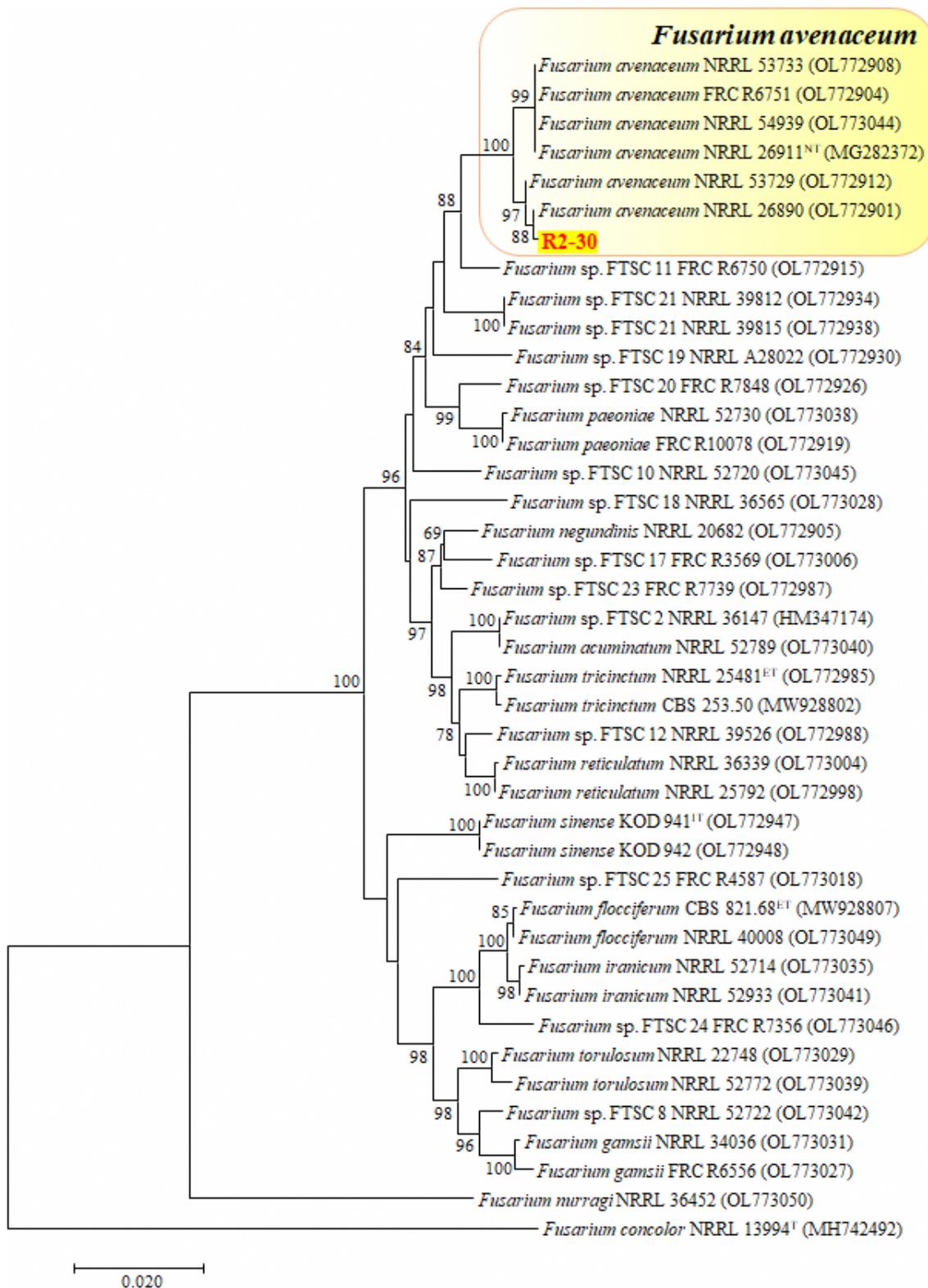
Supplementary Figure 1. Map of Okinawa, indicating the localizations of the greenhouses



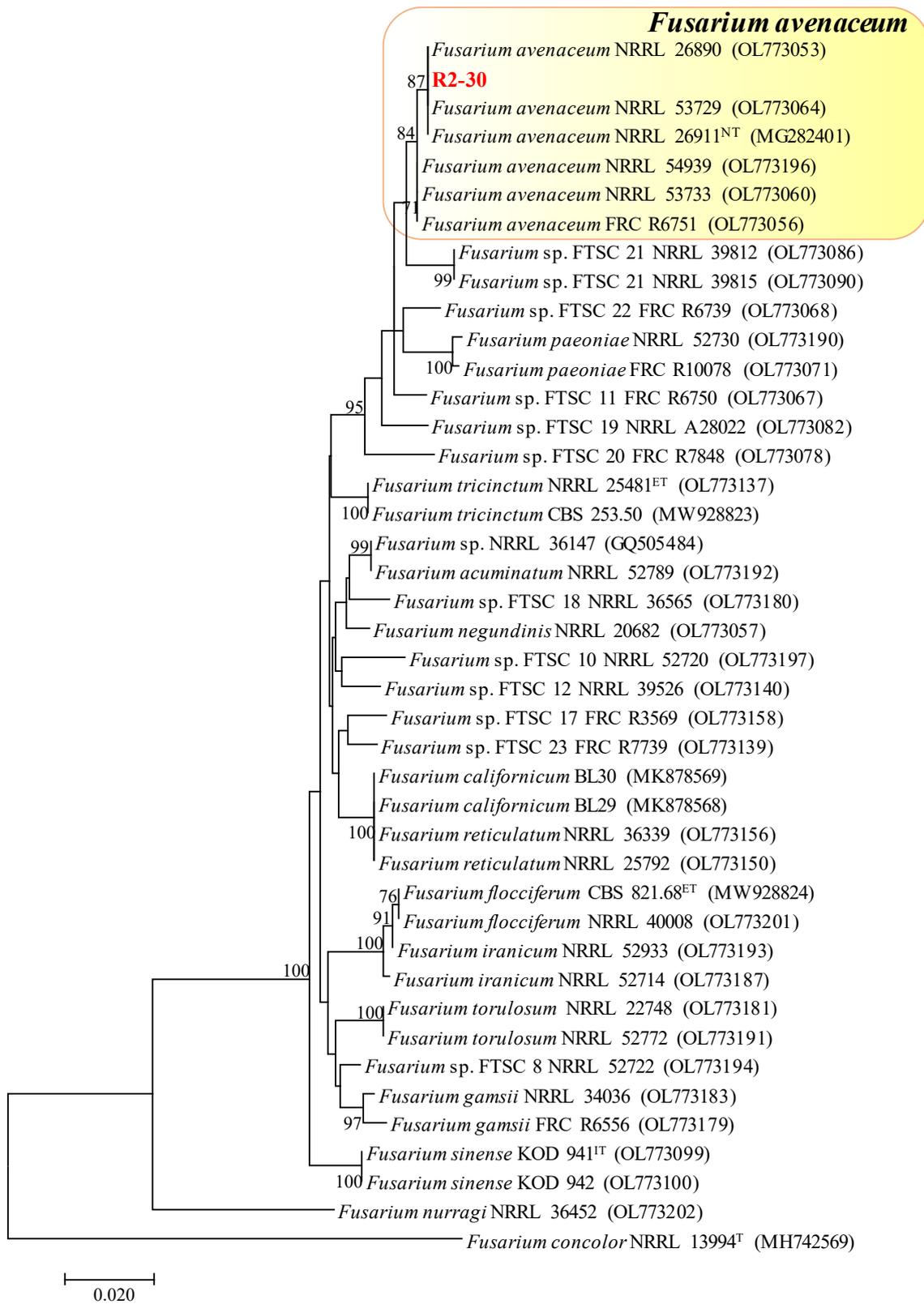
Supplementary Figure 2. Neighbor-Joining (NJ) phylogeny inferred from ITS sequences of R2-30, one strain from *Lisianthus*, and species of *Fusarium tricinctum* species complex. Numbers on the nodes are NJ Bootstrap values above 70%



Supplementary Figure 3. Neighbor-Joining (NJ) phylogeny inferred from *tef1* sequences of R2-30 and species of *Fusarium tricinctum* species complex. Numbers on the nodes are NJ Bootstrap values above 70%



Supplementary Figure 4. Neighbor-Joining (NJ) phylogeny inferred from *rpb1* sequences of R2-30 and species of *Fusarium tricinctum* species complex. Numbers on the nodes are NJ Bootstrap values above 70%



Supplementary Figure 5. Neighbor-Joining (NJ) phylogeny inferred from *rpb2* sequences of R2-30 and species of *Fusarium tricinctum* species complex. Numbers on the nodes are NJ Bootstrap values above 70%