Identification and Characterization of *Fusarium* Species Associated with Wilt of *Eleocharis dulcis* (Chinese water chestnut) in China

Zhixian Zhu, Lu Zheng, and Li Pan, The Key Lab of Plant Pathology of Hubei Province, Huazhong Agricultural University, Wuhan, 430070, China; Tom Hsiang, School of Environmental Sciences, University of Guelph, Guelph, ON N1G 2W1, Canada; and Junbin Huang, The Key Lab of Plant Pathology of Hubei Province, Huazhong Agricultural University

Abstract

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Fusarium wilt is one of the most important diseases of *Eleocharis dulcis* (Chinese water chestnut) in China. In order to characterize the pathogens responsible, 69 *Fusarium* isolates were collected from diseased plants in *E. dulcis* production areas of the Chinese provinces Anhui, Fujian, Hubei, Hunan, Jiangsu, and Zhejiang. These were then identified based on morphological and molecular characteristics. *F. commune* was the most common species (92.8%) and was widely distributed in the six provinces. A novel species within the *Gibberella fujikuroi* species complex (GFSC) was found in Hubei and Zhejiang provinces (5.8%), and an unidentified *Fusarium* sp. was found only in Hubei province (1.4%). Thirty *F. commune* isolates from different provinces and four GFSC isolates were selected for sequence analyses of the translation elongation factor 1- α (*EF-1 α*), the mitochondrial

small subunit (mtSSU) ribosomal DNA, and the nuclear ribosomal intergenic spacer region (IGS). Maximum parsimony and Bayesian analyses of the multilocus sequence data of these two species plus other taxa showed that the two species formed two distinct, well-supported clades among the three individual and combined gene genealogies. Isolates from different locations were scattered, with no evidence of geographic specialization. Pathogenicity assays showed that the two *Fusarium* spp., including the unidentified *Fusarium* sp., were pathogenic to *E. dulcis* 'Tuanfeng seven'. There was no relationship between the source of isolates and their pathogenicity. This is the first description of *F. commune*, a novel species within the GFSC, and an unidentified *Fusarium* sp. as causal agents of *Fusarium* wilt of *E. dulcis* in China.

Chinese water chestnut (*Eleocharis dulcis* (Burm. f.) Trin.) is a perennial herbaceous plant in the family Cyperaceae, which has been commercially cultivated in wet areas on low flat lands or lakes in China, India, Italy, Japan, and the United States (34). The corm of *E. dulcis* is commonly used as a vegetable or as a fruit. It also has been used as a folk medicine to treat hypertension, chronic nephritis, constipation, pharyngitis, laryngitis, and enteritis (42).

In China, *E. dulcis* has been cultivated mainly in Anhui, Fujian, Guangdong, Guangxi, Hubei, Hunan, Jiangsu, Jiangxi, and Zhejiang provinces. The planted area of *E. dulcis* was approximately 35,000 ha, producing approximately 750,000 tons in China in 1998 (10). The *E. dulcis* industry contributes significantly to the local economy. However, *Fusarium* wilt disease is widely distributed throughout areas producing *E. dulcis*. In recent years, with the expanding cultivation and continuous cropping of *E. dulcis*, *Fusarium* wilt has become a major limiting factor for *E. dulcis* production. From 2009 to 2012, the planted area of *E. dulcis* was over 1,700 ha and the production was approximately 30,000 tons during each growing season in Tuanfeng county of Hubei province, China. The disease incidence was between 20 and 35% in 2009, reaching 70% in some fields in 2012.

Fusarium wilt of *E. dulcis* was first reported by Jiang et al. (7) in Zhejiang province, and four *Fusarium* morphospecies—*Fusarium oxysporum*, *F. acuminatum*, *F. semitectum*, and *F. moniliforme* var. *intermedium*—were isolated and identified as the causal agents of *Fusarium* wilt based on morphological characteristics and pathogenicity tests. Their isolation frequencies were 80.8, 10.0, 5.83, and 3.33%, respectively; hence, *F. oxysporum* was considered to be the primary pathogen (7). Subsequently, various levels of inci-

dence of *Fusarium* wilt of *E. dulcis* caused by *F. oxysporum* have been reported from different production areas such as Anhui, Fujian, Guangdong, and Hubei provinces (24). Symptoms of the disease include yellowing of the plant stems, followed by whole plants slowly becoming chlorotic and stunted. White, tender, healthy stem bases turn brown and necrotic, and roots become black. In severe cases, pink mycelia are visible on the surface of stem bases. The disease can be found from planting to storage, and *E. dulcis* yield and quality can be significantly reduced. Thus far, the disease has only been reported in China, and limited studies on morphological identification and chemical control have been conducted. Research is needed on molecular characterization and virulence testing of *Fusarium* isolates causing *E. dulcis* wilt from different areas in China.

Fusarium spp. are ubiquitous fungi, which can cause diseases on a broad range of host plants with significant economic impacts (40). The morphological characteristics; the colony color and growth rate; the shape and size of macroconidia, microconidia, and chlamydospores; and the formation of conidiogenous cells have been the primary means for identifying *Fusarium* spp. (33). For example, morphological characteristics such as the formation of conidiogenous cells are used to differentiate *F. commune* from *F. oxysporum* (29). The genus *Fusarium* is complex, and different morphological characteristics at the species level may be subtle. A robust DNA-based analysis is needed for accurate, rapid identification and characterization of the species. Phylogenetic analysis based on DNA sequence data has made significant contributions to our understanding of the systematics of *Fusarium* spp. (2). Molecular data in combination with distinctive morphological characters usually have been used to distinguish new species of *Fusarium* (13,28,29). Loci used for phylogenetic analyses in *Fusarium* include nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS; 19), translation elongation factor 1- α (*EF-1 α* ; 21,22), ribosomal intergenic spacer (IGS; 16,20), mitochondrial small subunit (mtSSU) rDNA (14), the second largest subunit of the RNA polymerase II (29), the β -tubulin gene, and the calmodulin gene (28,38).

Corresponding author: J. Huang, E-mail: junbinhuang@mail.hzau.edu.cn

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An accurate understanding of the pathogen from different locations is crucial for efficient disease management. The objectives of this study were to (i) identify and characterize species of *Fusarium* associated with wilt of *E. dulcis* based on morphological and molecular characteristics, (ii) study the virulence of isolates from different areas and whether differences between isolates are related to geographic origin, and (iii) examine relationships among *Fusarium* isolates from *E. dulcis* using sequences of *EF-1 α* , mtSSU, and IGS to search for associations between phylogenetic structure, geographic origin, and pathogenicity.

Materials and Methods

Fungal isolation and preservation. From 2009 to 2012, immature *E. dulcis* with symptoms of Fusarium wilt were collected from commercial production fields of six provinces in China, including Anhui, Fujian, Hubei, Hunan, Jiangsu, and Zhejiang. Small (5 by 5 mm) sections were excised from margins of browning stem bases and surface-sterilized in 75% ethanol for 10 s, then sterilized in 5% (vol/vol) sodium hypochlorite for 1.5 min, followed by three rinses with sterile water. The fragments were then placed onto acidified potato dextrose agar (PDA; 2% dextrose, 1.5% agar, and 1 ml of 25% lactic acid per liter) and incubated at 25°C. Fungal growth was examined daily for up to 5 days. Isolates were transferred to fresh PDA and purified by single-spore culturing (23). All fungal isolates were placed on fresh PDA slants and stored at 4°C.

Morphological characterization. Monoconidial isolates were subcultured on PDA plates at 25°C under continuous white light for 5 days. A 6-mm-diameter plug from the colony margin was

placed in the center of a 90-mm-diameter PDA plate and grown at 25°C under light for examination of colony color, odor, and growth rates. All colors were assessed based on the Methuen Handbook of Colour (8). Colony diameters were measured after 3 days. The mean growth rate was calculated from three replicates. Isolates were incubated at 20°C in complete darkness for 14 days on synthetic nutrient-poor agar (SNA) to examine the production, type, and arrangement of microconidia and conidiogenous cells. Isolates were incubated at 25°C with a 12-h photoperiod for 14 days on carnation leaf agar (CLA) to examine the shape and size of macroconidia (9,17). Microconidia, macroconidia, and chlamydospores were measured based on 30 random selections.

DNA isolation, polymerase chain reaction amplification, and sequencing. Isolates were grown on PDA for 7 days at 25°C with a sterile cellophane membrane on the agar surface. Mycelia were harvested with a sterile blunt blade, and genomic DNA of the isolates was extracted by a cetyltrimethylammonium bromide method (36). *EF-1 α* , mtSSU, and IGS rDNA sequences were amplified with the primer pairs EF-1/EF-2 (21), NMS1/NMS2 (11), and CNS1/CNL12 (14), respectively. All primers were synthesized by SANGON. All polymerase chain reaction (PCR) amplifications were conducted in 50- μ l reaction volumes containing 1 $\times$ PCR buffer, 0.2 mM concentrations of each dNTP, 4 mM MgCl₂, 0.5 μ M concentrations of each primer, 0.5 units Ex Taq DNA polymerase (Takara), and 1 μ l of DNA (20 ng/ μ l).

Amplifications were performed in a Mycycler Thermal Cycler (Bio-Rad) using the following program for amplification of *EF-1 α* : an initial denaturation step at 95°C for 2 min; 35 cycles of 95°C for

Table 1. *Fusarium* spp. isolates from *Eleocharis dulcis* in China used in this study, and sequences deposited in GenBank

Isolate ^b	Geographic origin	Location	Species ^c	efFc100F/ efFc385R ^d	FOF1/ FOR1 ^e	GenBank accession ^a		
						IGS	mtSSU	<i>EF-1α</i>
TF3	Hubei	Tuanfeng	<i>F. commune</i>	+	–	KC430681	KC430704	KC430647
TF4	Hubei	Tuanfeng	<i>F. commune</i>	+	–	KC430682	KC430713	KC430648
TF5	Hubei	Tuanfeng	<i>F. commune</i>	+	–	KC430679	KC430717	KC430639
TF8	Hubei	Tuanfeng	<i>F. commune</i>	+	–	KC430684	KC430710	KC430637
TF10	Hubei	Tuanfeng	<i>F. commune</i>	+	–	ns	ns	ns
TF11	Hubei	Tuanfeng	<i>F. commune</i>	+	–	KC430687	KC430714	KC430632
Z141	Hubei	Tuanfeng	<i>F. commune</i>	+	–	ns	ns	ns
Z142	Hubei	Tuanfeng	<i>F. commune</i>	+	–	KC430686	KC430709	KC430658
Z352	Hubei	Tuanfeng	<i>F. commune</i>	+	–	ns	ns	ns
L221	Hubei	Tuanfeng	<i>F. commune</i>	+	–	ns	ns	ns
L222	Hubei	Tuanfeng	<i>F. commune</i>	+	–	ns	ns	ns
L232	Hubei	Tuanfeng	<i>F. commune</i>	+	–	ns	ns	ns
L253	Hubei	Tuanfeng	<i>F. commune</i>	+	–	ns	ns	ns
SY2	Hubei	Shayang	<i>F. commune</i>	+	–	KC430688	KC430707	KC430649
SY3	Hubei	Shayang	<i>F. commune</i>	+	–	KC430690	KC430711	KC430651
SY5	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY7	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY9	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY11	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY12	Hubei	Shayang	<i>F. commune</i>	+	–	KC430689	KC430696	KC430659
SY9106	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY9107	Hubei	Shayang	<i>Fusarium</i> sp.	n/a	n/a	KC430695	KC430730	KC430660
SY13	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY14	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY15	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY16	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
SY17	Hubei	Shayang	<i>F. commune</i>	+	–	ns	ns	ns
XG1	Hubei	Xiaogan	<i>F. commune</i>	+	–	ns	ns	ns
XG2	Hubei	Xiaogan	GFSC	n/a	n/a	KC430691	KC430728	KC430626
XGX2	Hubei	Xiaogan	<i>F. commune</i>	+	–	ns	ns	ns
XGX231	Hubei	Xiaogan	<i>F. commune</i>	+	–	KC430685	KC430720	KC430640

(continued on next page)

^a IGS = intergenic spacer region, mtSSU = mitochondrial small subunit, *EF-1 α* = translation elongation factor 1- α , and ns = not sequenced.

^b All isolates were obtained from stem bases of *E. dulcis*, except isolate QJ21, which was collected from soil, and *F. oxysporum* f. sp. *vasinfectum* was obtained from The Key Lab of Plant Pathology of Hubei Province, Huazhong Agricultural University.

^c GFSC = *Gibberella fujikuroi* species complex.

^d efFc100F/efFc385R: + indicates that amplicon was obtained with specific polymerase chain reaction (PCR) primers developed by Stewart et al. (31) for detection of *F. commune* and – indicates that no amplicon was detected; n/a = not detected.

^e FOF1/FOR1: + indicates that amplicon was obtained with specific PCR primers developed by Mishra et al. (15) for detection of *F. oxysporum* and – indicates that no amplicon was detected; n/a = not detected.

30 s, 57°C for 30 s, and 72°C for 1 min; and a final extension of 72°C for 10 min. Thermocycling parameters for amplification of mtSSU were 95°C for 2 min; 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 10 min. The IGS sequences were amplified by an initial denaturation of 94°C for 5 min; 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 5 min. PCR products were confirmed under UV light on 1.2% agarose gels stained with ethidium bromide, using the Gel Doc XR+ transilluminator and the software Image Lab 4.0.1 (Bio-Rad).

Products were directly sent to Sunny for purification and sequencing. The *EF-1α* and mtSSU genes were sequenced using the forward primers. To sequence the entire approximately 2.5-kb IGS regions, an additional six internal sequencing primers were used in this study: IGS1 (5'-ACCGACTATTTCAACCAAGG-3') and IGS4 (5'-CCTGCACCGTACCAGAATC-3') for XG2, ZJ3, ZJ5, and ZJ7; IGS5 (5'-TGCCTAGACACATAGAACA-3') and IGS6 (5'-TGTTCTATGTGTCTAGGCA-3') for SY9107; and IGS1, IGS2 (5'-GATACAAGCAGCCGACAC-3'), and IGS3 (5'-CTTCTC ATATACCTCCGAG-3') for the other sequenced isolates listed in Table 1.

Specific PCR diagnosis. A pair of *F. commune* diagnostic PCR primers (efFc100F and efFc385R) developed by Stewart et al. (31) to specifically detect isolates of *F. commune* and discriminate against *F. oxysporum* was used to screen isolates. In addition, specific primers (FOF1 and FOR1) developed by Mishra et al. (15) for detection of *F. oxysporum* were used to further screen out the *F. commune* isolates.

Pathogenicity trial. Thirty *F. commune* isolates were randomly selected from each sample site of the six provinces, and all isolates of the *Gibberella fujikuroi* species complex (GFSC) and an unidentified *Fusarium* sp. were used in pathogenicity assays. The pathogenicity of the isolates was tested using a root-dip inoculation method modified from that of Biles and Martyn (1). Inoculum was prepared by growing cultures in potato dextrose broth (PDB) on a rotating shaker (180 rpm, 25°C) for 5 days. The cultures were filtered through four layers of sterile cheesecloth, and a spore suspension containing 5×10^6 spores/ml was prepared.

Corms of *E. dulcis* 'Tuanfeng seven' were surface-sterilized by immersion in 70% ethanol for 30 s and 5% sodium hypochlorite for 5 min, then buried in sterile sand in uncovered plastic boxes (38 by 30 by 13 cm) and watered with distilled water for seedling growth. After 3 weeks, seedlings of *E. dulcis* were removed from the sand and the roots were washed in running tap water. The 5-mm tips of roots were trimmed off and the wounded roots were dipped into the spore suspension for 20 min, then transplanted into 22.5-cm-diameter pots containing a sterile mix of organic soil and vermiculite (3:2). The pots were placed inside a larger plastic box (38 by 30 by 13 cm) and sealed within a plastic bag for 24 h. They were then incubated in a growth chamber at 25°C with a 12-h photoperiod at a relative humidity of 90%, and watered to keep plants wet. Wounded roots treated with sterile water for 20 min served as control. Twenty plants were inoculated for each fungal isolate and the experiment was repeated three times.

Each plant was rated for disease severity 30 days after inoculation on a scale of 0 to 5, based on percentage of yellowing stems, where 0 = plants without visible symptoms and 1 = 1 to 10, 2 = 11

Table 1. (continued from preceding page)

Isolate ^b	Geographic origin	Location	Species ^c	efFc100F/ efFc385R ^d	FOF1/ FOR1 ^e	GenBank accession ^a		
						IGS	mtSSU	<i>EF-1α</i>
XGX232	Hubei	Xiaogan	<i>F. commune</i>	+	–	ns	ns	ns
XGH1	Hubei	Xiaogan	<i>F. commune</i>	+	–	KC430683	KC430705	KC430641
XGH2	Hubei	Xiaogan	<i>F. commune</i>	+	–	ns	ns	ns
CDY1	Hubei	Caidian	<i>F. commune</i>	+	–	ns	ns	ns
CDY2	Hubei	Caidian	<i>F. commune</i>	+	–	ns	ns	ns
CDY3	Hubei	Caidian	<i>F. commune</i>	+	–	KC430677	KC430700	KC430653
CDY4	Hubei	Caidian	<i>F. commune</i>	+	–	ns	ns	ns
CDY6	Hubei	Caidian	<i>F. commune</i>	+	–	KC430674	KC430722	KC430650
YC11	Hubei	Wuxue	<i>F. commune</i>	+	–	KC430662	KC430701	KC430644
YC21	Hubei	Wuxue	<i>F. commune</i>	+	–	ns	ns	ns
YL2	Anhui	Lujiang	<i>F. commune</i>	+	–	KC430663	KC430723	KC430656
YL3	Anhui	Lujiang	<i>F. commune</i>	+	–	ns	ns	ns
YL4	Anhui	Lujiang	<i>F. commune</i>	+	–	KC430668	KC430698	KC430642
YL5	Anhui	Lujiang	<i>F. commune</i>	+	–	KC430669	KC430718	KC430636
YL7	Anhui	Lujiang	<i>F. commune</i>	+	–	KC430665	KC430724	KC430635
YL9	Anhui	Lujiang	<i>F. commune</i>	+	–	ns	ns	ns
YL10	Anhui	Lujiang	<i>F. commune</i>	+	–	KC430664	KC430703	KC430630
SS2	Anhui	Wuwei	<i>F. commune</i>	+	–	ns	ns	ns
SS3	Anhui	Wuwei	<i>F. commune</i>	+	–	KC430671	KC430716	KC430633
SS5	Anhui	Wuwei	<i>F. commune</i>	+	–	KC430667	KC430699	KC430643
SS6	Anhui	Wuwei	<i>F. commune</i>	+	–	ns	ns	ns
SS12	Anhui	Wuwei	<i>F. commune</i>	+	–	KC430666	KC430708	KC430634
SS15	Anhui	Wuwei	<i>F. commune</i>	+	–	ns	ns	ns
SS16	Anhui	Wuwei	<i>F. commune</i>	+	–	KC430670	KC430702	KC430652
SS18	Anhui	Wuwei	<i>F. commune</i>	+	–	KC430676	KC430721	KC430654
QJ1	Hunan	Heshan	<i>F. commune</i>	+	–	KC430672	KC430719	KC430631
QJ20	Hunan	Heshan	<i>F. commune</i>	+	–	ns	ns	ns
QJ21	Hunan	Heshan	<i>F. commune</i>	+	–	KC430680	KC430706	KC430638
QJ23	Hunan	Heshan	<i>F. commune</i>	+	–	ns	ns	ns
ZJ1	Zhejiang	Jinhua	<i>F. commune</i>	+	–	ns	ns	ns
ZJ3	Zhejiang	Jinhua	GFSC	n/a	n/a	KC430693	KC430727	KC430628
ZJ5	Zhejiang	Jinhua	GFSC	n/a	n/a	KC430694	KC430729	KC430627
ZJ6	Zhejiang	Jinhua	<i>F. commune</i>	+	–	KC430673	KC430725	KC430645
ZJ7	Zhejiang	Jinhua	GFSC	n/a	n/a	KC430692	KC430726	KC430629
ZJ8	Zhejiang	Jinhua	<i>F. commune</i>	+	–	KC430675	KC430697	KC430655
JS1	Jiangsu	Danyang	<i>F. commune</i>	+	–	ns	ns	ns
JS6	Jiangsu	Danyang	<i>F. commune</i>	+	–	KC430678	KC430712	KC430646
FJ	Fujian	Longhai	<i>F. commune</i>	+	–	KC430657	KC430715	KC430657
Fov	Henan	Anyang	<i>F. oxysporum</i>	–	+	ns	ns	ns

to 30, 3 = 31 to 60, 4 = 61 to 90, and 5 = 91 to 100%. Disease index (DI) was calculated using the formula $20 \times (n1 + n2 \times 2 + n3 \times 3 + n4 \times 4 + n5 \times 5) / (n0 + n1 + n2 + n3 + n4 + n5)$, where $n0$, $n1$, $n2$, $n3$, $n4$, and $n5$ represent the number of plants with scores of 0, 1, 2, 3, 4, and 5, respectively. Attempts were made to reisolate the causal organisms from stem bases. Analysis of variance in SAS (version 8.1; SAS Institute) was used to assess differences in virulence among isolates and, when significant treatment differences were found, means were compared with the test of least significant

difference ($P < 0.05$). The criteria for classification of virulence were as follows: (i) DI values from 0 to 39 were considered as weakly virulent, (ii) DI values from 40 to 60 were considered as moderately virulent, and (iii) DI values from 61 to 100 were considered as highly virulent.

Phylogenetic analyses. Sequences were edited and assembled manually using DNAMAN (version 5.2.2; Lynnon Biosoft), then queried for similarity against the GenBank and FUSARIUM-ID databases using the BLAST search tool. Sequences were aligned

Fig. 1. Map of the locations of the collections of *Fusarium* spp. from six provinces in China for this study.

using CLUSTALX version 1.83 (37) using the following parameters: gap opening = 10, gap extension = 0.2, transition weight = 0.5, and delay divergent sequences = 30%. Alignments were checked and manual adjustments were made where necessary (39).

Unweighted parsimony analyses were performed using PAUP* (version 4.0b10; Sinauer Associates, Inc.) for the individual and combined *EF-1α*, mtSSU, and IGS data sets (35). The heuristic search option with 1,000 random addition sequence replicates and tree bisection reconnection branch swapping was used to infer maximum parsimony (MP). Clade stability was assessed with 1,000 bootstrap (BP) replicates. Tree length, consistency index, retention index, rescaled consistency index, and homoplasy index also were recorded for all MP analyses.

MrBayes v. 3.1.2 was used for Bayesian (BS) analyses to determine posterior probabilities (PP) (27). A hierarchical likelihood ratio test was implemented using MrModeltest version 3.7 (25), and BS analyses were performed according to the models. Metropolis-coupled Monte Carlo Markov chains with one million generations and four chains were calculated, keeping 1 of every 100th generation. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. Trees were visualized using TreeView (version 1.6.1; University of Glasgow). Topological incongruence was examined using a reciprocal 70% BP or a 95% PP threshold (26) in order to determine whether the sequences from the three genes should be combined in a single analysis.

Results

Fungal isolation and morphological characterization. In total, 227 plant samples exhibiting wilt symptoms were collected from 51 fields in six provinces in China, which included 11 fields in Tuanfeng county, 8 fields in Shayang county, 6 fields in Xiaogan city, 6 fields in Caidian district, and 2 fields in Wuxue city (all in Hubei province); 5 fields in Lujiang county and 5 fields in Wuwei county (both in Anhui province); 5 fields in Heshan district in Hunan province; 1 field in Danyang city in Jiangsu province; 1 field in Jinhua city in Zhejiang province; and 1 field in Longhai city in

Fujian Province. The fields were separated by at least 500 m (Fig. 1). Isolations from the browned stem bases onto acidified PDA only yielded species of *Fusarium*. At least one isolate from each field was chosen for further analysis. In total, 69 isolates were subjected to single-spore isolation (Table 1).

In all, 64 of the 69 isolates (Table 1) examined fit the description of *F. commune* (29) in the following features. On PDA, colony diameters after 3 days of incubation under light were 3 to 4.4 cm at 25°C ($\bar{x}$ = 3.7 cm). Aerial mycelia were abundant, densely floccose to fluffy, and pink to light violet, and colony undersides on PDA were violet with some pale orange. Orange sporodochia were present in most isolates. No odor was detected. All isolates produced abundant microconidia on SNA after 10 days. Microconidia were produced in false heads on monophialides or polyphialides, mostly 0-septate, oval, obovoid with a truncate base, elliptical or reniform in shape, measuring 5.0 to 13.8 by 2.5 to 3.8 μ m. Macroconidia were borne in sporodochia, typically three-septate, fusiform, with slightly curved apical cell and foot-shaped basal cell. Three-septate macroconidia formed in sporodochia, measuring 32.5 to 45 by 3.3 to 5.0 μ m. Chlamydospores were 8 to 15 μ m in diameter, smooth, intercalary or terminal, and produced in single or in pairs. Phialides of the conidiophores were cylindrical, consisting of short monophialides up to 10 μ m long and 4.0 μ m wide, or longer and more slender monophialides up to 41 μ m long and 2.5 μ m wide (Fig. 2).

Four isolates were morphologically indistinguishable from certain other species within GFSC following the descriptions of Nirenberg et al. (9) and Leslie et al. (18). On PDA, colony diameters after 3 days of incubation under light at 25°C were 2.1 to 2.8 cm ($\bar{x}$ = 2.5 cm). Aerial mycelia were abundant and off-white. Pale orange to violet pigmentation formed in agar and the colony centers often were heavily pigmented. Sporodochia were absent. Microconidia were abundant in false heads or chains from polyphialides, and less often from monophialides, mostly 0-septate, oval, obovoid with a truncate base, elliptical in shape, measuring 5.0 to 12.5 by 2.5 to 3.8 μ m. Macroconidia were extremely sparse and difficult to find but, where present, they were typically three-septate, fusiform with tapered apical cell and with poorly developed foot-shaped

Fig. 2. Morphological characteristics of *Fusarium commune*: a, macroconidia; b, microconidia; c, chlamydospores; d, polyphialidic conidiophores forming microconidia; e, false heads of microconidia on long and short monophialides; f, macroconidium borne on a monophialide; g, macroconidia on branched monophialide; and h, conidia in sporodochium formed on carnation leaf agar. Bars: a, b, d, e, f, and g = 10 μ m; c and h = 20 μ m.

basal cells. Three-septate conidia formed in CLA, measuring 21 to 36.5 by 2.5 to 4.5 μm . Chlamydospores were absent. Conidiophores were monophialidic or polyphialidic, up to 30 μm long and 3.2 μm wide (Fig. 3).

Isolate SY9107 was most similar morphologically to what has been described as *F. aquaeductuum* (17) but there were no sequence data in FUSARIUM-ID or in GenBank to confidently assign it to any known species. On PDA, colony diameter after 3 days of incubation under light was 4.1 to 4.2 cm at 25°C, and col-

ony were yeast-like. Aerial mycelia were rare, and salmon-orange pigmentation formed in agar. Microconidia were absent. Macroconidia were abundant, typically fusiform with slightly curved apical cells and foot-shaped basal cells, bending equally toward both ends. Three-septate conidia formed in CLA, measuring 32.5 to 45 by 3.3 to 5 μm (Fig. 4).

Molecular characterization. *F. commune* diagnostic PCR primers (efFc100F and efFc385R) were used to detected 64 isolates of *F. commune* which had been tentatively identified based on mor-

Fig. 3. Morphological characteristics of a novel species within the *Gibberella fujikuroi* species complex: **a**, macroconidia; **b**, microconidia; **c**, linear chains of microconidia; **d**, false heads of microconidia on monophialidic conidiophores; **e**, false heads of microconidia on polyphialidic conidiophore; and **f**, asci with ascospores. Bars: **a**, **b**, **d**, and **e** = 10 μm ; **c** and **f** = 20 μm .

Fig. 4. Morphological characteristics of an unclassified *Fusarium* isolate SY9107: **a**, macroconidia; **b**, macroconidia on monophialide on the surface of carnation leaf agar (CLA); **c**, mesoconidia on synthetic nutrient-poor agar; and **d**, sporodochial conidia formed on CLA. Bars: **a** and **b** = 10 μm ; **c** and **d** = 20 μm .

phological characteristics. The target bands were amplified for all 64 isolates. In addition, specific-PCR primers (FOF1 and FOR1) for detection of *F. oxysporum* were used to further characterize the *F. commune* isolates. Aside from positive control isolate of *F. oxysporum* f. sp. *vasinfectum*, no other species of *Fusarium* gave the target band (Table 1).

Pathogenicity testing. All 35 *Fusarium* isolates tested were pathogenic to *E. dulcis* 'Tuanfeng seven'. The disease symptoms observed on plants inoculated with any of the three *Fusarium* spp. were similar. Stems showed discoloration at 5 days after inoculation, and later, more stems became yellowed. Severely infected plants turned brown and became stunted, and pink to light violet mycelia grew abundantly around stem bases. These plants usually died within 20 days.

Virulence of the 30 isolates of *F. commune* from six provinces was significantly different, with a low to high DI range of 36.0 to 84.0. The average DI of Fujian, Anhui, Hunan, Hubei, Zhejiang, and Jiangsu provinces was 70.7, 69.6, 62.0, 61.6, 52.3, and 48.0, respectively. The virulence of *F. commune* from the same province and the same field also showed significant differences ($P < 0.05$). For example, the DI of isolate XGX231 from Xiaogan city, Hubei province was 84.0, whereas the DI of isolate YC11 from Wuxue city, Hubei province was 36.0. The DI of isolate TF5 from Tuanfeng county was 82.0, whereas the DI of isolates TF3 and TF4 from the same field were both 50.7. The 30 isolates of *F. commune* were separated into highly virulent (63.3%), moderately virulent (33.3%), and weakly virulent (3.3%) categories. Four isolates of GFSC also showed significant differences in pathogenicity. The DI values for ZJ5, ZJ3, and ZJ7 from the same field in Jinhua city were 70.7, 60.7, and 36.0, respectively. The DI of isolate XG2 from Xiaogan city was 41.3. The DI of SY9107 was 36.0 and it caused less severe symptoms, with only stem discoloration, but the bases remained healthy (Fig. 5). The average DI value for *Fusarium* sp. was the lowest (36.0), while *F. commune* was the highest (60.1) and GFSC isolates, moderate (52.2). The pathogens were reisolated from the inoculated plant stem bases, whereas the control set did not yield the pathogens.

Phylogenetic analyses. All DNA sequences generated for this study have been deposited in GenBank (Table 1). An isolate of *F. commune* (TF5) has been deposited in the China Center for Type Culture Collection. Initially, the comparison of the *EF-1 α* sequence of isolate SY9107 (KC430660) matched only *Fusarium* spp. (EU910074 at 91%, e-value = 0 and EU910073 at 91%, e-value = 0) from the GenBank non-redundant (NR) database, and no other *Fusarium* spp. were matched either from the GenBank NR database or from the FUSARIUM-ID database. The *EF-1 α* sequences of ZJ3, ZJ5, ZJ7, and XG2 showed 99% sequence identity with *F. andiyazi* (JF715933) and had 98.5% sequence identity with *F. andiyazi* (FD_01386) and 98.4% identity with GFSC (NRRL43304) in FUSARIUM-ID. The *EF-1 α* of other isolates showed 99% identity with *F. commune* and 99% identity with *F. oxysporum* in both GenBank and FUSARIUM-ID databases. Three regions (*EF-1 α* , mtSSU, and IGS) of the selected 30 *F. commune* isolates and four GFSC isolates were aligned with *F. commune*, GFSC selected from FUSARIUM-ID, and sequences of different formae speciales of the *F. oxysporum* species complex (FOSC) referred to by Mbofung et al. (14) and Wunsch et al. (41) (Table 2).

Summary statistics for the individual and combined data sets by MP and BS analyses are provided in Table 3. To assess whether DNA sequence data from the three gene partitions could be analyzed as a combined data set, we used a BP value of $\geq 70\%$ or a PP value of $\geq 95\%$ as threshold values to identify potential topological incongruence. No strongly conflicting nodes were detected for the same set of taxa among the *EF-1 α* , mtSSU, and IGS phylogenies, indicating that the data sets could be combined for the MP and BS analyses. Because the topology of the BS analysis of the combined data set was nearly identical to that of the MP consensus tree, only the MP tree is shown with BP (from MP analysis) above and PP (from BS analysis) below the corresponding nodes (Fig. 6).

The dendrogram of the combined data set contained three main clades (Fig. 6). Within the *F. commune* clade, 30 isolates clustered with *F. commune* (NRRL22903 and NRRL28387) supported by a BP value of 100% and a PP value of 100%, clearly distinct from

Fig. 5. Disease index of *Fusarium* isolates on 'Tuanfeng seven'. Isolates from XGX231 to YC11 were identified as *Fusarium commune*. Isolates from ZJ5 to ZJ7 were identified as a novel species within the *Gibberella fujikuroi* species complex. Isolate SY9107 was identified as an unclassified *Fusarium* sp.

the clade of FOSC. High BP and PP values support the separation of the isolates into nine subclades. Four isolates were clustered within GFSC in a separate clade supported by 100% BP and 100% PP. The four isolates formed two subclades with 100% BP, 100% PP and 61% BP, 61% PP support, respectively. The two subclades were close related to *F. verticillioide*s (NRRL 22172) and distinct from other species within GFSC (Fig. 6).

Topologies were similar for each partition and the combined date set but the IGS rDNA provided better resolution for *F. commune*. The IGS dendrogram had six subclades in *F. commune* clade and the *EF-1 α* dendrogram had five subclades; however, there was only one subclade in the mtSSU dendrogram. Some isolates showed variation and moved between subclades in individual or combined data set phylogenetic trees. However, the 30 isolates of *F. commune* and the four isolates of GFSC identified based on morphological characteristics formed separate clades by species with strong support in all dendrograms (*data not shown*). MP analyses of the individual partitions also revealed that IGS contributed more polymorphic sites (20.7%) than *EF-1 α* (14.7%) and mtSSU (5.8%) (Table 3).

Discussion

Previous attempts at identification of *Fusarium* spp. associated with Fusarium wilt of *E. dulcis* was based mainly on morphology, and there has also been a lack of systematic research on the disease. This study represents the first attempt to characterize pathogens of Fusarium wilt of *E. dulcis* in an extensive collection of isolates, and with pathogen identification based on morphological, pathogenicity, and phylogenetic analyses of *EF-1 α* , mtSSU, and

IGS regions. In this study, *F. commune*, a novel species within the GFSC, and *Fusarium* spp. were isolated from *E. dulcis* and found to be pathogenic on this plant. *F. commune* was the most common *Fusarium* sp. associated with Fusarium wilt of *E. dulcis*, and this pathogen was widely distributed in all six provinces. The novel species within the GFSC was found in Hubei and Zhejiang provinces, while the unclassified *Fusarium* sp. was not found in provinces outside of Hubei (Fig. 1).

Four *Fusarium* spp. (*F. oxysporum*, *F. acuminatum*, *F. semitectum*, and *F. moniliforme* var. *intermedium*) obtained from 11 counties in Zhejiang province were reported responsible for Fusarium wilt of *E. dulcis* by Jiang et al. (7). The occurrence of Fusarium wilt of *E. dulcis* attributed to *F. oxysporum* was also found in Anhui, Fujian, Guangdong and Hubei provinces (24). The difference in results between this study and previous reports might be explained from two aspects. First, the origin of isolates differs between the studies and, because several species of *Fusarium* have been reported as potential causes of Fusarium wilt of *E. dulcis*, the species causing *E. dulcis* wilt may vary in different locations. Second, there may be misidentifications in the studies because the morphological differences may be subtle. For example production of longer monophialides (>25 μ m) and polyphialides distinguished *F. commune* from *F. oxysporum* (29), and DNA-based identification techniques were lacking or not conducted during other studies (32).

Sixty-four isolates were identified as *F. commune* by the production of longer monophialides, and the majority of the isolates produced polyphialides. All of our sequenced *F. commune* isolates formed a clade with 100% BP and 100% PP support with the pre-

Table 2. Additional *Fusarium* spp. used in the phylogenetic study downloaded from GenBank

Isolate ^b	Species	Geographic origin	Accession number ^a		
			IGS	mtSSU	<i>EF-1α</i>
NRRL22903	<i>Fusarium commune</i>	Denmark	HM057285	U34509	HM057341
NRRL28387	<i>F. commune</i>	Denmark	HM057283	AF250560	HM057338
NRRL22538	<i>F. oxysporum</i> f. sp. <i>cepae</i>	Germany	DQ831891	DQ831929	DQ837681
BMP1375	<i>F. oxysporum</i> f. sp. <i>lactuca</i>	Wellton	DQ831878	DQ831920	DQ837673
Fo069	<i>F. oxysporum</i> f. sp. <i>loti</i>	Clinton Co., NY	EU313436	EU313473	EU313509
FOLR2	<i>F. oxysporum</i> f. sp. <i>lycopersic</i>	California	DQ831894	DQ831941	DQ837692
NRRL22546	<i>F. oxysporum</i> f. sp. <i>medicaginis</i>	South East Asia	DQ831901	DQ831937	DQ837690
TX388	<i>F. oxysporum</i> f. sp. <i>melonis</i>	Texas	DQ831887	DQ831943	DQ837696
FOV14	<i>F. oxysporum</i> f. sp. <i>vasinfectum</i>	California	DQ831885	DQ831942	DQ837695
FoChz10c	<i>F. oxysporum</i>	Clinton Co., NY	EU313440	EU313477	EU313533
FoStL1a	<i>F. oxysporum</i>	Addison Co., VT	EU313454	EU313491	EU313534
NRRL25446	<i>F. brevicatenulatum</i>	Madagascar	U61649	U61597	AF160265
NRRL13566	<i>F. fujikuroi</i>	Taiwan	AY249382	U34499	AF160279
NRRL25200	<i>F. lactis</i>	<i>Ficus carica</i>	U61655	U61603	AF160272
NRRL13604	<i>F. napiforme</i>	South Africa	AF160266	U34570	AF160266
NRRL13617	<i>F. phyllophilum</i>	Italy	AF160274	U34516	AF160274
NRRL25206	<i>F. pseudoanthophilum</i>	Zimbabwe	U61657	U61605	AF160264
NRRL22946	<i>F. pseudocircinatum</i>	Ghana	U34540	U34511	AF160271
NRRL13592	<i>F. pseudonygamai</i>	Nigeria	AF160263	U34505	AF160263
NRRL22944	<i>F. proliferatum</i>	Germany	AF160280	U34500	AF160280
NRRL25208	<i>F. ramigenum</i>	California	U61658	U61606	AF160267
BMP1462	<i>F. subglutinans</i>	Wellton, AZ	DQ831904	DQ831946	DQ837698
NRRL22045	<i>F. thapsinum</i>	South Africa	U34531	U34502	AF160270
NRRL22172	<i>F. verticillioide</i> s	Germany	AY249379	U34497	AF160262

^a IGS = intergenic spacer region, mtSSU = mitochondrial small subunit, and *EF-1 α* = translation elongation factor 1- α .

^b Designation of isolates and culture collections: NRRL = National Center for Agricultural Utilization Research, Peoria; FOV = Plant Sciences, University of Arizona; BMP = Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona; Fo = Department of Plant Pathology, Cornell University; and PUF = Clinical Microbiology, Peking Union Medical College Hospital, Shuaifuyuan, Beijing.

Table 3. Summary of tree statistics from translation elongation factor 1- α (*EF-1 α*), the mitochondrial small subunit (mtSSU) ribosomal DNA, the nuclear ribosomal intergenic spacer region (IGS), and combined sequence analyses^a

Locus	Character	MPTs length	Synapomorphies	Autapomorphies	CI	HI	RI	RC
<i>EF-1α</i>	702	269	103 (14.7%)	75 (10.7%)	0.784	0.216	0.914	0.717
mtSSU	707	75	41 (5.8%)	13 (18.4%)	0.760	0.240	0.959	0.729
IGS	2,772	1,593	574 (20.7%)	246 (8.9%)	0.646	0.354	0.895	0.578
Combined	4,181	1,930	718 (17.2%)	334 (8.0%)	0.672	0.328	0.902	0.606

^a MPTs = most-parsimonious trees, CI = consistency index, HI = homoplasy index, RI = retention index, and RC = rescaled consistency index.

viously-identified *F. commune* isolates from FUSARIUM-ID, thus confirming our morphological species identification. All *F. commune* isolates were characterized using specific PCR primers (efFc100F and efFc385R) to distinguish them from *F. oxysporum*, and specific PCR primers (FOF1 and FOR1) for detection of *F. oxysporum* were used for further characterization. Molecular detection results were consistent with morphological identification. Four isolates of GFSC were most closely related to *F. verticillioides* in the combined data set dendrogram but differed from *F. verticillioides* with the production of microconidia in false heads from monophialides and the absence of sporodochia (9). The *EF-1 α* sequences of XG2, ZJ3, ZJ5, and ZJ7 had 99% sequence identity with *F. andiyazi* (JF715933), and the MP and BS analyses inferred

from *EF-1 α* also showed that the four isolates formed a distinctive clade closely related to *F. andiyazi* (data not shown). However, the production of polyphialides by the four isolates distinguishes them from *F. andiyazi* (13). All dendrograms showed the four isolates forming a strongly supported clade distinct from other species within the GFSC, and the four isolates were morphologically indistinguishable from certain other species within GFSC. Therefore, it was identified as a novel *Fusarium* sp. within the GFSC. SY9107 is morphologically similar to *F. aquaeductum* (17); however, no sequences were available for comparison. Blast of mtSSU or IGS sequences in GenBank resulted in matches with sequences from unnamed *Fusarium* isolates, as well as a match with *F. cf. incarnatum*. However, *F. cf. incarnatum* differs from SY9107 by the

Fig. 6. Phylogram of maximum parsimony tree generated from combined sequence analyses of elongation factor 1- α , mitochondrial small subunit ribosomal DNA, and ribosomal intergenic spacer sequence data. Isolates sequenced are shown in bold. Upper numbers at major nodes indicate parsimony bootstrap values greater than 50% (1,000 replications) and lower numbers indicate Bayesian posterior probabilities.

production of polyphialides, and with single chlamydospores borne terminally or multiple intercalary chlamydospores formed in the aerial mycelium (5). Furthermore, the ITS region of SY9107 was sequenced and compared against GenBank, which showed 99% sequence identity with *Fusarium* spp. (GQ352485). The ITS sequence of *F. aquaeductum* (accession number FJ624268.1) did not appear in the matches of the blast result (*data not shown*). Thus, isolate SY9107 is described as an unclassified *Fusarium* sp.

DNA sequences from the mtSSU or *EF-1 α* regions were useful to distinguish *F. commune* from *F. oxysporum* on host plants of soybean, Douglas-fir, tomato, and horseradish (4,6,31,32). Phylogenetic analyses of the individual or the combined mtSSU and *EF-1 α* sequences have identified *F. commune* as a sister taxon of *F. oxysporum* (4,6,31,32). The phylogenetic analyses of three regions (mtSSU, *EF-1 α* , and IGS) in this study all indicated that *F. commune* is in a separate clade, clearly distinct from the FOSC (Fig. 6). Within the *F. commune* clade, isolates from different locations were scattered, with no evidence of phylogenetic structure with respect to geographic origin. Pathogenicity of *F. commune* isolates from different geography was significantly different. There was no evidence of geographic specialization, because there was no relationship between the geography of isolates and pathogenicity. Pathogenicity differentiation was also not concordant with intra-specific variation. For example, the combined gene dendrogram showed that highly virulent isolates TF5 and SS12 were clustered in different subclades, and highly virulent isolate TF5 was clustered with the lowly virulent isolate QJ21. The trees generated from the mtSSU data set in this study revealed only one distinct subclade among the *F. commune* isolates used in our study. All of the *F. commune* isolates from *E. dulcis* had identical sequences for mtSSU. Although, in many organisms, mitochondrial DNA has a higher rate of evolution than nuclear DNA (12), the mtSSU rDNA is highly conserved and inadequate to resolve the phylogenetic structure of *F. commune* isolated from *E. dulcis*. The *efF*c100F and *efF*c385R primers were found to be able to differentiate *F. commune* from *F. oxysporum* (31), although there is some risk in the specificity of detection because primer *efF*c385R showed a 100% identity with *EF-1 α* sequences of *F. fujikuroi* and other *Fusarium* spp. Because our studies suggest that the mtSSU sequences of *F. commune* in this study are highly conserved, and there is considerable sequence variation among closely related taxa, the mtSSU sequences may be used to differentiate *F. commune* from other *Fusarium* spp.

From the results of our study, a disease complex may be responsible for the Fusarium wilt of *E. dulcis*. According to the frequency of isolates obtained, it seems that *F. commune* is the primary causal agent for the disease in China. *F. commune* was the most aggressive species in the pathogenicity assays. The GFSC isolates also caused severe symptoms in artificial inoculations, and the pathogenicity of SY9107 was the lowest. However, the isolation frequency of these two latter species was comparatively low, and *F. commune* should retain primary consideration for disease management. Because *E. dulcis* is an aquatic plant, it will be difficult to control wilt of *E. dulcis* by chemicals. Management of wilt diseases caused by *Fusarium* spp. typically relies on crop rotations to reduce soil inoculum levels (3) and the use of genetically resistant cultivars (3). Due to high genetic variation and the range of virulence of different *F. commune* isolates, the development of resistant cultivars should consider the diversity of *F. commune*, making it essential that isolates do not overcome the resistance easily. Further research will focus on developing a real-time PCR assay for quantification of *F. commune* in plants and soil, which be of great value for better understanding of Fusarium wilt epidemiology, as well as developing an efficient disease management system through prediction of disease risk and evaluation of resistant cultivars.

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