

Incidence of *Fusarium* spp. on the invasive *Spartina alterniflora* on Chongming Island, Shanghai, China

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Abstract *Fusarium palustre* is an endophyte/pathogen of *Spartina alterniflora*, a saltmarsh grass native to North America that has been associated in the USA with a saltmarsh decline known as Sudden Vegetation Dieback (SVD). Since the intentional introduction of *S. alterniflora* to stabilize mud flats on Chongming Island, Shanghai, China, *S. alterniflora* has become invasive, but shows no symptoms of dieback even though *F. palustre* can be isolated from the plant. When declining *S. alterniflora* from SVD sites in the northeastern USA were assayed for *Fusarium* species, an

average of 8 % of tissues sampled gave rise to a species of *Fusarium* of these, 64 % were *F. palustre* and 16 % were *F. incarnatum*, a nonpathogenic species. To determine if low densities of *F. palustre* could explain the lack of dieback symptoms on *S. alterniflora* from Chongming Island, we assessed the incidence and distribution of *Fusarium* spp. on *S. alterniflora* from 12 sites on Chongming Island. On average, 26 % of the stem and root tissues sampled were colonized by a *Fusarium* species. Of 196 isolates recovered from *S. alterniflora*, 44 % were *F. incarnatum* and 41 % were *F. palustre*. Species determinations were confirmed for a subset of these isolates using a phylogenetic analysis of partial sequences of the translation elongation factor (*tef*) gene. The observation that *Fusarium* incidence on *S. alterniflora* was much greater on Chongming Island than in the USA survey raises the question as to why *S. alterniflora* on Chongming Island is showing no dieback. Other factors, such as predator release, enhanced nutritional, edaphic and/or other unidentified environmental constraints on Chongming Island may afford *S. alterniflora* protection from dieback.

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Introduction

The introduction of non-native plants into new habitats can result in successful invasion as well as many

other unintended consequences, including loss or decline of native species (Wilcove et al. 1998; Mack et al. 2000). In other instances, pests, saprophytes, or pathogens that accompany the non-native, invading plants may undergo changes in their relationship with the host facilitated by the new environment or may impact the native flora, possibly resulting in expanded host ranges, especially under conducive environmental conditions (Flory and Clay 2013; Strauss et al. 2012). For example, two plant pathogens, *Cryphonectria parasitica*, associated with chestnut blight, and *Ophiostoma ulmi*, associated with Dutch elm disease, were inadvertently introduced into the USA on plants, which had substantial impacts on the native host species (Anagnostakis 1987; Potter et al. 2011).

Spartina alterniflora Loisel was intentionally introduced to Chongming Island near Shanghai, China, in 2001 to stabilize mud flats. The source of the *S. alterniflora* germplasm was vegetative ramets from Yancheng in Jiangsu Province that had originated from an intentional introduction in 1979 via seeds and ramets obtained from coastal marshes from the southeastern USA (Morehead City, NC, Altamaha Estuary and Sapelo, GA and Tampa Bay, FL) (An et al. 2007; Li et al. 2009). Coincident with the invasion of *S. alterniflora* on Chongming Island was the decline of native sedges (*Scirpus mariqueter* Wang et Tang and *Carex* spp.) (Chen et al. 2004) and dieback of common reed grass (*Phragmites australis* Cav. Trin. ex Steud. (Chen et al. 2004; Xu and Zhao 2005).

While *S. alterniflora* continues to flourish on Chongming Island, it has undergone a major decline along the east and Gulf coasts of the USA where it is native. Known as Sudden Vegetation Dieback (SVD), this saltmarsh decline was first reported about 15 years ago (Alber et al. 2008; Elmer et al. 2013). Many hypotheses have been proposed, but no explanation adequately explains the cause of the occurrence. In 2011, a new fungal pathogen, *Fusarium palustre* Elmer and Marra, was isolated and described from declining *S. alterniflora* (Elmer and Marra 2011). The fungus was subsequently found in every USA marsh where SVD occurred, but pathogenicity tests never demonstrated that *F. palustre* could alone cause dieback without a contribution from other environmental stressors such as drought (Elmer et al. 2011, 2013). In a survey of salt marshes along the eastern and Gulf seaboard of the USA, more than half (55 %) of all *Fusarium* isolates recovered from SVD-affected

S. alterniflora ($n = 504$) were identified as *F. palustre* (Elmer et al. 2013). Also recovered in high numbers (20 %) in the USA were isolates belonging to a group of species known as the *F. incarnatum-equiseti* species complex (FEISC) (O'Donnell et al. 2009); these isolates are collectively identified in this report as *F. incarnatum*. Species in this group were rarely found to be pathogenic on *S. alterniflora* (Elmer and Marra 2011; Elmer et al. 2011) or other plants (Castellá and Cabañes 2014), but given their ubiquity in salt marshes and other niches, their ecological contribution may be significant as secondary decomposers or modulators of infection.

A dieback of the native reed *Phragmites australis* has been well documented on Chongming Island (Chen et al. 2004; Li et al. 2013; Xu and Zhao 2005). Li et al. (2014) isolated *F. palustre* from both *S. alterniflora* and *P. australis* sampled from Chongming Island and demonstrated in greenhouse tests that it was pathogenic to both plants by satisfying Koch's postulates. Despite proof of pathogenicity in the greenhouse, no symptoms of dieback have ever been observed on *S. alterniflora* on Chongming Island. Even though the presence of *F. palustre* was confirmed by Li et al. (2014), we questioned whether the relative incidence of *F. palustre* on Chongming Island on *S. alterniflora* was comparable to *S. alterniflora* in the USA. A survey conducted along the coast of Connecticut and Massachusetts, USA, found the mean incidence of *Fusarium* spp. isolated from *S. alterniflora* tissue to be 8 % in sites with SVD, but <1 % from tissue from marshes where no SVD was evident (Elmer et al. 2011). Of 240 isolates recovered in that survey, 65 % were *F. palustre*.

The relationship between inoculum density and disease expression can often dictate whether or not symptoms appear (Seem 1984), therefore, the presence of a lower incidence of *F. palustre* on *S. alterniflora* on Chongming Island may explain the lack of disease symptoms. Furthermore, it is not clear if *Fusarium* species composition could influence *S. alterniflora* dieback. Therefore, a detailed examination of the *Fusarium* community colonizing *S. alterniflora* may provide information on why the invasive *S. alterniflora* exhibits no symptoms of dieback. If *S. alterniflora* supports different incidences and distributions of *Fusarium* species in China than the USA, it may suggest some competitive interaction with resident species of *Fusarium* in China. Our

objective in this study was to assess *Fusarium* species composition on *S. alterniflora* on Chongming Island and compare results with a survey done in the USA (Elmer et al. 2011).

Materials and methods

Field sampling

The Dongtan wetland on Chongming Island (31°25′–31°38′ N 121°50′–122°05′ E) is a 32,600 ha salt marsh once dominated by native sedges (e.g., *Scirpus* spp. and *Carex* spp.) and *P. australis*. Over the past decade, *S. alterniflora* has invaded large areas. Twelve sites were sampled where *S. alterniflora* had invaded a marsh area (Table 1). From each site, three to five plants were sampled within a 5 m radius. Plants were uprooted so that crown roots were still attached along with about 60 cm of the above ground tissue. Tissue was thoroughly washed in tap water to remove all soil, pressed between absorbent paper towels, and allowed to dry at room temperature until examination. Plants were processed within 72 h of being sampled.

Isolation and Identification

Eighty pieces of stem and 20 pieces of root tissue (0.5 cm long) were cut from the three to five bulked plants from each site. Tissue was surface-disinfested in 10 % household bleach (0.053 % Na hypochlorite),

and placed on Peptone PCNB agar (PPA) (10 pieces per plate). Plates were incubated at 22–25 °C for 5–7 days whereupon colonies were counted, then sub-cultured to carnation leaf agar (CLA), and grown for 7–10 days under cool white fluorescent lights set at 12-h photoperiods. Colonies were examined under 100× magnification and all *Fusarium* colonies were sub-cultured again by placing a single conidium onto CLA as described above. Colonies were identified to species based on spore morphology (Leslie and Summerell 2006; Elmer and Marra 2011).

PCR and Sequencing

Sixteen monosporic *Fusarium* cultures—thirteen morphologically identified as *F. palustre* and three as *F. incarnatum*—were chosen for molecular phylogenetic species confirmation using partial sequences of the translation elongation factor (*tef*) gene (Table 2). Mycelium was grown for 1 week at room temperature in potato dextrose broth (PDB). Following centrifugation and rinsing in sterile distilled water, mycelium was then lyophilized, and genomic DNA extracted using the Omniprep-for-Fungi kit (G-Biosciences, St. Louis, MO). Conditions and primers for PCR were as previously published (Elmer and Marra 2011). Briefly, concentrations of approximately 0.1 ng DNA/μL were combined in 30 μL volumes with 0.5 units of Invitrogen (Carlsbad, CA) High-Fidelity Platinum Taq, 2.5 mM MgSO₄, 0.2 mM each of the four nucleotides and 0.25 mM each primer (EF1 and EF2; O'Donnell

Table 1 Percent *Fusarium* incidence on *Spartina alterniflora* and coordinates of sampling sites

Site number	Percent <i>Fusarium</i> incidence ^x	Coordinates
1	71.6 ^b	N31°33.704′ E121°56.089′
2	46.7 ^b	N31°30.945′ E121°57.431′
3	31.7 ^a	N31°30.944′ E121°57.441′
4	1.7 ^a	N31°31.370′ E121°58.887′
5	13.3 ^a	N31°31.483′ E121°57.594′
11	8.3 ^a	N31°34.338′ E121°55.400′
12	30.0 ^a	N31°34.334′ E121°55.409′
13	16.7 ^a	N31°34.323′ E121°55.368′
8	25.0 ^a	N31°31.477′ E121°57.583′
16	43.3 ^b	N31°33.702′ E121°56.084′
17	18.3 ^a	N31°33.719′ E121°56.080′
19	20.0 ^a	N31°33.721′ E121°56.071′
Mean	26 %	

^x *Fusarium* incidence based on the number of colonies of *Fusarium* growing from 100 pieces of plant tissue (80 stem pieces and 20 root pieces); values followed by differing letters are statistically different by Tukey's HSD Test at $P = 0.05$

Table 2 Isolate codes, origin, and morphological designation for isolates of *Fusarium* recovered from *Spartina alterniflora* used in genetic analysis of *Tef* gene

Isolate number	Isolate (alternate name)	Origin	Species
ChF 34 ^x	C15R 3	Shanghai, China	<i>F. palustre</i>
ChF 35 ^x	C15S 2	Shanghai, China	<i>F. palustre</i>
ChF 36	C16S 6	Shanghai, China	<i>F. palustre</i>
ChF 35b	C17S 1	Shanghai, China	<i>F. palustre</i>
ChF 37	C17S 1	Shanghai, China	<i>F. palustre</i>
ChF 30	C1S23	Shanghai, China	<i>F. palustre</i>
ChF 31	C3S 2	Shanghai, China	<i>F. palustre</i>
Fp 117	ChF48, C9S 3	Shanghai, China	<i>F. palustre</i>
Fp 121	ChF52, C9S 3	Shanghai, China	<i>F. palustre</i>
Fp 112	ChF46	Shanghai, China	<i>F. palustre</i>
Fp 125	ChF55, C11S 13	Shanghai, China	<i>F. palustre</i>
Fp 124	ChF 54, C17S 4	Shanghai, China	<i>F. palustre</i>
Fp 119	ChF 19, C4S X	Shanghai, China	<i>F. palustre</i>
Fp 135	PI 4D Heathy 2-4	Poplar Island, MD USA	<i>F. palustre</i>
Fp 137	PI 4D Heathy 1-2	Poplar Island, MD USA	<i>F. palustre</i>
Fp 134	PI 4D Healthy1-4	Poplar Island, MD USA	<i>F. palustre</i>
CaesSaCT4 ^y	NRRL 54063	Madison, CT USA	<i>F. palustre</i>
CaesSaME1	–	Wells, ME USA	<i>F. palustre</i>
Fp 136	Pi Cell 4D SVD 3-1	Poplar Island, MD USA	<i>F. palustre</i>
Fp 127	Site E BG Brown 6	LA, USA	<i>F. palustre</i>
Fp 128	Site E BG Green 7	LA, USA	<i>F. palustre</i>
ChF 11	C2R 4	Shanghai, China	<i>F. incarnatum</i>
ChF 14	C7S 5	Shanghai, China	<i>F. incarnatum</i>
ChF 5	C16R 6	Shanghai, China	<i>F. incarnatum</i>
CaesSaVA3	–	VA, USA	<i>F. incarnatum</i>
CaesSaVA6 ^y	NRRL 54083	VA, USA	<i>F. incarnatum</i>
CaesSaGA4	–	GA, USA	<i>F. incarnatum</i>

^x Recovered from *Phragmites australis*

^y Partial *tef* sequences for *F. palustre* (CaesSaCT4, NRRL 54063) and *F. incarnatum* (CaesSaVA6 NRRL 54083) were deposited in GenBank database under accession number GQ856943 and GQ856977, respectively

et al. 1998) in the accompanying PCR Buffer. Cycling parameters, amplicon sequencing, and alignment of the raw sequence data were as previously published (Elmer and Marra 2011), and as follows.

Partial *tef* sequences from the 16 Chongming isolates were aligned using MEGA 6.06 (Tamura et al. 2013) along with partial *tef* sequences from eight USA *F. palustre* isolates. Phylogenetic analysis was performed in MEGA using the Maximum Likelihood method (Guindon and Gascuel 2003; Tamura et al. 2013) with 500 bootstrap replicates based on the Kimura 2-parameter evolutionary model, which was chosen because it had the lowest Bayesian Inference Criterion (Schwarz 1978) score in a test of six evolutionary models (General Time Reversible; Kasegawa-Kishino-Yano; Tamura-Nei; Tamura 3-parameter; Kimura 2-parameter; Jukes-Cantor). From an initial alignment of 528 nucleotides, all positions with

less than 95 % site coverage were eliminated, resulting in a total of 498 informative positions in the final dataset. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach (Tamura et al. 2004), then selecting the topologies with superior log likelihood values.

Results

The relative incidence of *Fusarium* species recovered from *S. alterniflora* averaged 23 % and 29 % for the roots and stems pieces, respectively (Table 1). Of the 196 *Fusarium* isolates recovered from Chongming Island, 44 % were *F. incarnatum*, 41 % were *F. palustre*, 12 % were *F. chlamyosporum*-like, and

3 % were *F. proliferatum*. The distribution between the root and stems differed in that *F. palustre* was found in greater incidence on stems where *F. incarnatum* composed a greater percentage in the roots (Fig. 1).

Species determinations were confirmed for a subset of these isolates using a molecular phylogenetic analysis of partial sequences of the *tef* gene using the Maximum Likelihood method (Table 2). This confirmed morphology-based species determinations, showing that those Chinese isolates identified as *F. palustre* based on morphology clustered as expected with isolates of *F. palustre* from the USA (Fig. 2). Partial *tef* sequences from the US isolates were deposited in Genbank database. Similarly, isolates from Chongming Island identified morphologically as *F. incarnatum* clustered with *F. incarnatum* from the USA.

Discussion

These findings show that the incidence of *Fusarium* species on the invasive *S. alterniflora* samples from Chongming Island is much higher than that found on native *S. alterniflora* in the USA where plants exhibit SVD. What was not clear is why *S. alterniflora* exhibit no symptoms of dieback on Chongming Island, despite the presence of very high densities of the *F. palustre*. An earlier report by Li et al. (2014) showed that *F. palustre* isolated from *S. alterniflora* was capable of inciting disease on *S. alterniflora* using artificial inoculations under greenhouse conditions (Li et al. 2014). Although Li et al. (2014) implicated *F.*

palustre as a contributing factor in the Phragmites dieback, there was no indication that *S. alterniflora* was adversely affected by the presence of *F. palustre* or any other biotic pests (Li et al. 2014). In the USA, the opposite has occurred, where *F. palustre* has been associated with SVD of *S. alterniflora* (Elmer et al. 2013). However, despite the appreciable differences in the relative incidences of colonization of *S. alterniflora* by *Fusarium* spp. between Chongming Island (26 %) and the USA survey (8 %), the overall distribution of *Fusarium* spp. on *S. alterniflora* was similar in that *F. palustre* totaled 41 and 65 % of the isolates recovered from Chongming Island and the USA, respectively.

This study does not provide any convincing explanation for why no dieback occurs on *S. alterniflora* on Chongming Island. Release from unknown environmental stressors may have allowed *S. alterniflora* to proliferate. Nutritional and edaphic factors may also govern whether or not symptoms appear on plants (Datnoff et al. 2007). Li et al. (2014) demonstrated that *F. palustre* was recovered from *P. australis* in areas where *S. alterniflora* had invaded, and they associated *F. palustre* with the dieback on *Phragmites*. These observations do support a hypothesis that *S. alterniflora* may be mediating the spread of *F. palustre* on Chongming Island.

Species determinations of putative *F. palustre* and *F. incarnatum* isolates from Chongming Island were confirmed through genetic analysis of partial *tef* gene sequences; all isolates identified morphologically as *F. palustre* or *F. incarnatum* clustered with known *F. palustre* and *F. incarnatum* isolates from eastern marshes of the USA. We note that there is less variation in *F. palustre* compared to *F. incarnatum*. The dominance of these two fungal species in two geographically distinct marshes is intriguing. Both species may have evolved in salt marsh habitats. Elmer and LaMondia (2014) demonstrated that both species have increased tolerance to NaCl. Hyphae of *F. palustre* and *F. incarnatum* could grow unimpeded on agar amended with NaCl at levels (0.27 M) equivalent to marsh water, whereas genetically similar terrestrial species of *Fusarium* showed substantial inhibition.

It is tempting to speculate that *F. palustre* may have been introduced to Chongming Island on the invasive *S. alterniflora*. However, data presented here do not provide sufficient evidence to establish the origin of

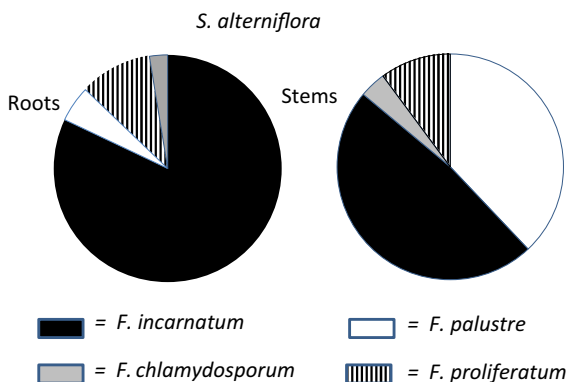
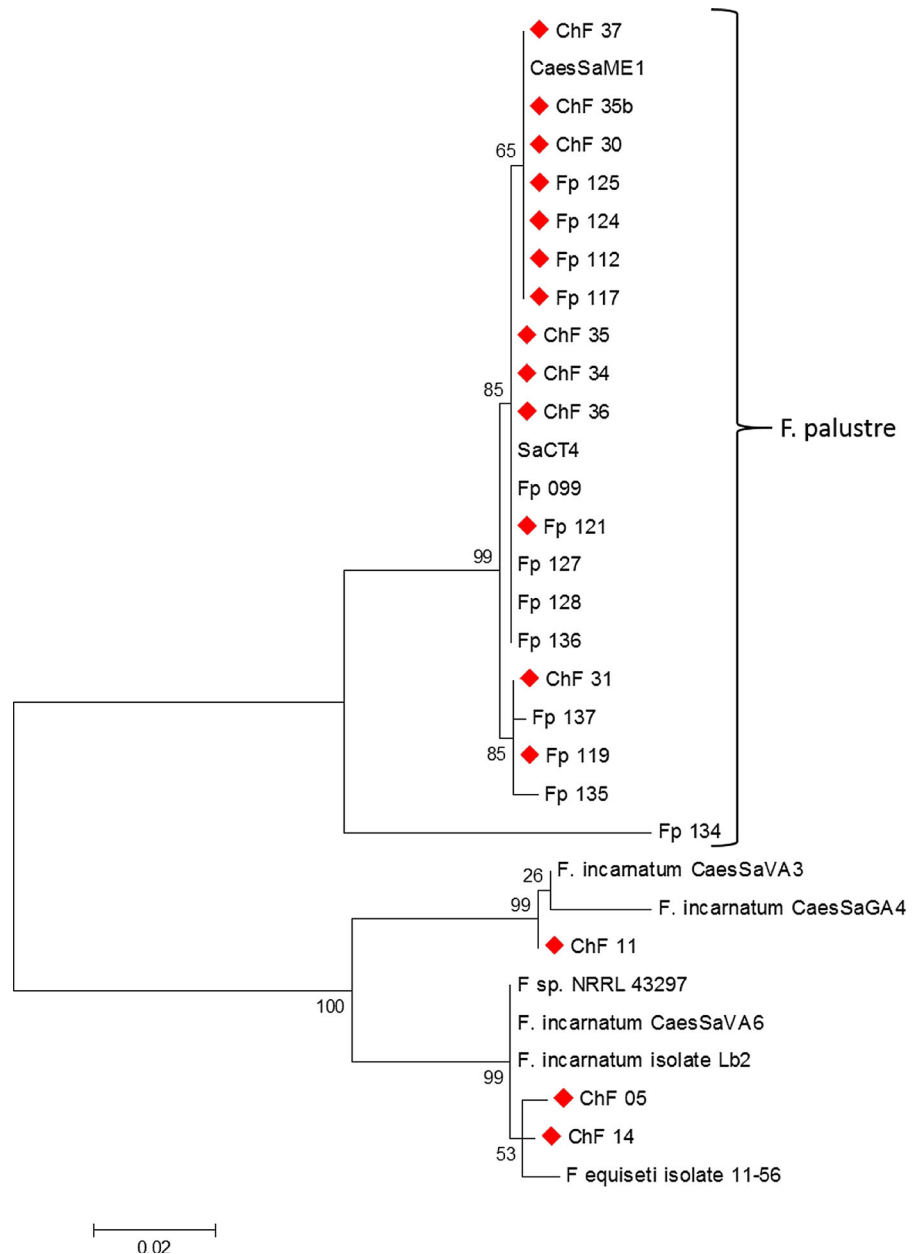


Fig. 1 Total distribution of *Fusarium* species isolated from roots ($n = 42$) and stems ($n = 84$) from *Spartina alterniflora*

Fig. 2 Phylogenetic relationships among taxa of *Fusarium* associated with *Spartina alterniflora* on Chongming Island, China, and marshes of the eastern USA, inferred using the Maximum Likelihood method. Isolates ChF34, ChF35 were isolated from *Phragmites australis*. Based on an analysis of partial gene sequences from translation-elongation factor 1- α , the tree shown had the highest log-likelihood value among the 500 bootstrap trees generated in the analysis. The percentage of trees in which the associated taxa clustered together is shown at the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. See text for further details. Red triangles represent Chinese isolates



Chinese strains of *F. palustre*; i.e., whether *F. palustre* was introduced from the USA with *S. alterniflora*, or was already present at the time that *S. alterniflora*'s was introduced. Testing these hypotheses would require population genetic analyses using molecular markers and a large set of isolates from each continent.

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