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Fusarium oxysporum and *Fusarium avenaceum* associated with yield-decline of pyrethrum in Australia

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Abstract *Fusarium* species were isolated from roots, crowns, basal petioles but rarely from the leaves of infected pyrethrum (*Tanacetum cinerariifolium*) plants showing poor growth and stunting in yield-decline sites of northern Tasmania and the Ballarat region of Victoria, Australia. Multigene phylogenetic analyses using the internal transcribed spacer (ITS), the polymerase II second largest subunit (*RPB2*) and the partial translation elongation factor 1-α (*EF1*) sequences, identified *F. oxysporum* and *F. avenaceum* as the most frequently isolated species associated with root and crown diseases of pyrethrum, and *F. equiseti* and *F. venenatum* at a low incidence. Pathogenicity trials confirmed that *F. oxysporum* and *F. avenaceum* significantly affected growth of pyrethrum plants causing Fusarium crown rot; however, *F. avenaceum* was less pathogenic than *F. oxysporum*. *Fusarium oxysporum* and *F. avenaceum* may be part of a complex of pathogens that are involved in pyrethrum yield-decline.

Key words: *Fusarium avenaceum, Fusarium oxysporum*, Multigene phylogeny, *Tanacetum cinerariifolium*, Yield-decline

Introduction

Pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz. Bip. is a perennial plant cultivated commercially for the extraction of natural pyrethrin insecticides contained in the oil glands on the surface of the achenes. In Australia, pyrethrum production occurs in northern Tasmania and near Ballarat in Victoria where the majority of the world's natural pyrethrum is grown (Hay et al. 2015).

Pyrethrum seed is sown from July to September (winter) with first harvest occurring in December (summer) and January of the following year (15 to 17 months after planting). Thereafter, harvests are implemented annually in summer (Pethybridge et al. 2008b). Ideally, under this intensive production system, pyrethrum fields should persist without replanting for at least four harvests (Hay et al. 2015). However, over the last 10 years there has been increasingly poor regrowth of plants after the first harvest, or lower yield after the second growing season. Plants affected by this "yield-decline" had weak root growth and in some cases had no feeder roots at all. Internal crown discoloration was observed in most affected plants and brown necrotic leaf lesions could be found on both sides of the leaves. Pycnidia and perithecia were observed at the bases of the flower stems, where they were attached to the crown (Moslemi et al. 2016a).

Pyrethrum is known to be subjected to a range of potentially damaging foliar and soil-borne diseases caused by both nematodes and fungi. Several foliar pathogens including *Stagonosporopsis tanaceti*, causing ray blight (Vaghefi et al. 2012), *Didymella tanaceti* the cause of tan spot (Pearce et al. 2015) and *Colletotrichum tanaceti* that causes anthracnose (Barimani et al. 2013) have also been shown to reduce pyrethrum growth. Hay et al. (2009) identified the root rot nematodes, *Pratylenchus penetrans* and *P. crenatus*, and Scott et al. (2014) identified *Sclerotinia sclerotiorum* the cause of Sclerotinia flower blight and crown rot in poorly growing plants. More recently, Moslemi et al. (2016b) identified *Paraphoma vinacea,* as the cause of Paraphoma crown rot of pyrethrum. However, no study has conclusively shown any one of these pathogens to be the principal cause of poor plant growth and subsequent yield-decline of pyrethrum. Hay et al. (2002) could not attribute yield-decline to a single cause and concluded that a combination of biotic and abiotic factors was likely to be responsible for the problem.

Fusarium species might also be implicated in yield-decline as they are important soil-borne pathogens with a wide host range. Crown rot of cereals occurs in most regions of the world including Australia, Europe, South Africa and South America (Smiley et al. 2005). Smiley et al. (2005) reported that a combination of *Fusarium* species including *F. pseudograminearum*, *F. culmorum* and *F. avenaceum* were associated with root rot of barley and wheat in Pacific Northwest, USA. Species of *F. avenaceum* have been reported as causal agents of head blight, crown and root rot of several cereals, mostly in cool and temperate areas of the world including Canada and Central Europe (Uhlig et al. 2007; Stakheev et al. 2016). They have also been reported as root and crown rot pathogens of ornamental plants including lisianthus (*Eustoma grandiflorum*) and carnation (*Dianthus caryophyllus*) (Nalim et al. 2008). Fusarium dry rot is a significant disease of potato tubers caused predominately by *F. sulphureum (syn. F. sambucinum)* in North America and Europe (Leslie et al. 2006); and *F. coeruleum, F. avenaceum, F. sulphureum* and *F. culmorum* in the UK (Cullen et al. 2005).

Not many species of *Fusarium* have been reported as causing disease in pyrethrum, although *F. oxysporum* was reported to be a pathogen of pyrethrum in South Africa and Kenya, where it caused damping-off of seedlings, and wilt, chlorosis and necrosis of larger plants. However, *F. oxysporum* has never been reported as a pathogen of pyrethrum in Tasmania (Pethybridge et al. 2008b). *Fusarium oxysporum* is generally regarded as the most widely distributed species causing vascular wilt in many plant species throughout the world (Leslie and Summerell 2006; Laurence et al. 2014) damaging plants by blocking the xylem vessels and producing mycotoxins (O'Donnell et al. 2013). *Fusarium oxysporum* also causes damping-off, root and crown rot, and eventually wilting, with or without the involvement of toxins or enzymes (Singh and Kumar 2014). Saprophytic strains of *F. oxysporum* are also abundant in soil, and generally cause no disease (Leslie and Summerell 2006; Ellis et al. 2014). *Fusarium oxysporum* is often referred to as the "*F. oxysporum* species complex" as it consists of multiple cryptic species with varying pathogenicity and host ranges (Ellis et al. 2014). Within the complex, form species (*formae speciales*) that can infect particular host species but not others, have been described for over 150 specific hosts (O'Donnell et al. 1998; Ellis et al. 2014).

Fusarium taxonomy is very fluid with the number of recognised species fluctuating from nine to over 1000 depending on which species concept (morphological, biological and phylogenetic) has been adopted (Summerell et al. 2003). However, none of the species concepts have been able to adequately describe variation within the genus. For instance the morphological concept fails in some aspects of species identification as the number of morphological characters is far less than the number of species to be distinguished (Summerell et al. 2010). Species complexes within *Fusarium* have been shown to be polyphyletic making species identification difficult. For instance, species of the *F. chlamydosporum* species complex fall within the *F. sambucinum* and *F. triticinum* species complexes in the phylogenetic study conducted by O'Donnell et al. (2013) using RNA polymerase II subunit 1 (*RPB1*) and subunit 2 (*RPB2*) gene sequences. Based on the *RPB1*, *RPB2* and the partial translation elongation factor 1-α (*EF1*) genes sequences, most *Fusarium* phytopathogens have been classified into four important *Fusarium* complexes: the *F. fujikuroi* species complex (FFSC), the *F. sambucinum* species complex (FSAMSC), the *F. oxysporum* species complex (FOSC), and the *F. solani* species complex (FSSC; (Hyde et al. 2014; O'Donnell et al. 2015). K. O'Donnell et al. (2010) reported that all three genes were equally informative in identification of species within *Fusarium*.

The aims of this study were i) to identify *Fusarium* species associated with pyrethrum yield-decline in Australia ii) to assess their pathogenicity and effect on growth of pyrethrum plants.

Materials and methods

Sample collection

All isolates were recovered from different tissues of pyrethrum plants collected from nine yield-decline affected fields in 2014, 2015 and 2016. The first sampling in June 2014 was a preliminary survey of *Fusarium* spp. with samples collected from all nine sites (one plant per site) (Table 1). Pyrethrum plants were randomly selected from sites 49206, 49207, 49301, 64209, 86901, 46410 (located near Devonport, Tasmania); 76406 (located near Deloraine, Tasmania); and 47110 and 74201 (located near Launceston, Tasmania). These fields had been sown in September 2012 and harvested in the summer of 2013 and 2014.

Plants showing stunting and poor regrowth were uprooted with intact sub-crown and partial root systems and transferred to the laboratory. Roots were washed gently under tap water for 5 min to remove excess soil. Tissues from the upper roots, crowns, petiole bases and leaves were cut into 3 to 5 cm sections, surface sterilised in 80% ethanol for 30 s, 1% active ingredient sodium hypochlorite for 1 min and then rinsed twice in sterilised water for 1 min. They were blotted on a sterilised paper towel and small 5 mm sub-sections of the tissue were placed on both water agar (WA) and ethanol potassium amoxicillin agar (EPAA) (Mansoori 2011). Plates were incubated under a 12 h photoperiod at 23-25°C for 3-4 days (Leslie and Summerell 2006). Mycelium from developing colonies was subsequently subcultured onto potato dextrose agar (PDA) and incubated for a further 5 days at 23°C under cool white fluorescent light with a 12 h photoperiod (Burgess and Summerell 1992).

The second sampling was in January 2015 from three of the sites sampled previously, 49301 and 76406 (located near Devonport, Tasmania) and 64209 (located near Deloraine, Tasmania) (Table 2). The remaining six Tasmanian sites that had been previously sampled, were not available having been ploughed-in due to the poor regrowth after harvest. Seven plants from each site were selected based on visual observation of yield-decline symptoms, such as necrotic crown tissue and curled, silvery and necrotic leaves. Similar steps to the first sample collection were followed to transfer and culture the plants.

Two further samplings were carried out in September 2015 and February 2016 from yield-decline affected pyrethrum fields near Ballarat, Victoria, Australia (Table 3). In each sample collection, seven pyrethrum plants from three fields were uprooted, transferred to the laboratory and tissues incubated following the same techniques as described for the previous collections. Cultures of the isolates were deposited at the Queensland Plant Pathology Herbarium (BRIP), Brisbane, Australia.

Morphological identification of *Fusarium* spp.

Single spore colonies were obtained from isolates grown on PDA and incubated for 10 days with conditions as previously described. *Fusarium* cultures were identified based on colony morphology and pigmentation on PDA. The microscopic characters of spore shape and size, presence or absence of chlamydospores, micro- and macroconidia, sporodochia and mono- or poly-phialides (Leslie and Summerell 2006) were studied after subculturing for 10-12 days on synthetic poor nutrient agar (SNA) with pieces of sterilised filter paper on the surface of the agar as a source of carbon to induce sporulation (Crous et al. 2009).

Micromorphological features were viewed, measured and photographed using a DM2900 *Leica* compound microscope (×100 magnification). The size (length × width) of 30 micro- and macroconidia, and chlamydospores were measured for each isolate (the mean and standard deviation (SD) of the mean were calculated). Colony growth rate was measured for three plates per isolate at two-day intervals for eight days. Presence or absence of sporodochia was assessed after one month's growth on SNA with sterilised filter paper.

Molecular identification

DNA extraction and PCR amplification

Nine Tasmanian and six Victorian isolates of *Fusarium* from a range of morphotype groups were selected for identification to species level using multi-gene phylogenetic analysis. Mycelia were scraped directly from 7-day-old single spored cultures on PDA and DNA extracted using the DNeasy Plant Mini Kit (Qiagen Pty. Ltd., Australia) following the manufacturer's instructions. PCR was used to amplify the internal transcribed spacer (ITS) region (500 bp) using primer pairs ITS1 and ITS4 (White et al. 1990). Approximately 410 bp of the *EF1* gene was amplified using primers EF-728f (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998); and >1800 bp of the *RPB2* gene was amplified using 5F2 and 7cR, 7cF and 11aR, respectively (O'Donnell et al. 2010). The PCR conditions for amplification of the ITS region were as described by White et al. (1990), for the *RPB2* gene as described by Reeb et al. (2004) and for the *EF1* gene, touchdown PCR was used following conditions described by Rehner at http://www.aftol.org/pdfs/EF1primer.pdf, with annealing temperatures of 56, 62 and 53-63°C, respectively. DNA amplification was performed using an Eppendorf thermal cycler (Eppendorf Pty. Ltd. Australia). PCR products were purified using a QIAquick

PCR purification kit (Qiagen Pty. Ltd. Australia) and sent to the Australian genome research facility (AGRF) (Melbourne, Australia) for sequencing.

Multigene phylogenetic analysis

Purified amplicons sequences were viewed using Chromas Lite MFS software (Technylesium Pty Ltd, South Brisbane, Australia). Isolate sequences were tentatively identified after BLAST searching in the FUSARIUM-ID database (Geiser et al. 2004). Sequences were aligned both manually using the sequence alignment editing program MEGA6 (Tamura et al. 2013), and in MAFFT (http://mafft.cbrc.jp/alignment/software/) separately. Consensus sequences were obtained from forward and reverse sequences using "*DeNovo* assembly" in Geneious v 7.06 (Kearse et al. 2012). Consensus sequences were pairwise aligned and annotated with the closely related reference sequences derived from the GenBank database within Geneious. Phylogenetic trees were constructed for each individual gene, and in combination, using MEGA6. Both maximum likelihood (ML) and maximum parsimony (MP) statistical methods were used to construct individual and multigene trees. The best substitution model for each analysis was selected using MEGA6. Support of branches was checked by bootstrap analysis with 1000 pseudoreplicates. Gaps were treated as missing data. Consensus sequences were deposited in the GenBank database (accession numbers are shown in Table 4) and trees were deposited in TreeBASE (www.treebase.org).

Pathogenicity tests

Pathogenicity of *Fusarium* species isolated from pyrethrum plants was assessed in three trials. For each trial, seedlings of cultivar Pyrate were germinated from steam sterilised seeds and germinated in a seed raising mix in Tasmania (BRA Pty. Ltd.). Seedlings were transferred to 10 cm diameter pots containing potting mix (Debco Pty. Ltd., Victoria, Australia), fertilised with 5 g of Osmocote Plus (Scotts Pty. Ltd., New South Wales, Australia) per pot and grown in a glasshouse for two months at 25-27°C under natural light.

Inoculum of the different isolates of *Fusarium* was prepared from 5-day-old, single-spored cultures by adding 10 mL of sterile water to each plate and gently scraping the colony surface with a glass spreader and filtering the spore suspension through cheesecloth. The concentration of each spore suspension was standardised to 10^5 spore/mL using a haemocytometer. Two drops of 0.1% Tween-20 solution were added to each spore suspension to improve adherence of the spores to the surface of the inoculated tissue (Pethybridge et al. 2008a).

For inoculation, three-month-old pyrethrum plants at the rosette stage of growth were removed from the soil in the pots, and the roots washed in running tap water for 5 min to remove excess soil. The roots were immersed in 200 mL of the spore suspension for 10 min, and each plant was subsequently replanted in the same pot. Controls were dipped in sterilised water but otherwise treated identically. Pots were maintained in the glasshouse for two months at 25-27°C under natural light. Two weeks after inoculation, senesced and curled petioles were randomly selected and cultured on WA and PDA as described previously to check for infection and identify pathogens.

Two months after inoculation plants were destructively sampled, tissues were plated on WA and any colonies transferred to PDA for isolate identification. Above-ground, below-ground and total biomass of the harvested plants in each trial were measured by drying individual plants in an oven at 71°C for three days.

The first trial involved screening of *F. oxysporum* (BRIP 64441) and *F. avenaceum* (BRIP 64442) isolated from fields in Tasmania in a four replicate trial. The second trial included *F. oxysporum* (BRIP 64449) and *F. avenaceum* (BRIP 64443) in a 10 replicate (per pathogen treatment) trial.

A third trial assessed pathogenicity of *F. oxysporum*, (BRIP 64441) *F. avenaceum* (BRIP 64442) and *F. venenatum* (BRIP 64446). All trials were performed in a completely randomised design (CRD). *Fusarium equiseti* was only isolated from one pyrethrum plant in Tasmania; therefore it was not considered to be associated with yield-decline of pyrethrum and hence was not included in the pathogenicity bioassays.

Dry weight data (above ground, below ground and total) were analysed using a mixed model (the MIXED procedure in SAS) and applying the residual maximum likelihood (REML) estimation method. This permitted data from all three trials to be combined validly in the one analysis. The linear mixed model fitted was:

$$Y_{ijk} = \mu + \tau_i + \gamma_j + \tau\gamma_{ij} + \varepsilon_{ijk}$$

Where μ is the overall mean, τ_i is the fixed effect of treatment i, γ_j is the random effect of trial j, $\tau\gamma_{ij}$ is the random interaction between treatment i and trial j and ε_{ijk} is an independent random error. The variable ε is assumed to be normally distributed, with fixed variance within a trial, but may vary between trials. Comparison of means was performed by pairwise t-tests (α=0.05). SAS was used for all analyses (SAS V9.4, SAS Institute Inc., Cary, NC, USA).

Results

Isolation of *Fusarium* spp.

From the first sample collection in Tasmania in 2014, plants from all the sites except 64209 were found to be infected with various *Fusarium* spp. At three out of the nine sites plants were infected with *F. oxysporum* which was frequently recovered from crown, upper root and basal petiole tissues of poorly growing plants. *Fusarium avenaceum* was also recovered from the upper roots, crowns, basal petioles and leaves of plants from three different sites. *Fusarium venenatum* was recovered only from crown tissue of one plant at one site, and *F. equiseti* from the crown and petiole tissues of two plants at a different site (Table 1).

Stagonosporopsis tanaceti, the cause of ray blight of pyrethrum was recovered from basal petiole and leaf tissues of all sampled plants (100% of the plants) and at a lower but still considerable frequency from the crown and upper root tissues (50% of the plants). *Didymella tanaceti* and *P. vinacea* were isolated from six of the nine sites (67%). *Didymella tanaceti* was recovered only from basal petiole and leaf tissues, while *P. vinacea* was isolated mostly from upper root and crown tissues.

From the second sampling (January 2015), all seven plants from site 49301, six plants from site 64209 and six plants from site 76406 were shown to be infected with *F. oxysporum, F. avenaceum* and/or *F. venenatum*, (Table 2). One plant at each of the 49301 and 64209 sites was infected with all three *Fusarium* species. Infected crown tissues were discoloured. At site 64209, *F. oxysporum* was most frequently isolated from the upper roots, crowns and basal petioles of infected plants. At site 74604, *F. avenaceum* was most frequently isolated from the upper root, crown and basal petiole tissues of infected plants. At site 49301, *F. oxysporum* and *F. avenaceum* were isolated from the upper roots, crowns and basal petioles of the infected plants; *F. venenatum* was isolated less frequently from the upper roots. Two cases of crown infection with *P. vinacea* were also observed at site 64209.

From the first sampling in Ballarat (September 2015), all seven plants at sites 92409 and 92205 were infected with *F. oxysporum*, which was isolated mostly from the upper roots and crowns and in low percentages from the basal petioles; and *F. avenaceum,* which was isolated frequently from the crowns, upper roots and in lower percentages from the basal petioles (Table 3). At all three sites, two to five plants had crown tissues infected with *F. oxysporum* and *F. avenaceum*.

From the second sampling near Ballarat (February 2016), all seven plants at each site were infected with *F. oxysporum* with a high incidence at sites 91006 and 90707. At all three sites, three to five plants had crown and upper root tissues infected with both *F. oxysporum* and *F. avenaceum* (Table 3).

Fusarium species identification

Individual and combined Maximum Parsimony (MP) trees were developed using the sub-tree pruning regrafting (SPR) algorithm. For the maximum likelihood analyses, K2+G was determined as the best substitution model. The combined multilocus maximum parsimony phylogenetic tree, consisting of aligned nucleotide sequences for 28 isolates, was 1803 bp in length (ITS: 464, *RPB2*: 1009, *EF1*: 330) in the final dataset of which 412 nucleotide sites (ITS: 108, *RPB2*: 183, *EF1*: 121) were variable. In the MP and ML individual and combined phylogenetic trees the two *F. oxysporum* isolates from the infected pyrethrum plants from Tasmania and four from Ballarat clustered with the *F. oxysporum* reference isolates and formed a monophyletic clade in the *F. oxysporum* species complex (FOSC); four *F. avenaceum* isolates from Tasmania and two from Ballarat formed a well-supported clade with reference isolates in the *F. triticinum* species complex (FTSC); one isolate from Tasmania was identical to *F. venenatum* in the *F. sambucinum* species complex (FSAMSC); and two isolates from Tasmania were nested within the *F. incarnatum-equiseti* species complex (FIESC). A combined maximum parsimony tree of ITS, *EF1* and *RPB2* is shown in Fig. 1 and the phylogenetic tree for *EF1* is presented (Supplementary Figure 1). Maximum likelihood bootstrap values were very similar to those in maximum parsimony analyses.

Taxonomy

Morphological characters of the isolates conformed to those described for *F. oxysporum* and *F. avenaceum* species studied by Leslie and Summerell (2006) (Table5).

Pathogenicity of *Fusarium* species

In the first glasshouse bioassay with *F. oxysporum* and *F. avenaceum*, the petioles, crowns and upper roots were infected by both species in all the four replicates, two months after root dip inoculation. *Fusarium oxysporum* and *F. avenaceum* were isolated from basal petioles sampled two weeks after inoculation showing that infection rapidly became established. In the second bioassay, the crowns and upper roots of all 10 replicates were infected with *F. oxysporum*; and in seven replicates the crowns and upper roots were infected with *F. avenaceum*. In the third experiment, of the eight replicates, crowns and upper roots of 50% (4 out of 8) of the plants were infected with *F. oxysporum* and *F. avenaceum* and the crown and upper roots of 25% of the plants (2 out of 8) with *F. venenatum*.

Further analyses of the effect of *Fusarium* spp. on dry weight of infected plants were only undertaken for *F. oxysporum* and *F. avenaceum* as they caused a high incidence of infection. *Fusarium venenatum* was excluded, as it infected only two of the inoculated plants in the third trial. The mixed model analysis of growth estimates of the treatment by trial interaction variance were zero for above-ground and total biomass and not statistically significant for below-ground. Thus a reduced model was used omitting the interaction. The F-tests indicated there were highly significant differences between treatments for all of the variables, below-ground, above ground and total biomass. Plants infected with *F. oxysporum* invariably had significantly lower mean dry weight when compared to the control plants for all three variables. *Fusarium avenaceum* infected plants had intermediate mean dry weights, significantly less than the control for both total and below-ground biomass, but above-ground biomass was not significantly different compared to the control (Table 6). Thus both species are pathogens capable of reducing growth of pyrethrum plants, but *F. oxysporum* appears to be the more pathogenic of the two.

Discussion

Fusarium oxysporum and *F. avenaceum* were frequently isolated from the crown and upper root tissue of infected pyrethrum plants in yield-decline affected fields of northern Tasmania and Ballarat, Victoria, Australia. *Fusarium equiseti* and *F. venenatum* were isolated from only two fields in Tasmania and hence, were not considered to be generally involved in crown and root rot of pyrethrum.

Stagonosporopsis tanaceti was isolated from crown and/or roots of 50% of the infected plants (89% of the fields) as well as from the leaf and petiole tissues in the first samples collected in Tasmania indicating that *S. tanaceti* was an important pathogen of pyrethrum in yield-decline affected fields. In four fields, crown tissue, and in one field upper root tissue was infected with the pathogen. This is the first report of crown and root infection of pyrethrum plants with *S. tanaceti* and this will have serious implications for the management of the disease. More investigations are required to determine the interaction between *S. tanaceti* and *Fusarium* spp. and their effect on growth and regrowth of pyrethrum plants.

The diversity of *Fusarium* spp. is greatly dependent on environmental conditions and geographical locations of the susceptible crops (Doohan et al. 2003). Cool temperatures in summer affect incidence of *F. oxysporum, F. graminearum, F. acuminatum* and *F. solani* as the causal agents of soybean root rot in Iowa, while, wet conditions induced damping-off of soybean by various *Fusarium* spp. (Arias et al. 2013).

Popovski and Clear (2013) reported that temperature and rainfall played the most important role in the population of *Fusarium* species in the soil, and both factors significantly influenced the growth and interaction between *Fusarium* species such as *F. graminearum* and *F. verticillioides*. Burgess and Summerell (1992) also found that the diversity of *Fusarium* species was related to environmental conditions including rainfall, vegetation and temperature; but correlation between temperature and rainfall made it difficult to separate the effects of each factor. Certain species of *Fusarium* can be restricted to areas with different temperatures and rainfall regimes. Very little is known about the biogeography of these pathogens in soils in relation to climate (Burgess and Summerell 1992). Hence, the association and importance of *Fusarium* species infecting pyrethrum plants in additional geographical areas need to be investigated.

Although, a *formae speciales* has not been described for *F. oxysporum* causing root and crown rot of pyrethrum plants, more knowledge is needed regarding the host range of the *F. oxysporum* holotypes isolated from pyrethrum. Singh and Kumar (2014) reported *F. oxysporum* f.sp. *chrysanthemi* as causing wilt of *Chrysanthemum,* in which the xylem was obstructed resulting in stunted growth and abnormal growth of buds and flowers. However, the pathogen was also reported to infect other *Asteracea*e including Paris daisy (*Argyranthemum frutescens*) and African daisy (*Osteospermum* sp.), thus raising doubts about the status of this isolate of *F. oxysporum* as a host-specific form species. More investigation needs to be performed in order to determine if *F. oxysporum* strains isolated from the root and crown of pyrethrum plants in Tasmania are host specific, or have a broader host range.

Pathogenicity bioassays showed that *F. oxysporum* and *F. avenaceum* caused significant reduction in plant growth two months after inoculation of young pyrethrum plants however, *F. avenaceum,* was less pathogenic. Further bioassays are needed to determine the importance of the interaction of these two species in crown rot and yield-decline of pyrethrum, especially in combination with abiotic stresses that may predispose pyrethrum to infection by soil-borne pathogens. The root dip inoculation technique which was successful in establishing crown infection by *F. oxysporum* has been used for studying pathogenicity of the soil-borne pathogens *Verticillium dahlia* causing Verticillium wilt of olive and potato; and for *F. oxysporum* f. sp. *niveum* and *F. oxysporum* f. sp. *melonis* causing wilt disease of cucurbits (Campbell and Griffiths 1973; Freeman and Rodriguez 1993; Trapero et al. 2013). Trapero et al. (2013) suggested that disease severity progressed faster in plants inoculated by roots dipped in spore suspension and the time needed to obtain the maximum disease severity was considerably shorter compared to other inoculation methods that have been used, including stem injection.

This is the first report of the isolation of *F. oxysporum, F. avenaceum, F. equiseti* and *F. venenatum* from the root, crown and basal petiole tissue of pyrethrum plants; and of the pathogenicity of *F. oxysporum* and *F. avenaceum* on pyrethrum plants sampled from poorly growing pyrethrum in northern Tasmania and the Ballarat region of Victoria, Australia. *Fusarium oxysporum* was shown to be an important pathogen of pyrethrum.

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Table 1 Site number, type of tissue sampled from which *Fusarium* spp. were isolated in nine fields of pyrethrum affected with symptoms of yield-decline in June 2014

Site Number	Infected tissues	No. of infected tissues from which *Fusarium* spp. were isolated			
		F. oxysporum	*F. avenaceum*	*F. venenatum*	*F. equiseti*
47110	Upper root	1	0	0	0
	Crown	0	0	0	0
	Basal petiole	0	0	0	0
	Leaf	0	0	0	0
46410	Upper root	0	0	0	0
	Crown	0	0	1	0
	Basal petiole	0	0	0	0
	Leaf	0	0	0	0
74201	Upper root	0	1	0	0
	Crown	0	1	0	0
	Basal petiole	0	0	0	0
	Leaf	0	0	0	0
64209	Upper root	0	0	0	0
	Crown	0	0	0	0
	Basal petiole	0	0	0	0
	Leaf	0	0	0	0
49301	Upper root	1	0	0	0
	Crown	1	0	0	0
	Basal petiole	0	0	0	0
	Leaf	0	0	0	0
76406	Upper root	0	0	0	0
	Crown	0	1	0	0
	Basal petiole	0	0	0	0
	Leaf	0	1	0	0
49206	Upper root	1	0	0	0
	Crown	1	0	0	0
	Basal petiole	0	0	0	0
	Leaf	0	0	0	0
49207	Upper root	0	0	0	0
	Crown	0	0	0	0
	Basal petiole	0	1	0	0
	Leaf	0	0	0	0
86901	Upper root	0	0	0	0
	Crown	0	0	0	1
	Basal petiole	0	0	0	1
	Leaf	0	0	0	0

Table 2 Site number and type of tissue samples and plants from which *Fusarium* spp. were isolated in three fields of pyrethrum affected with symptoms of yield-decline in January 2015

Site Number	Infected tissues	No. of infected tissues from which *Fusarium* spp. were isolated				
		F. oxysporum	*F. avenaceum*	*F. venenatum*	*F. oxysporum,F. avenaceum,F. venenatum*[a]	Total No. of infected plants (of the 7 replicates)
49301	Upper root	1	0	1	0	7
	Crown	2	1	0	1 B	
	Basal petiole	0	1	0	1 A	
	Leaf	0	0	0	1 C	
	No. of plants infected with each *Fusarium* sp.	3	2	1	1	
64209	Upper root	4	0	0	1 B	6
	Crown	1	0	0	1 A	
	Basal petiole	3	0	0	1 C	
	Leaf	0	0	0	0	
	No. of plants infected with each *Fusarium* sp.	5	0	0	1	
76406	Upper root	0	2	0	0	6
	Crown	1	2	0	0	
	Basal petiole	0	1	0	0	
	Leaf	0	0	0	0	
	No. of plants infected with each *Fusarium* sp.	1	5	0	0	

[a] A= infection with *F. avenaceum*; B = infection with *F. oxysporum*; C = infection with *F. venenatum*

Table 3 Site number and type of tissue samples and plants from which *Fusarium* spp. were isolated in three fields of pyrethrum afflicted with symptoms of yield-decline in Ballarat, Victoria, in September 2015 and February 2016

Sampling date	Site Number	Infected tissue	No. of infected tissues from which *Fusarium* spp. were isolated			
			F. oxysporum	*F. avenaceum*	*F. oxysporum* and *F. avenaceum*[a]	Total No. of infected plants (7 replicates)
Sep-15	92413	Upper root	0	0	0	3
		Crown	1	0	2 A,B	
		Basal petiole	0	0	0	
		Leaf	0	0	0	
		No. of plants infected with each *Fusarium* sp.	1	0	2	
	92409	Upper root	0	0	1 A,B	7
		Crown	2	2	2 A,B	
		Basal petiole	0	0	0	
		Leaf	0	0	0	
		No. of plants infected with each *Fusarium* sp.	2	2	3	
	92205	Upper root	1	0	1 A,B	7
		Crown	2	0	3 A,B	
		Basal petiole	0	0	0	
		Leaf	0	0	0	
		No. of plants infected with each *Fusarium* sp.	3	0	4	
Feb-16	92205	Upper root	0	1	1 A,B	7
		Crown	1	0	4 A,B	
		Basal petiole	0	0	0	
		Leaf	0	0	0	
		No. of plants infected with each *Fusarium* sp.	1	1	5	
	91006	Upper root	3	0	1 A,B	7
		Crown	2	0	1 A,B	
		Basal petiole	0	0	0	
		Leaf	0	0	0	
		No. of plants infected with each *Fusarium* sp.	5	0	2	
	90707	Upper root	2	0	0	7
		Crown	2	0	3 A,B	
		Basal petiole	0	0	0	
		Leaf	0	0	0	
		No. of plants infected with *Fusarium* sp.	4	0	3	

[a] A= infection with *F. avenaceum*; B = infection with *F. oxysporum*.

Table 4 Accession numbers of *Fusarium* isolates used in this research

Fusarium isolates	Culture accession No.	Location	Isolate source	Accession No.		
				RPB2	*EF1*	ITS
F. avenaceum[a]	PUF034	China	Unknown	HQ165829	HQ165865	HQ165937
F. avenaceum	UoM002; BRIP 64445	Tasmania; Australia; site 47201	*Tanacetum cinerariifoilum*,	KU529170	KU529161	KU529152
F. avenaceum	UoM0012; BRIP 64442	Tasmania; Australia; site 76406	*Tanacetum cinerariifoilum*,	KU529171	KU529162	KU529153
F. avenaceum	UoM0014; BRIP 64443	Tasmania; Australia; site 76406	*Tanacetum cinerariifoilum*,	KU529172	KU529163	KU529154
F. avenaceum	UoM0016; BRIP 64444	Tasmania; Australia;site 49207	*Tanacetum cinerariifoilum*,	KU529173	KU529164	KU529155
F. avenaceum	UoM0024; BRIP 64450	Ballarat; Australia; sites 91006, 92205	*Tanacetum cinerariifoilum*,	KX058559	KX058553	KX058547
F. avenaceum	UoM0025; BRIP 64451	Ballarat; Australia; sites 70909, 92409	*Tanacetum cinerariifoilum*,	KX058560	KX058554	KX058548
F. concolor [d]	NRRL 13459	South Africa	Plant debris	GQ505852	GQ505763	GQ505674
F. culmorum[c]	NRRL 25475	England	Unknown	JX171628	AF212463	AF006342
F. equiseti[d]	NRRL 20697	Chile	Beet	JX171595	GQ505595	GQ505683
F. equiseti[d]	NRRL 26419	Germany	Soil	GQ505777	GQ505599	GQ505688
F. equiseti[d]	NRRL 36136	Unknown	Unknown	GQ505822	GQ505644	GQ505733
F. equiseti	UoM0022; BRIP 64447	Tasmania; Australia; site 86901	*Tanacetum cinerariifoilum*,	KU529174	KU529165	KU529156
F. equiseti	UoM0023; BRIP 64448	Tasmania; Australia; site 86901	*Tanacetum cinerariifoilum*,	KU529175	KU529166	KU529157
F. oxysporum[b]	NRRL 13604	Namibia	Millet	EF470117	HE984150	U34570
F. oxysporum[b]	NRRL 43539	Missouri	Cornea	DQ790596	DQ790508	DQ790552
Fusarium sp.[b]	NRRL 43504	Pennsylvania	Cornea	EF469994	EF459955	EF453107
Fusarium sp.[b]	NRRL 43466	Ohio	Contact lens case	EF469978	EF452939	EF453091
Fusarium sp.[b]	NRRL 43521	Florida	Cornea	EF470001	EF452962	EF453114
F. oxysporum[b]	NRRL 43668	New Jersey	Cornea	EF470038	EF452999	EF453151
F. oxysporum[b]	NRRL 43431	Connecticut	Cornea	DQ790579	DQ790491	DQ790535
F. oxysporum	UoM005; BRIP 64449	Tasmania; Australia; site 49301	*Tanacetum cinerariifoilum*,	KU529168	KU529159	KU529150
F. oxysporum	UoM0011; BRIP 64441	Tasmania; Australia; sites 49301, 47110, 49206	*Tanacetum cinerariifoilum*,	KU529169	KU529160	KU529151
F. oxysporum	UoM0026; BRIP 64452	Ballarat; Australia; sites	*Tanacetum cinerariifoilum*,	KX058555	KX058549	KX058543

		70909 , 92413				
F. oxysporum	UoM0027; BRIP 64453	Ballarat; Australia; sites 92409, 92205	*Tanacetum cinerariifoilum,*	KX058556	KX058550	KX058544
F. oxysporum	UoM0028; BRIP 64454	Ballarat; Australia;sites 92205, 92409	*Tanacetum cinerariifoilum,*	KX058557	KX058551	KX058545
F. oxysporum	UoM0029; BRIP 64455	Ballarat; Australia; sites 91006,	*Tanacetum cinerariifoilum,*	KX058558	KX058552	KX058546
F. venenatum[e]	NRRL 22196	Japan	-[f]	JX171607	GQ915515	U85543
F. venenatum	UoM0020; BRIP 64446	Tasmania; Australia; site 46410	*Tanacetum cinerariifoilum,* n	KU529167	KU529158	KU529149

[a] Wang et al. (2011), [b] O'Donnell et al. (2007), [c]Bakan et al. (2002), [d] O'Donnell et al. (2009), [e] O'Donnell et al. (2013), [f] Isolate source unknown.
NRRL=Agricultural Research Service Culture Collection; FRC= Fusarium Research Centre, Pennsylvania State University, PA; UoM= University of Melbourne

Table 5 Key morphological features of isolates of *F. oxysporum* and *F. avenaceum* from pyrethrum characterised and measured in this study. SD=standard deviation

Fusarium species	Growth rate after 7 d (mm)	Phialides	Septa	Macroconidia length × width (SD)	Microconidia length × width (SD)	Chlamydospore	Sporodochium
F. oxysporum (BRIP 64441)	40	Monophialides	3-4 septate	16-44 (SD 7.9) µm × 2-4.5 (SD 0.76) µm	2-12 (SD 3.39) µm × 2-5 (SD 0.95) µm	Single and in chains	Present
F. avenaceum (BRIP 64442)	35	Mono and ployphialides present	4-7 septate	15-57 (SD 12.61) µm × 3-4 (SD 3.67) µm	3-17 (SD 6.26) µm × 1.5 -3.5 (SD 0.53) µm	Absent	Present

Table 6 The effect of two *Fusarium* species on the dry weights of the above and below ground portions of pyrethrum plants. Means with different letters in the same column are significantly different by pairwise t-tests (α=0.05). In all cases the F ratio for testing the treatment effect has 2 and 61 df.

Treatment	Dry weight component (g)		
	Above ground	Below ground	Total
Control	4.57 a	4.59 a	9.28 a
F. avenaceum	4.15 a	3.58 b	6.69 b
F. oxysporum	3.50 b	3.43 b	6.90 b
F-ratio (P-value)	12.81 (<0.001)	8.56 (<0.001)	11.46 (<0.001)

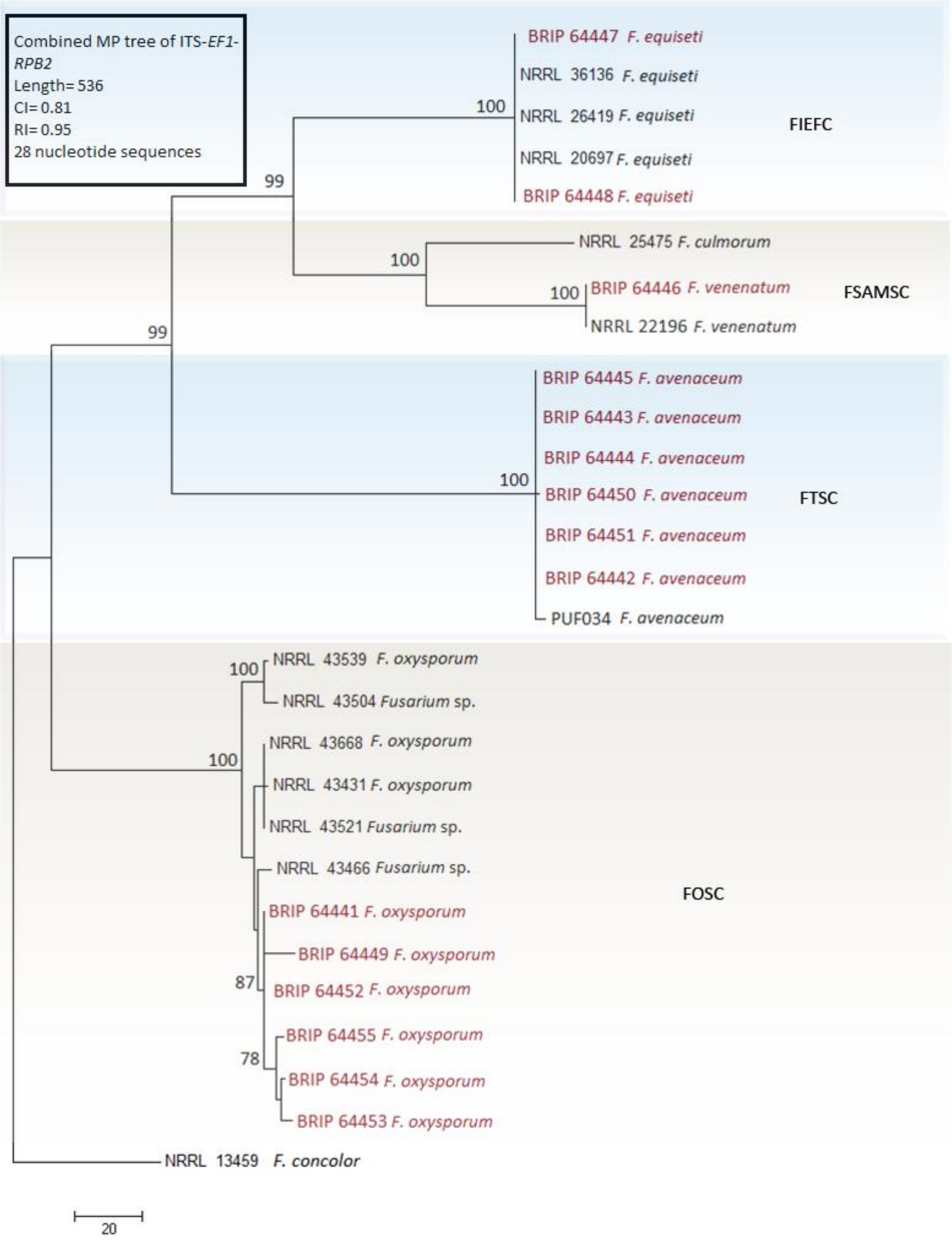

Fig. 1 One of the eight most parsimonious trees inferred from ITS, *RPB2* and *EF1* sequence data. CI= consistency index, RI= retention index. MP bootstrap values are indicated above internodes. Bootstrap values less than 70% are not shown. The highest log likelihood in the ML tree was (-5023.6236). The tree is rooted with *F. concolor* NRRL 13459 (O'Donnell et al. 2009). The scale bar shows the number of nucleotide changes per site. Strains included in this study are shown in red. FIEC= *F. incarnatum-equiseti* species complex; FSAMSC= *F. sambucinum* species complex; FTSC= *F. triticinum* species complex; FOSC= *F. oxysporum* species complex.

Electronic Supplementary Material

Fusarium oxysporum and *Fusarium avenaceum* associated with yield-decline of pyrethrum in Australia

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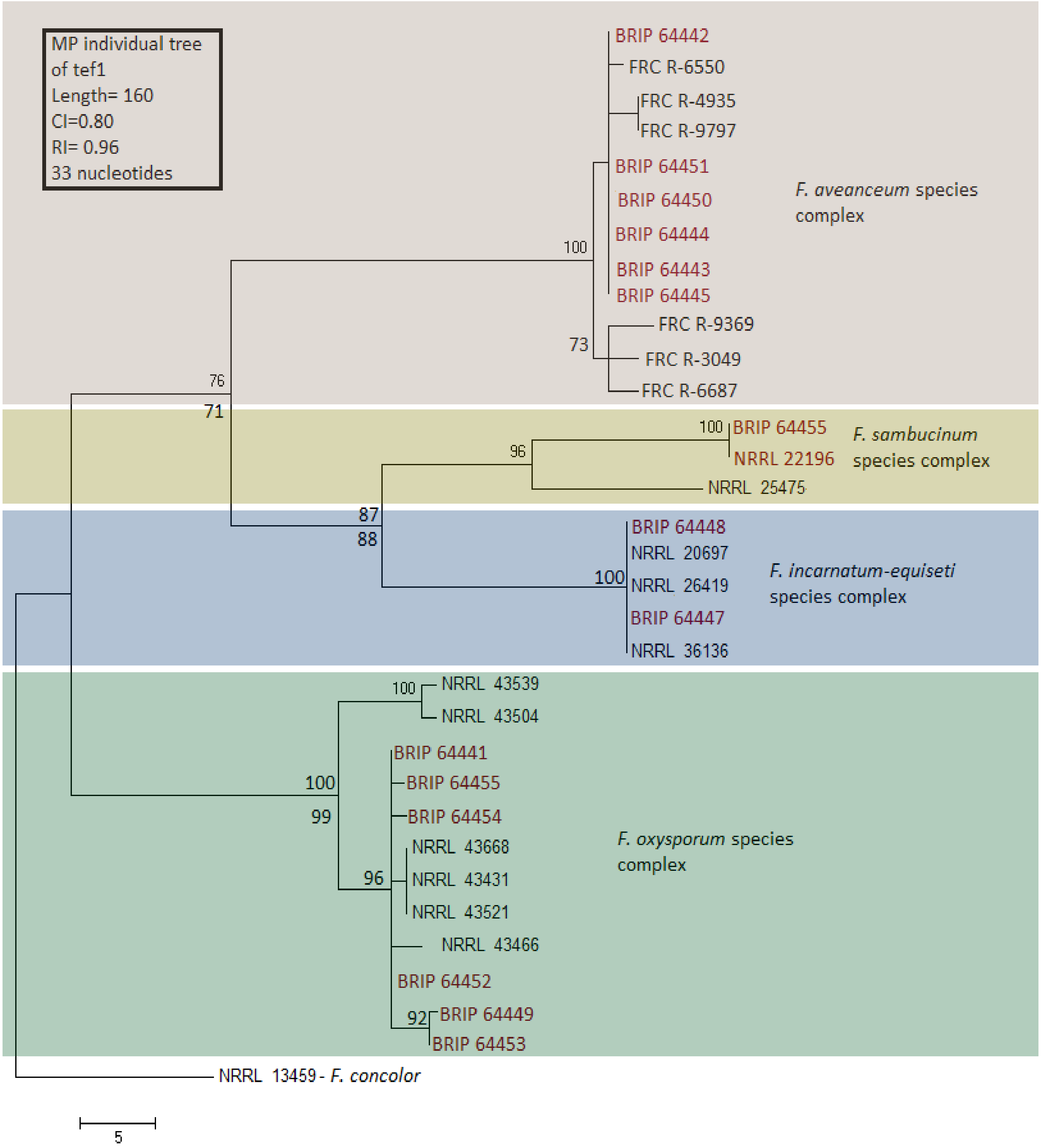

Electronic Supplementary Material Fig. 1. One out of nine most parsimonious trees of *EF1*. CI= consistency index, RI= retention index. MP bootstrap values are indicated above internodes. ML values are indicated below internodes. Bootstrap values less than 70% were removed. The highest log likelihood in ML tree was (-236.9984). The tree is rooted with *F. concolor* NRRL 13459 (O'Donnell et al. 2009). The scale bar shows the number of changes per site.