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Multilocus sequence analysis of *Fusarium pseudograminearum* reveals a single phylogenetic species

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ABSTRACT

Fusarium pseudograminearum causes crown rot of wheat in Australia and most other wheat growing regions, but its evolutionary history is largely unknown. We demonstrate for the first time that *F. pseudograminearum* is a single phylogenetic species without consistent lineage development across genes. Isolates of *F. pseudograminearum*, *F. graminearum sensu lato*, and *F. cerealis*, were collected from four countries and four single copy, nuclear genes were partially sequenced, aligned with previously published sequences of these and related species, and analysed by maximum parsimony and Bayesian inference. Evolutionary divergence varied between genes, with high phylogenetic incongruence occurring between the gene genealogies. The absence of geographic differentiation between isolates indicates that the introduction of new fungal strains to a region has the potential to introduce new pathogenic and toxigenic genes into the native population through sexual recombination.

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Introduction

The fungal species *Fusarium pseudograminearum*, is the principal causal agent of crown rot, a disease of increasing concern for wheat producers in many parts of the world, including Australia, North America (Mishra *et al.* 2006; Smiley *et al.* 2005), Turkey (Nicol *et al.* 2004) and New Zealand (Monds *et al.* 2005). In Australia crown rot is a chronic problem of wheat production inflicting a loss of >A$ 56 million annually. It is particularly damaging in Queensland, northern New South Wales and parts of South Australia (Williams *et al.* 2002), and its significance in South Australia has risen with the increasing importance of durum varieties that are highly susceptible (Wallwork *et al.* 2004). In the USA the disease occurs in the states of Idaho, Oregon, Washington, Colorado, Wyoming and Texas where it is variously named as crown rot, dryland foot rot, foot rot, dryland root rot and root rot; all have a complex aetiology with *F. pseudograminearum*, *F. culmorum* and *F. avenaceum* as the dominant pathogens (Paulitz *et al.* 2002). Additionally, *F. pseudograminearum* has been responsible for major epidemics in Australia (Burgess *et al.* 1987) of Fusarium head blight (FHB; McMullen *et al.* 1997), a disease that is emerging as a sporadic, but significant, threat to global wheat production (Goswami & Kistler 2004). Field isolates of *F. pseudograminearum* from both wheat spikes and crowns have been shown to cause FHB in greenhouse tests (Akinsanmi *et al.* 2004; Monds *et al.* 2005). Epidemics of these diseases result in high economic losses through reduced grain quality and quantity. Of particular concern is the contamination of infected grains with mycotoxins, such as the trichothecenes dexoynivalenol (DON) and nivalenol (NIV), which pose threats to both animal and human health upon consumption (Snijders 1990).

Fusarium pseudograminearum was first identified as a morphologically and culturally indistinguishable population within the species *F. graminearum* (Purss 1969); designated

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F. graminearum Group 1 (Burgess *et al.* 1975). Based on variation in DNA sequence (Aoki & O'Donnell 1999a), RFLP (Benyon *et al.* 2000) and isozymes (Laday *et al.* 2000), *F. pseudograminearum* has been formally accepted as a separate species. A key factor differentiating the two species is the heterothallic nature of *F. pseudograminearum* (teleomorph: *Gibberella coronicola*) compared to the homothallic *F. graminearum* (teleomorph: *Gibberella zeae*) (Aoki & O'Donnell 1999b).

Due in part to its recent description as an independent species, little work has been undertaken on the evolutionary history of *F. pseudograminearum*. Conversely, the former *F. graminearum* Group 2 has been subject to in depth study in recent times probably because of its close association with FHB worldwide. Cumulative sequencing and phylogenetic comparison of 11 nuclear genes has demonstrated that *F. graminearum* (hereafter referred to as the *Fg* clade) consists of nine distinct lineages (O'Donnell *et al.* 2000; O'Donnell *et al.* 2004; Ward *et al.* 2002), each of which have been proposed for formal species status (O'Donnell *et al.* 2004). The presence of nine phylogenetic species within the *Fg* clade, begs the question of whether a similar lineage development/speciation has occurred within its close relative, *F. pseudograminearum*. Significant genetic diversity has been recorded in Australian populations of *F. pseudograminearum*, based on AFLP markers, and was equivalent to the levels of diversity among Australian isolates from the *Fg* clade (Akinsanmi *et al.* 2006). However, the sexual stage of *F. pseudograminearum* is rarely observed in the field (Summerell *et al.* 2001) and sexual recombination is rare in laboratory studies (Aoki & O'Donnell 1999b). This would suggest that clonal reproduction dominates the evolutionary history of *F. pseudograminearum*, which could allow this diversity to develop into distinct lineages over time.

Barriers to sexual recombination, such geographic separation, permit the development of new species. In the *Fg* clade, lineage structure was related to geographic origin, and was seen as a cause of lineage development (O'Donnell *et al.* 2000). Relationships between genotypes and geography can also be indicative of factors such as climatic preference, and/or host genotype interactions.

This study aimed to provide answers to the question of whether *F. pseudograminearum* contains distinct evolutionary lineages. Secondary questions were: (1) is the evolutionary structure of *F. pseudograminearum* indicative of recombination?; and (2) is there a biogeographic relationship with the evolutionary structure of *F. pseudograminearum*? The answers to these questions are necessary to better understand the ecology of the pathogen and improve control strategies for the diseases it causes.

Materials and methods

Isolate collection

Wheat plants infected with crown rot and/or FHB were collected from fields in Queensland and New South Wales in Australia (Table 1). Two millimetre sections were cut from symptomatic head or crown tissue, surface sterilised in bleach (1 % available chlorine) for 5 min, followed by two 5 min rinses in sterile water and placed onto ¼ strength potato dextrose agar (PDA) plates containing 100 $\mu g ml^{-1}$ streptomycin sulphate and 10 $\mu g ml^{-1}$ tetracycline hydrochloride. Plates were incubated under ambient light and temperature for seven to ten days. Following incubation, plates were flooded with sterile distilled water, and the resultant suspension streaked onto water agar plates, that were maintained under ambient conditions for 12 to 24 h. A single germinating macroconidium was then transferred to a fresh, ¼ strength PDA plate. Monoconidial isolates were maintained at 25 °C for 14 d and stored as agar plugs under 15 % glyercol at −80 °C. Additional isolates were obtained from collaborators in Australia and internationally, where CR and/or FHB epidemics associated with *F. pseudograminearum* have been recently reported (Table 1). All Australian isolates used in this study were deposited in the Queensland Plant Pathology Herbarium, Brisbane.

DNA extraction

DNA was extracted using a modified CTAB protocol (Möller *et al.* 1992). Briefly, 50 mg of fungal mycelia was scraped from 10-d-old PDA cultures and manually ground in 1.5 ml microcentrifuge tubes. Five hundred microlitres of pre-warmed (60 °C) TES lysis buffer (100 mM Tris, pH 8.0; 10 mM ethylenediaminetetraacetate (EDTA), pH 8.0; 2 % (w/v) sodium dodecyl sulphate), and 50 μg of Proteinase K, were added to the ground material, and the resultant suspension incubated at 60 °C for 60 min. One hundred and forty microlitres of 5 M NaCl and 64 μl of 10 % (w/v) cetyltrimetholammonium bromide (CTAB) were added and the suspension incubated at 65 °C for 10 min. DNA was extracted by twice adding an equal volume of chloroform:isoamyl alcohol (24:1), centrifuging at 14 000 ***g*** for 10 min and transferring the aqueous phase to a fresh tube. DNA was precipitated by adding 0.6 vol of cold isopropanol and 0.1 vol of 3 M sodium acetate (pH 5.2) and maintaining tubes at −20 °C overnight. DNA was pelleted by centrifugation at 14 000 ***g*** for 30 min, washed twice with cold 70 % ethanol and suspended in 100 μl TE (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0). RNA was digested by adding 10 mg/ml of RNAse A and incubating at 37 °C for 45 min. Extractions were stored at −20 °C prior to use.

Species confirmation

All isolates were identified to species level using a two-step screening process, involving a combination of morphological and molecular techniques (Akinsanmi *et al.* 2004) to identify suitable subset of isolates for phylogenetic analysis.

Isolates were initially identified based on morphological and cultural characteristics on both PDA and carnation leaf agar (CLA). Isolates were grown on PDA and CLA at 25 °C for 10 to 14 d, under alternating periods of 12 h of combined black light (F20T9BL-B 20W FL20S.SBL-B NIS, Japan) and standard fluorescent light (35098 F18E/33 General Electric, USA), and 12 h darkness. Colony morphology and pigmentation on PDA, and spore type, shape and size on CLA, were compared to the published descriptions of Burgess *et al.* (1994) and Aoki & O'Donnell (1999a). For each isolate, 50 macroconidia were examined at 400× magnification and their dimensions and morphology recorded. Mean spore dimensions were used for comparison with published descriptions.

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Table 1 – Isolates used in this study

Isolate	BRIP No.[a]	Species[b]	Origin[c]	Source[d]	GenBank accession No.			
					TEF	*PHO*[e]	*RED*	*BT*
CS3002	5168	*F. pseudograminearum*	QLD, Australia	CPI	DQ382109	DQ382045	DQ381981	DQ382173
CS3043	47669	*F. pseudograminearum*	QLD, Australia	CPI	DQ382110	DQ382046	DQ381982	DQ382174
CS3060	47670	*F. pseudograminearum*	QLD, Australia	CPI	DQ382111	DQ382047	DQ381983	DQ382175
CS3064	47671	*F. pseudograminearum*	QLD, Australia	CPI	DQ382112	DQ382048	DQ381984	DQ382176
CS3066	47672	*F. pseudograminearum*	QLD, Australia	CPI	DQ382113	DQ382049	DQ381985	DQ382177
CS3074	47673	*F. pseudograminearum*	QLD, Australia	CPI	DQ382114	DQ382050	DQ381986	DQ382178
CS3077	47674	*F. pseudograminearum*	QLD, Australia	CPI	DQ382115	DQ382051	DQ381987	DQ382179
CS3085	47675	*F. pseudograminearum*	NSW, Australia	CPI	DQ382116	DQ382052	DQ381988	DQ382180
CS3096	47676	*F. pseudograminearum*	NSW, Australia	CPI	DQ382117	DQ382053	DQ381989	DQ382181
CS3121	47677	*F. pseudograminearum*	NSW, Australia	CPI	DQ382118	DQ382054	DQ381990	DQ382182
CS3173	47679	*F. pseudograminearum*	NSW, Australia	CPI	DQ382119	DQ382055	DQ381991	DQ382183
CS3269	47684	*F. pseudograminearum*	NSW, Australia	CPI	DQ382120	DQ382056	DQ381992	DQ382184
CS3270	47685	*F. pseudograminearum*	NSW, Australia	CPI	DQ382121	DQ382057	DQ381993	DQ382185
CS3286	47686	*F. pseudograminearum*	NSW, Australia	CPI	DQ382122	DQ382058	DQ381994	DQ382186
CS3324	47687	*F. pseudograminearum*	NSW, Australia	CPI	DQ382123	DQ382059	DQ381995	DQ382187
CS3348	47688	*F. pseudograminearum*	NSW, Australia	CPI	DQ382124	DQ382060	DQ381996	DQ382188
CS3406	47690	*F. pseudograminearum*	NSW, Australia	CPI	DQ382125	DQ382061	DQ381997	DQ382189
CS3417	47691	*F. pseudograminearum*	NSW, Australia	CPI	DQ382126	DQ382062	DQ381998	DQ382190
CS3441	47692	*F. pseudograminearum*	QLD, Australia	CPI	DQ382127	DQ382063	DQ381999	DQ382191
CS3452	47693	*F. pseudograminearum*	SA, Australia	AM	DQ382128	DQ382064	DQ382000	DQ382192
CS3454	47694	*F. pseudograminearum*	NSW, Australia	CPI	DQ382129	DQ382065	DQ382001	DQ382193
CS3507	47695	*F. pseudograminearum*	QLD, Australia	CPI	DQ382130	DQ382066	DQ382002	DQ382194
CS3590	47696	*F. pseudograminearum*	VIC, Australia	GH	DQ382131	DQ382067	DQ382003	DQ382195
CS3616	47697	*F. pseudograminearum*	VIC, Australia	GH	DQ382132	DQ382068	DQ382004	DQ382196
CS3632	47698	*F. pseudograminearum*	VIC, Australia	GH	DQ382133	DQ382069	DQ382005	DQ382197
CS3645	47699	*F. pseudograminearum*	VIC, Australia	GH	DQ382134	DQ382070	DQ382006	DQ382198
CS3685	47700	*F. pseudograminearum*	VIC, Australia	GH	DQ382135	DQ382071	DQ382007	DQ382199
CS3687	47701	*F. pseudograminearum*	VIC, Australia	GH	DQ382136	DQ382072	DQ382008	DQ382200
CS3890	47702	*F. pseudograminearum*	SA, Australia	AM	DQ382137	DQ382073	DQ382009	DQ382201
CS3891	47703	*F. pseudograminearum*	SA, Australia	AM	DQ382138	DQ382074	DQ382010	DQ382202
CS3892	47704	*F. pseudograminearum*	SA, Australia	AM	DQ382139	DQ382075	DQ382011	DQ382203
CS3893	47705	*F. pseudograminearum*	SA, Australia	AM	DQ382140	DQ382076	DQ382012	DQ382204
CS3894	47706	*F. pseudograminearum*	SA, Australia	AM	DQ382141	DQ382077	DQ382013	DQ382205
CS3898	47707	*F. pseudograminearum*	SA, Australia	AM	DQ382142	DQ382078	DQ382014	DQ382206
CS3899	47708	*F. pseudograminearum*	SA, Australia	AM	DQ382143	DQ382079	DQ382015	DQ382207
CS3900	47709	*F. pseudograminearum*	SA, Australia	AM	DQ382144	DQ382080	DQ382016	DQ382208
CS3901	47710	*F. pseudograminearum*	SA, Australia	AM	DQ382145	DQ382081	DQ382017	DQ382209
CS3907	47711	*F. pseudograminearum*	SA, Australia	AM	DQ382146	DQ382082	DQ382018	DQ382210
CS3911	47712	*F. pseudograminearum*	QLD, Australia	CPI	DQ382147	DQ382083	DQ382019	DQ382211
CAN41		*F. pseudograminearum*	Alberta, Canada	RC	DQ382148	DQ382084	DQ382020	DQ382212
CAN42		*F. pseudograminearum*	Alberta, Canada	RC	DQ382149	DQ382085	DQ382021	DQ382213
CAN43		*F. pseudograminearum*	Alberta, Canada	RC	DQ382150	DQ382086	DQ382022	DQ382214
CAN44		*F. pseudograminearum*	Saskatchewan, Canada	RC	DQ382151	DQ382087	DQ382023	DQ382215
CAN47[f]		*F. pseudograminearum*	Alberta, Canada	RC	DQ382152	DQ382088	DQ382024	DQ382216
CAN48[f]		*F. pseudograminearum*	Saskatchewan, Canada	RC	DQ382153	DQ382089	DQ382025	DQ382217
CAN50[f]		*F. pseudograminearum*	Saskatchewan, Canada	RC	DQ382154	DQ382090	DQ382026	DQ382218
NRRL28062		*F. pseudograminearum*	Australia	KOD	DQ382155	DQ382091	DQ382027	DQ382219
10902001		*F. pseudograminearum*	Minnesota, USA	RDM	DQ382156	DQ382092	DQ382028	DQ382220
10902002		*F. pseudograminearum*	Minnesota, USA	RDM	DQ382157	DQ382093	DQ382029	DQ382221
10902003		*F. pseudograminearum*	Minnesota, USA	RDM	DQ382158	DQ382094	DQ382030	DQ382222
10902005		*F. pseudograminearum*	Minnesota, USA	RDM	DQ382159	DQ382095	DQ382031	DQ382223
NZ32		*F. pseudograminearum*	North Island, New Zealand	MC	DQ382160	DQ382096	DQ382032	DQ382224
NZ67		*F. pseudograminearum*	North Island, New Zealand	MC	DQ382161	DQ382097	DQ382033	DQ382225
NZ69		*F. pseudograminearum*	South Island, New Zealand	MC	DQ382162	DQ382098	DQ382034	DQ382226
CS3040	47668	*F. graminearum*	NSW, Australia	CPI	DQ382164	DQ382100	DQ382036	DQ382228
CS3147	47678	*F. graminearum*	NSW, Australia	CPI	DQ382165	DQ382101	DQ382037	DQ382229
CS3179	47680	*F. graminearum*	NSW, Australia	CPI	DQ382166	DQ382102	DQ382038	DQ382230
CS3196	47681	*F. graminearum*	NSW, Australia	CPI	DQ382167	DQ382103	DQ382039	DQ382231
CS3246	47682	*F. graminearum*	NSW, Australia	CPI	DQ382168	DQ382104	DQ382040	DQ382232

(continued on next page)

Table 1 (continued)

Isolate	BRIP No.[a]	Species[b]	Origin[c]	Source[d]	GenBank accession No.			
					TEF	*PHO*[e]	*RED*	*BT*
CS3257	47683	*F. cortaderiae*	NSW, Australia	CPI	DQ382169	DQ382105	DQ382041	DQ382233
CS3384	47689	*F. graminearum*	NSW, Australia	CPI	DQ382170	DQ382106	DQ382042	DQ382234
CS4491	47713	*F. cerealis*	NSW, Australia	DB	DQ382171	DQ382107	DQ382043	DQ382236
R6693		*F. cerealis*	NSW, Australia	DG	DQ382172	DQ382108	DQ382044	DQ382235
NRRL28065		*F. pseudograminearum*	South Africa	NCBI	AF212469	AF212506 AF212543	AF212579	AF107870
NRRL28334		*F. pseudograminearum*	South Africa	NCBI	AF212470	AF212507 AF212544	AF212580	AF107880
NRRL28338		*F. pseudograminearum*	NSW Australia	NCBI	AF212471	AF212508 AF212545	AF212581	AF107882
NRRL3288		*F. culmorum*		NCBI	AF212462	AF212499	AF212573	U85569
NRRL25475		*F. culmorum*	Denmark	NCBI	AF212463	AF212500	AF212574	AF006362
NRRL13721		*F. cerealis*	Poland	NCBI	AF212464	AF212504	AF212575	U85568
NRRL25491		*F. cerealis*	Netherlands	NCBI	AF212465	AF212502	AF212576	AF006360
NRRL25805		*F. cerealis*	Columbia	NCBI	AF212466	AF212503	AF212577	AF006361
NRRL2903		*F. austroamericanum*	Brazil	NCBI	AF212438	AF212475 AF212512	AF212549	AF212733
NRRL28436		*F. meridonale*	New Caledonia	NCBI	AF212435	AF212472 AF212509	AF212546	AF212730
NRRL26916		*F. boothii*	South Africa	NCBI	AF212444	AF212481 AF212518	AF212555	AF212738
NRRL25797		*F. mesoamericanum*	Honduras	NCBI	AF212441	AF212478 AF212515	AF212552	AF006364
NRRL26754		*F. acaciae-mearnsii*	South Africa	NCBI	AF212448	AF212485 AF212522	AF212559	AF212742
NRRL13818		*F. asiaticum*	Japan	NCBI	AF212451	AF212488 AF212525	AF212562	AF212745
NRRL5883		*F. graminearum*	Ohio, USA	NCBI	AF212455	AF212492 AF212529	AF212566	U34436
NRRL29297		*F. cortaderiae*	New Zealand	NCBI	AY225885	AY225889 AY225891	AY225888	AY225893
NRRL31281		*F. brasilicum*	Brazil	NCBI	AY452964	AY452916 AY452924	AY452932	AY452940

a All Australian isolates are deposited with the Queensland Plant Pathology Herbarium, Brisbane, Australia.
b Species based PCR, morphology and DNA sequences.
c Australian state abbreviations: NSW = New South Wales; QLD = Queensland; SA = South Australia; VIC = Victoria.
d CPI = CSIRO Plant Industry; KOD = Kerry O'Donnell (ARS-USDA, USA); RDM = Ruth Dill-Macky (University of Minnesota, USA); RC = Randy Clear (Cereal Research Centre, Canada); AM = Alan McKay (SARDI, Australia); GH = Grant Hollaway (DPI, Victoria, Australia); MC = Matthew Cromey (Crop & Food Research, New Zealand); DB = David Backhouse (University of New England, Armidale, Australia); DG = David Geiser (Fusarium Research Centre, USA); NCBI = Isolates sequenced by O'Donnell *et al.* (2000), sequences obtained from NCBI database (http://www.ncbi.nlm.nih.gov/).
e *PHO* locus sequences were obtained separately for phosphate permease 1 and 2 genes from the NCBI database for *F. pseudograminearum* and *F. graminearum* complex isolates, and concatenated into a single sequence prior to alignment. Accession numbers listed in table refer to phosphate permease 1 and 2 sequences, respectively.
f Isolates CAN47, CAN48 and CAN50 are synonymous with isolates A2-98-11, S2A-00-3 and S9B-00-2, respectively (Mishra *et al.* 2006).

The identity of each isolate was confirmed by PCR using species-specific primers, previously validated against Australian isolates (Table 2; Akinsanmi *et al.* 2004). All amplification reactions contained PCR reaction buffer (67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.45 % w/v Triton X-100; 0.2 mg mL^{-1} gelatin), 3.0 mM $MgCl_2$, 240 nM of each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (Biotech Int.) and 25 to 50 ng of DNA. Reaction volumes were made up to 25 μl with sterile distilled water. Amplifications were carried out using an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 2 min. Amplifications were concluded with a final extension phase at 72 °C for 7 min, and soaked at 4 °C. Amplicons were separated by gel electrophoresis in 1 % agarose gels in 0.5× TBE (45 mM Tris-borate; 1 mM EDTA), containing 0.5 mg l^{-1} ethidium bromide and visualised under UV light.

DNA sequencing

DNA from sections of the translation elongation factor-1α gene (*TEF*), β-tubulin gene (*BT*), putative reductase gene (*RED*), and the combined phosphate permease 1, phosphate permease 2 and intergenic region between these two genes (*PHO*), were amplified by PCR using gene specific primers (Table 2). Amplifications were carried out as described

Table 2 – PCR primers for species identification and gene sequencing

Target species/Gene region	Primer name[a]	Direction[b]	Primer sequence (5′-3′)	Annealing temperature (°C)
F. pseudograminearum	Fp1-1	sense	CGGGGTAGTTTCACATTTCCG	57.0
	Fp1-2	antisense	GAGAATGTGATGACGACAATA	
Fg clade	Fg16NF	sense	ACAGATGACAAGATTCAGGCACA	57.0
	Fg16NR	antisense	TTCTTTGACATCTGTTCAACCCA	
Translation elongation factor-1α (*TEF*)	EF1T	sense	GTGGGGCATTTACCCCGCC	60.0
	EF2T	antisense	ACGAACCCTTACCCACCTC	
Phosphate permease (*PHO*)	PHO1	sense	ATCTTCTGGCGTGTTATCATG	54.0
	PHO6	antisense	GATGTGGTTGTAAGCAAAGCCC	
β-tubulin (*BT*)	T1	sense	AACATGCGTGAGATTGTAAGT	55.0
	T22	antisense	TCTGGATGTTGTTGGGAATCC	
	T2	antisense	TAGTGACCCTTGGCCCAGTTG	
Putative reductase (*RED*)	RED1d	sense	TCTCAGAAAGACGCATATATG	54.0
	RED2	antisense	CGTAACTGCGTCATTCGGC	

a Primer references: Fp1-1/Fp1-2 (Aoki & O'Donnell 1999a); Fg16NF/FgN16R: (Nicholson *et al.* 1998); EF1T/EF2T, PHO1/PHO6, T1/T22/T2 and RED1d/RED2: (O'Donnell *et al.* 2000).
b Amplification of *BT* gene was undertaken using the primer pair T1/T22. Antisense sequencing reactions were undertaken with T2. All other gene amplifications and sequencing reactions undertaken using the same primer pair.

previously, with the following exceptions: amplifications of *RED* were carried out with a $MgCl_2$ concentration of 2.5 mM, and the final volume of all sequencing amplifications was 50 μl, with three units of *Taq* polymerase added per reaction.

Following amplification, amplicons were cleaned using QIAQuick™ PCR Cleanup Kits (QIAGEN, Hilden, Germany), according to manufacturer's specifications. Amplicon concentration was quantified by gel electrophoresis, as described previously, relative to DNA standards of known concentration. Amplicons were directly sequenced using BigDye Terminator v3.1 chemistry in a 96-capillary automated DNA sequencer (ABI 3730xl, Applied Biosystems, Foster City, CA, USA) in both sense and anti-sense directions using the relevant primers (Table 2), and visually compared to construct a consensus sequence.

Sequence identity of each gene region for all isolates sequenced was confirmed by BLAST searches (Altschul *et al.* 1997) of the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast/index.shtml).

Additional sequences were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/) and added to the relevant data sets (Table 1). Sequence data was downloaded for each of the ex-holotype strains of the nine phylogenetic species within the *Fg* clade, plus isolates of *F. pseudograminearum, F. culmorum* and *F. cerealis* (syn. *F. crookwellense*) sequenced by O'Donnell *et al.* (2000).

Phylogenetic analysis

Sequence data for each gene were aligned using CLUSTAL W v1.4 (Thompson *et al.* 1994) and edited manually using BIOEDIT v7.0.5.2 (Hall 1999). Data for each gene were analysed separately, and together as a combined multilocus sequence (*MLS*). GC content, variable substitution sites, parsimony information sites and informative indels were calculated using MEGA v3.1 (Kumar *et al.* 2004), excluding the *Fg* clade, *F. cerealis* and *F. culmorum* isolates.

Data sets were coded to remove phylogenetic bias of indels introduced to improve alignment. Sites of multibase indels of uniform length were deleted from alignments, with the exception of the first character of the indel site, to indicate a single mutation event. When nucleotide variation occurred at an indel site, sequences were edited manually to indicate this variation.

Congruence between individual gene data sets was tested using the partition homogeneity test (Farris *et al.* 1995) implemented in PAUP*. One thousand replicates were analysed in a heuristic search with the addition of ten random sequences and one tree saved per replicate. Invariant characters were deleted prior to analysis and 0.01 was used as a significance threshold (Cunningham 1997).

Maximum parsimony analyses (MP) were conducted using PAUP* v4.0b10 (Swofford 2003), using a heuristic search with 10 random sequence additions and tree bisection-reconnection branch swapping. A maximum of 20,000 trees were stored and ACCTRAN optimisation was used. The remaining indels present in data sets were treated as fifth character states. Tree topology was supported by analysing 1000 bootstrap pseudo-replicates (Felsenstein 1985) using the same MP settings. Bootstrap values greater than or equal to 70 % were considered significant (Hillis & Bull 1993).

Bayesian analyses (BI) were conducted using a Metropolis-coupled Markov chain Monte Carlo (MC^3) methodology as implemented in MRBAYES v3.1 (Ronquist & Huelsenbeck 2003). Indels remaining in data sets following initial coding were coded as present (0)/absent (1). To distinguish between sequence variations aligned with indel positions, nucleotide characters were coded (1 to *n*, where *n* is the total number of nucleotide sequence variations) and data sets were divided into coded and nucleotide partitions. As sequenced loci in this work were complex sequences consisting of both introns and exons, a simple model of evolution, the Kimura-2-parameter model (Kimura 1980), was used for Bayesian analysis of the nucleotide partitions. Coded partitions were analysed using the standard discrete evolutionary model implemented in

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MRBAYES. Two concurrent analyses of four chains (one cold and three heated) were both run for 1 M generations, ensuring analyses were not trapped at local optima (Leache & Reeder 2002), using random starting trees, and sampled every 100 generations. The number of samples discarded as ''burn-in'' prior to stationarity being reached was determined by plotting the log-likelihood scores of samples in an *x-y* graph against generation, with stationarity deemed to occur when log-likelihood values reached stable equilibrium. A majority rule consensus tree was constructed from the remaining trees from both analyses and the posterior probabilities of clades calculated, with values equal to 0.95 or greater considered significant (Leache & Reeder 2002).

Phylogenetic trees were drawn using the MrEnt v. 1.2 software (Zuccon & Zuccon 2006), with *F. cerealis* as an outgroup.

Results

Sequence characteristics

Aligned sequence length of *Fusarium pseudograminearum* isolates varied within two of the four genes. The sequences in the *TEF* data set varied between 616 and 623 base pairs (bp), while sequences in the *RED* locus ranged from 838 to 851 bp (Table 3). Sequences in the *PHO* and BT loci were all 800 and 541 bp in length, respectively. The percentage of variable characters in individual data sets also varied. The *RED* gene had the greatest percentage of variable sites (9.2 %), while the *BT* data set had the lowest percentage (1.1 %). The percentage of parsimony informative sites varied between 0.6 % (*BT*) and 7.5 % (*PHO*), while phylogenetically informative indels were present only in the *TEF* (four) and *RED* (one) genes.

BLAST searches using individual sequences confirmed the identity of all isolates by PCR and morphology (Table 1). The isolate CS3257, identified as *Fg* clade by PCR and morphological comparison, showed the greatest sequence homology in all four loci with isolates of *F. cortaderiae* O'Donnell, Aoki, Kistler & Geiser (former *Fg* clade lineage 8) and has been tentatively named as *F. cortaderiae* in this work. All other *Fg* clade isolates had the greatest sequence homology with *F. graminearum* isolates (former *Fg* clade lineage 7).

Partition homogeneity test

The partition homogeneity test indicated significant incongruence in phylogenetic signal between the combined four gene data sets ($P = 0.001$). Comparisons between all possible three data set combinations found significant incongruence ($P = 0.001$) between all combinations. Pairwise comparisons indicated that the phylogenetic signal of *BT* locus was not significantly incongruent with any other loci (*BT/TEF*, $P = 0.131$; *BT/PHO*, $P = 0.363$; *BT/RED*, $P = 0.013$). All other pairings indicated significant incongruence between loci (*TEF/PHO*, $P = 0.002$; *TEF/RED*, $P = 0.001$; *PHO/RED* $P = 0.001$).

Phylogenetic analysis of multilocus sequence

The coded *MLS* consisted of 2777 characters. BI produced a tree topology that distinctly separated *Fusarium culmorum*, *F. pseudograminearum* and *F. graminearum* isolates into three well-supported species groups (Bayesian posterior probability (PP) = 1.00; Fig 1). MP analysis produced 223 equally parsimonious trees, each 622 steps long with consistency indices (CI) of 0.678 and retention indices (RI) of 0.954. The majority rule consensus of these trees produced a tree of identical topology to that of BI. Six clades, supported by both BI and MP (PP = 1.00, BS≥77 %), were observed each comprising within three to seven isolates (Fig 1). Clades Fps 1 and Fps 5 contained only isolates from Australia, while clade Fps 3 contained only Canadian isolates. The remaining clades contained isolates from multiple regions; clade Fps 2 contained Australian and US isolates, clade Fps 4 a Canadian and New Zealand and isolates, and clade Fps 6 a Canadian, South African and Australian isolates. In addition to these clades, 28 Australian and one New Zealand isolate were not differentiated into well-supported clades within the *F. pseudograminearum* species group.

Phylogenetic analysis of individual gene sequences

BI of the *BT* (537 characters), *TEF* (620 characters), *PHO* (800 characters) and *RED* (820 characters) genes differentiated all *Fusarium pseudograminearum* isolates from other *Fusarium* species in this study (PP = 1.00; Fig 2b,c). MP produced two (38 steps, CI = 0.921, RI = 0.987), 288 (102 steps, CI = 0.863, RI = 0.983), 34 (190 steps, CI = 0.700, RI = 0.962), and 1296 (205 steps, CI = 0.810, RI = 0.978) equally parsimonious trees for the *BT*, *TEF*, *PHO*, and *RED* genes, respectively. The majority-rule consensus of the MP trees of all loci had identical topologies to the trees produced by BI (Fig 2a–d). Within *F. pseudograminearum* one, three, two and five well-supported groups were observed at the *BT*, *PHO*, *TEF* and *RED* loci, respectively. The only grouping maintained across multiple loci was the isolates NZ32 and NZ67: group T2 in the *TEF* locus (Fig 2c), which were

Table 3 – Sequence characteristics and phylogenetic information for *TEF*, *PHO*, *RED* and *BT* gene loci, and combined multilocus sequence (*MLS*), data sets based on *F. pseudograminearum* isolates only

Data set	Sequence range (bp)	GC%	No. variable sites (%)	No. informative sites (%)	No. informative indels
BT	541	52.6	6 (1.1 %)	3 (0.6 %)	0
PHO	800	47.1	71 (8.9 %)	60 (7.5 %)	0
TEF	616-623	52.8	20 (3.2 %)	15 (2.4 %)	4
RED	838-851	47.4	78 (9.2 %)	61 (7.2 %)	1
MLS	2796-2815	49.5	175 (6.2 %)	139 (4.9 %)	5

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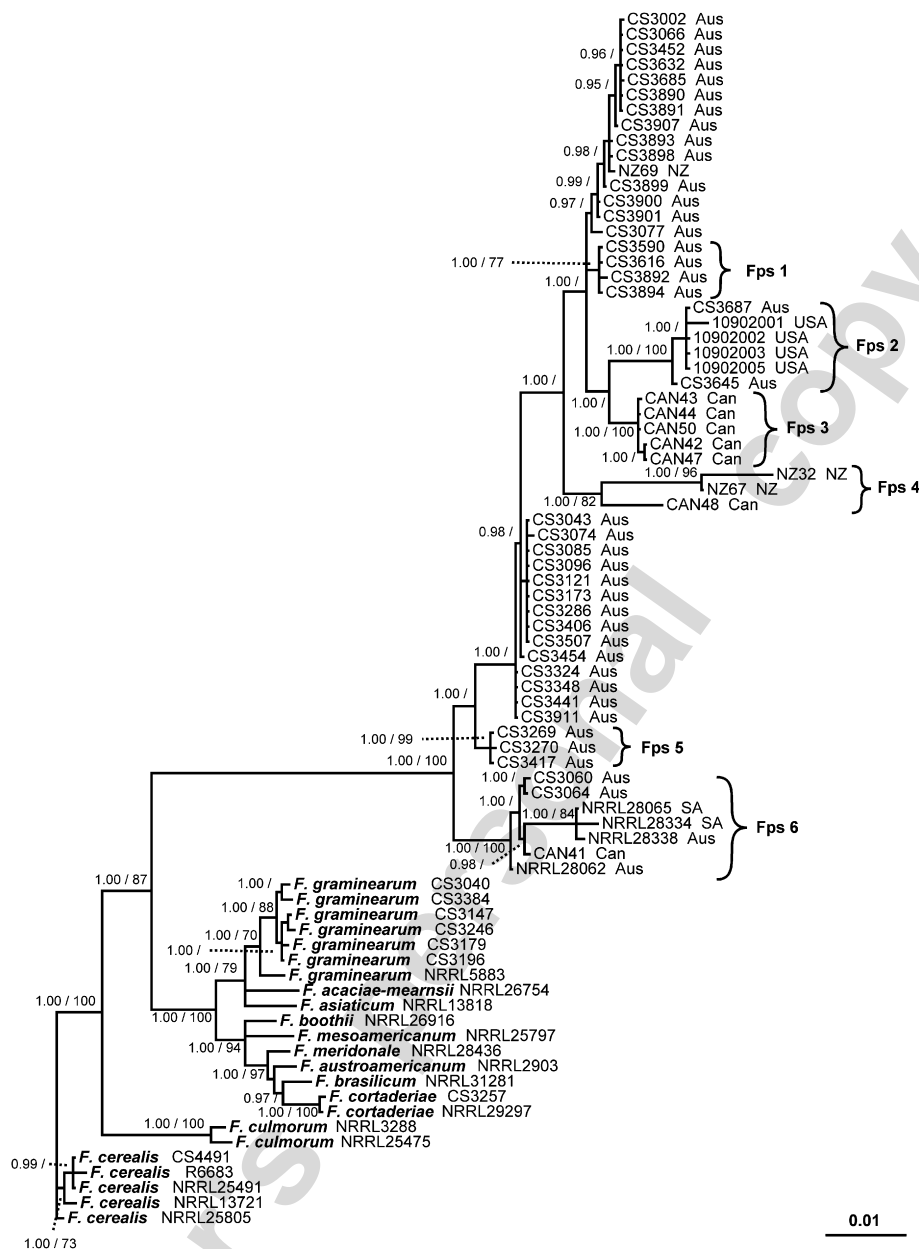

Fig 1 – Bayesian inference tree based on multilocus sequence data set ($-\ln L = 8185.30$). Where species is not indicated isolates belong to *Fusarium pseudograminearum*. Brackets indicate well-supported clades within *F. pseudograminearum*. Values at branch nodes indicate branch support, with posterior probabilities (PP; values $\geq$0.95 shown) and bootstrapping percentages based on maximum parsimony analysis (BS; values $\geq$70 % shown) separated by a forward slash (ie., PP/BS). Dashed lines are extrapolations of branches to indicate clade support. Scale bar represents expected substitutions per site. All trees rooted with *F. cerealis* as the outgroup.

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also grouped with the isolate CAN48 in the *BT* (group B1; Fig 2a) and *PHO* (group P2; Fig 2b).

Discussion

This study, for the first time, investigated the evolutionary relationships within *Fusarium pseudograminearum* isolates from Australia, Canada, New Zealand, South Africa and the USA. In addition to partially sequencing four single copy nuclear genes from these isolates, previously published sequence data of *F. pseudograminearum* and related species has been included for a comprehensive analysis of its phylogeny. Multilocus sequence analysis show limited lineage development in this species with no evidence of geographic separation of the putative lineages, pointing to a genetically recombining, single phylogenetic species.

The extent of lineage development within *F. pseudograminearum* varied with the gene locus in question. For both the *BT* and *TEF* loci very little divergence was observed, with

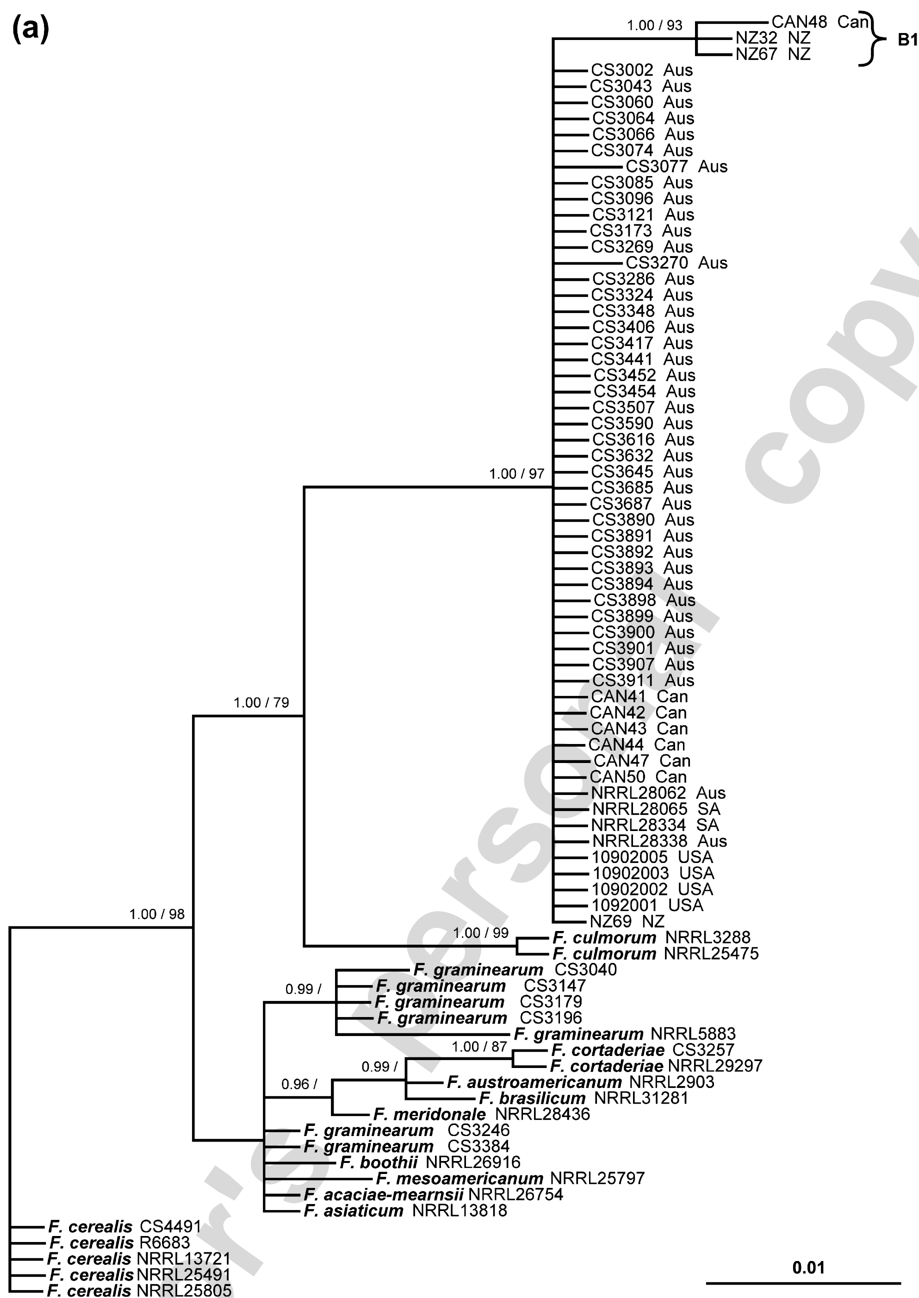

Fig 2 – Bayesian inference tree based on partial sequences of the (a) *BT* (−ln *L* = 1117.65), (b) *PHO* (−ln *L* = 2360.04), (c) *TEF* (−ln *L* = 1619.74), and (d) *RED* (−ln *L* = 2632.23) loci. Where species is not indicated isolates belong to *Fusarium pseudograminearum*. Brackets indicate well-supported groups within *F. pseudograminearum*. Values at branch nodes indicate branch support, with posterior probabilities (PP; values ≥0.95 shown) and bootstrapping percentages based on maximum parsimony analysis (BS; values ≥70 % shown) separated by a forward slash (ie., PP/BS). Dashed lines are extrapolations of branches to indicate grouping support. Scale bars represent expected substitutions per site. All trees rooted with *F. cerealis* as the outgroup.

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only one and two groups, respectively, supported by both BI and MP formed within the *F. pseudograminearum* clade. Additionally, at these loci only 0.6 (*BT*) and 2.4 % (*TEF*) of characters were phylogenetically informative amongst *F. pseudograminearum* isolates. Conversely, 7.5 and 7.2 % of characters were phylogenetically informative in the *PHO* and *RED* loci. The *PHO* gene genealogy differentiated the majority of *F. pseudograminearum* isolates into three groups that were supported by both BI and MP, with only the isolates NRRL28338, NRRL28334 and NRRL28065 not placed within these groupings. In contrast, *RED* genealogy spread *F. pseudograminearum* isolates over five groups supported by both BI and MP, with only the isolate, NZ32, excluded. Previous studies involving *F. pseudograminearum* isolates have also demonstrated similar variation in evolutionary divergence between genes. In their initial description of *F. pseudograminearum* as a species

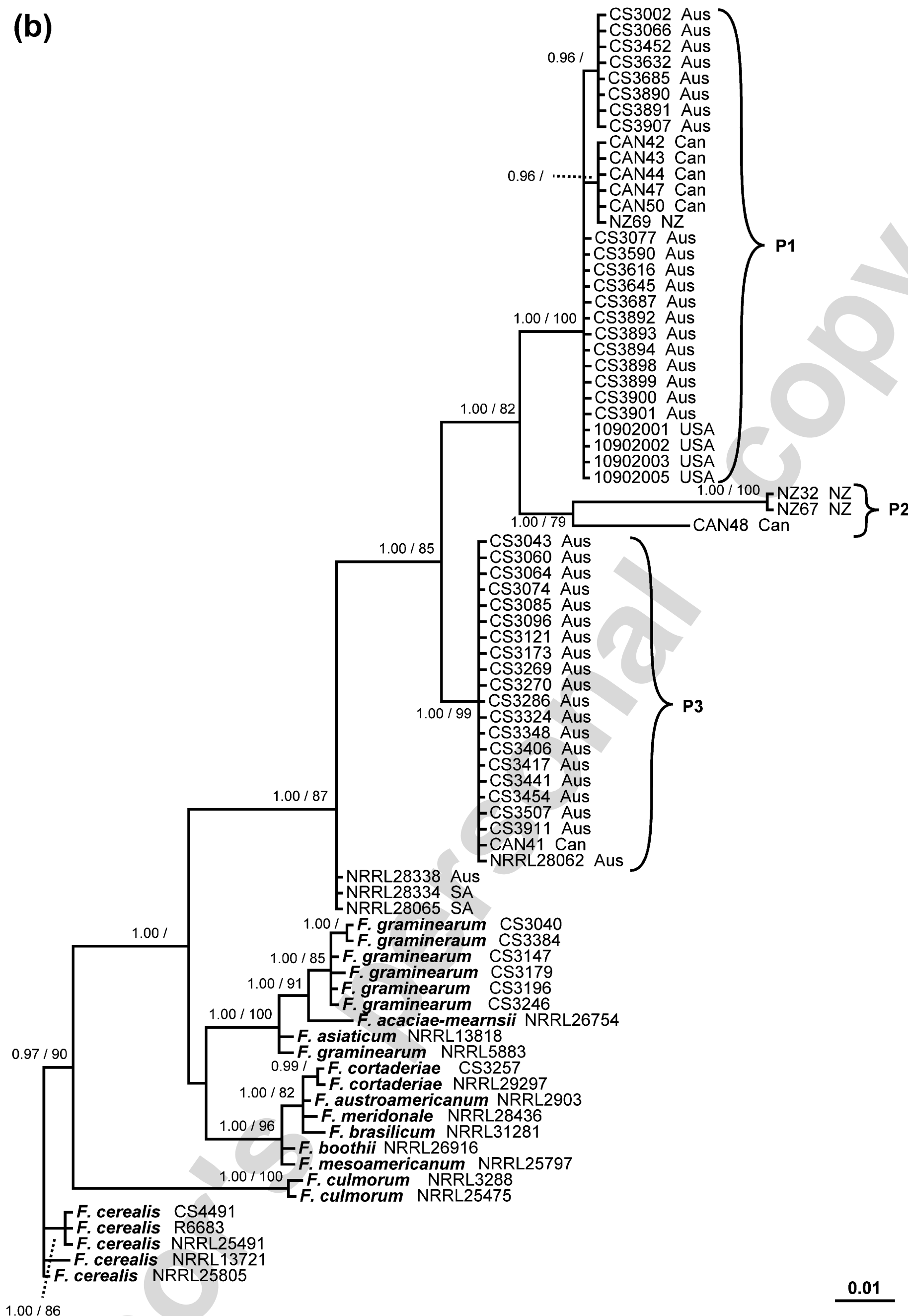

Fig 2 *(continued)*

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separate from the *Fg* clade, Aoki & O'Donnell (1999a) found minimal evolutionary divergence in *F. pseudograminearum* based on the *BT* gene genealogy. Conversely, in a comparison of New Zealand and USA isolates, Monds *et al.* (2005) displayed significant variation in the *PHO* gene genealogy, which differentiated isolates into three well-supported clades, using distance based phylogeny.

In addition to individual gene genealogy comparison, sequences were combined into a *MLS* for analysis. All four loci were combined into the *MLS*, despite significant incongruence between genes as growing evidence indicates that combining all genes can improve the accuracy of phylogenies relative to individual gene phylogenies, or those based on only congruent gene sequences, even in the presence of significant incongruence (Cunningham 1997; Darlu & Lecointre 2002; Yoder *et al.* 2001). Both BI and MP analysis of the MLS placed the species *F. cerealis*, *F. culmorum*, *F. pseudograminearum* and the *Fg* clade into separated clades with strong statistical support (PP = 1.00, BS = 100 %). Within *F. pseudograminearum* a further six clades were formed with strong bootstrap support, however these contained only half the isolates studied, with the remainder placed in clades poorly supported by either BI or MP (or both), or ungrouped within the species. It should be noted that 87 % of phylogenetically informative sites within the *MLS* of *F. pseudograminearum* isolates were contributed by the *PHO* and *RED* loci, and thus the structure of the *MLS*

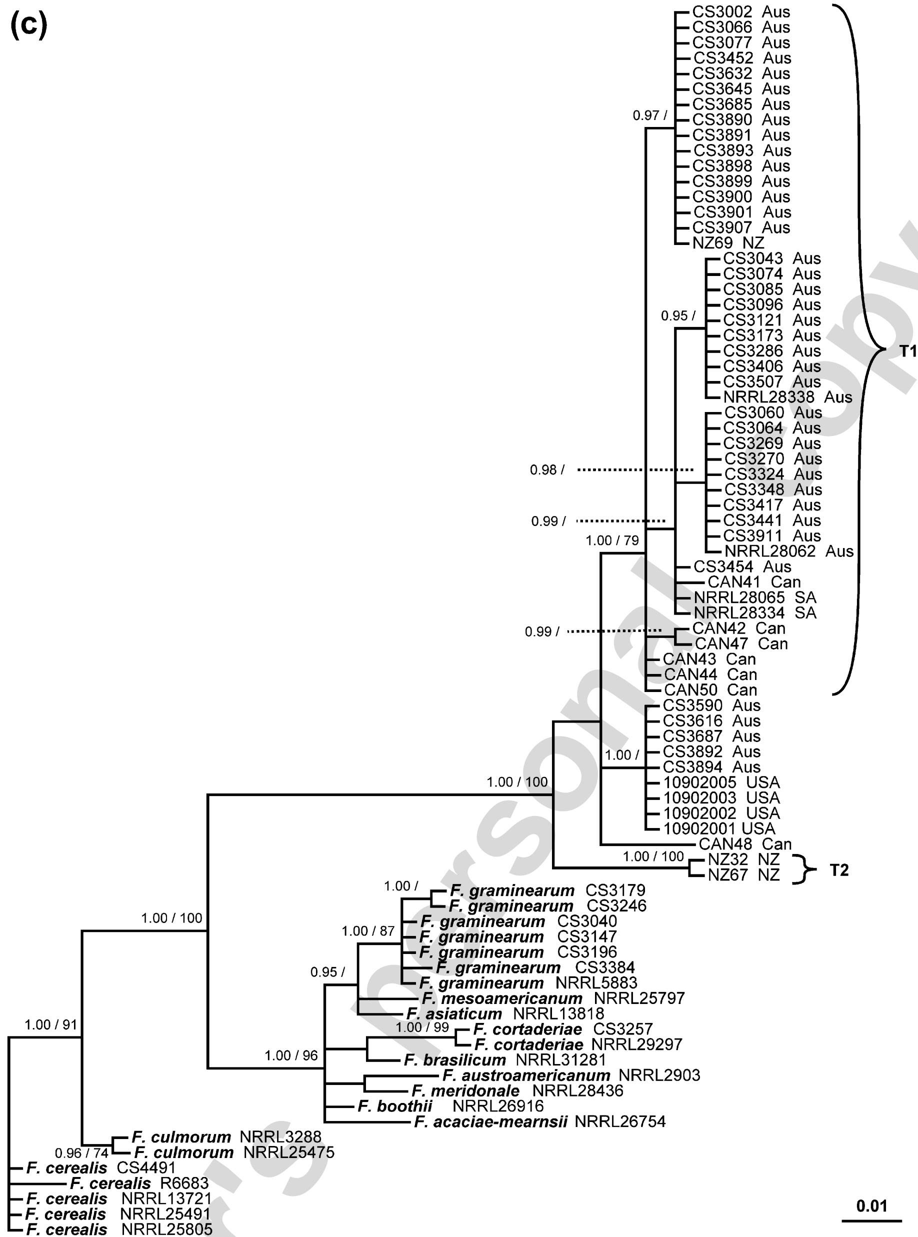

Fig 2 (*continued*)

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phylogeny obtained is heavily weighted towards the evolutionary history of those two loci. Also, only limited numbers of isolates were obtained from regions outside Australia, thus greater genetic variation may exist in sampled regions than was observed in this study.

Incongruence in phylogenetic signal between genes, and the different degrees of genetic variation between these genes suggests different evolutionary histories for these genes. Congruence was only observed in loci pairings that included the *BT* locus; probably due to the minimal sequence variation in this locus relative to the other loci. In addition to the results of the partition homogeneity test, visual observation of the individual gene genealogies indicated only one significant group within *F. pseudograminearum*, containing the isolates NZ32 and NZ67, indicated by three genes, but not the *RED* gene. Several processes, including incomplete lineage sorting, variable evolutionary rates, parasexual recombination and sexual recombination, could generate these differences. Recently, comparison of phylogenetic tree topologies of multiple genes has been promoted as a method of species recognition, termed Genealogical Concordance Phylogenetic Species Recognition (GCPSR; Taylor *et al.* 2000) in the genus *Fusarium* (O'Donnell *et al.* 2004). Under GCPSR, congruent topologies over multiple gene loci is considered indicative of lineages that have developed under recombinational isolation, and are thus recognised as phylogenetic species. Conversely, incongruence, as seen in this study, is indicative of a genetically recombining, single phylogenetic species, with limited lineage development. This situation is the reverse of that observed in the closely related, and morphologically similar, *Fg* clade.

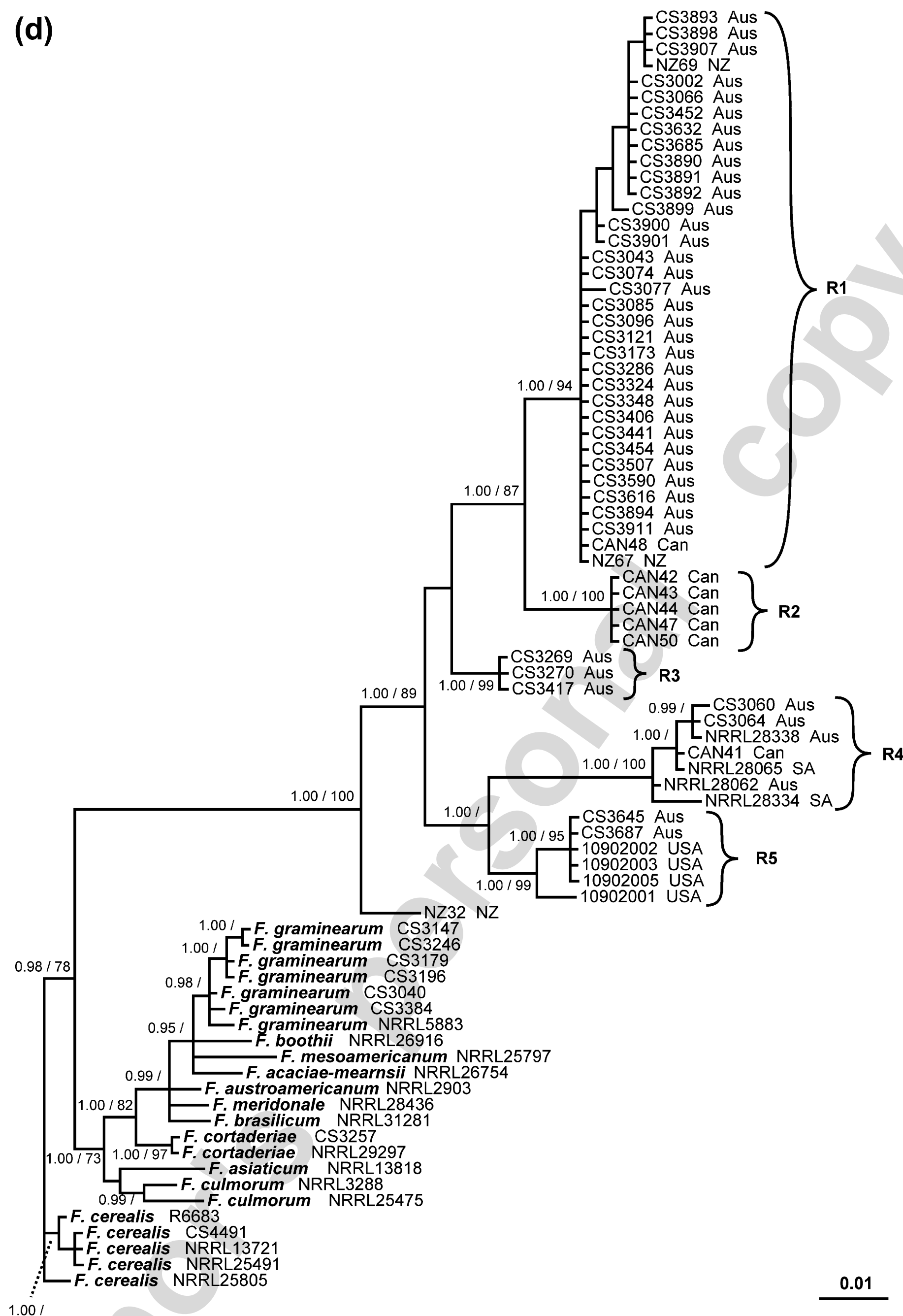

Fig 2 (*continued*)

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In the *Fg* clade, GCPSR has been used to show congruence in phylogenetic signal over 11 genes (O'Donnell *et al.* 2000; O'Donnell *et al.* 2004; Ward *et al.* 2002), and it has been formally proposed that the *Fg* clade be redefined into nine phylogenetic species (O'Donnell *et al.* 2004).

In the *Fg* clade, phylogenetic lineages are linked to the geographic origin of isolates (O'Donnell *et al.* 2000; O'Donnell *et al.* 2004; Ward *et al.* 2002), but this is not the case with *F. pseudograminearum* as seen in the current study. In the tree topology generated from the *MLS* data set, clades Fps 2 (Australia and USA), Fps 4 (New Zealand and Canada) and Fps 6 (Australia, Canada and South Africa) contained isolates from multiple regions. Only the four isolates from USA (Fps 2) and the two from South Africa (Fps 6), were placed together in single clades, and in both cases were not significantly differentiated from the Australian isolates present in those clades. The story is similar in the genetically variable *RED* and *PHO* loci used in this study. For the *PHO* locus, isolates from each of Australia, New Zealand and Canada were spread across two of the three groups. Tree topology of the *RED* locus spread the Australian isolates across four of five groups and the Canadian isolates across three. This contrasts recent results, where a 644 bp portion of the *PHO* locus separated 10 isolates from New Zealand and the USA into distinct clades (Monds *et al.* 2005).

Considering the geographic barriers between the sampled regions, international trade and tourism may be influencing the evolution of this species by aiding the movement of pathogen genotypes and nullifying the effects of geographic isolation on evolution.

Crown rot caused by *F. pseudograminearum* is a chronic problem in Australia and is becoming recognised as more widely dispersed pathogen of wheat worldwide (Mitter *et al.* 2006; Nicol *et al.* 2004). The ability of strains and/or species from geographically separated locations to recombine, poses the danger of introducing virulence and/or toxigenic genes into local pathogen populations. Increasing toxigenic potential is of particular concern, as *F. pseudograminearum* has been associated with major FHB epidemics in Australia (Burgess *et al.* 1987) and all isolates of this species can cause FHB (Akinsanmi *et al.* 2004), a disease closely associated with the production of DON and NIV mycotoxins. Factors such as aggressiveness and toxigenic potential of the global *F. pseudograminearum* population require careful consideration. This information is vital for the development of improved control strategies for the diseases *F. pseudograminearum* causes.

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