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Fusarium graminearum, *F. cortaderiae* and *F. pseudograminearum* in New Zealand: molecular phylogenetic analysis, mycotoxin chemotypes and co-existence of species

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Fusarium graminearum and *F. pseudograminearum* are important plant pathogens in New Zealand and around the world. Headblight and crown rot diseases of cereals caused by these species are responsible for large economic losses due to reduction in seed quality and contamination of grain with tricothecene mycotoxins. In the current study we have used two different molecular phylogenetic approaches, AFLPs and gene genealogies, to gain insight into the evolutionary relationships between *F. graminearum*, and *F. pseudograminearum* in New Zealand. The worldwide genetic diversity of *F. graminearum* clade is represented by at least eight biogeographically distinct species (previously designated as lineages of *F. graminearum*). Our analysis demonstrated that this clade is represented by *F. graminearum* (=*F. graminearum* Lineage 7) and *F. cortaderiae* (=*F. graminearum* Lineage 8) in New Zealand. Through our analysis we also confirm the presence of *F. pseudograminearum* in New Zealand as a first record for this organism. Information on species is necessary for preventing the inadvertent intercontinental introduction of genetically unique foreign pathogens associated with world trade. The ability to place species information into a worldwide context enabled postulation that the New Zealand representatives of *F. graminearum* clade originated from at least two regions, and probably on at least two hosts. Correlation of species descriptions with biogeographical and host information revealed evidence for co-localisation of *F. graminearum* clade species with potential for genetic outcrossing in the field. Mycotoxin analysis showed *F. graminearum* (=lineage 7) isolates produce either nivalenol (NIV) or deoxnivalenol (DON). In contrast, *F. cortaderiae* isolates produced only NIV. These findings support earlier observations that mycotoxin production in the *F. graminearum* clade is not species specific, but suggest maintenance of chemotype diversity through speciation may have been restricted to a subset of species.

INTRODUCTION

In recent years, diseases caused by *Fusarium* spp. (foot or crown rot and Fusarium Head Blight (FHB)) have become an increasing concern in cereal crops in New Zealand and in other parts of the world (Braithwaite, Alexander & Adams 1998). Epidemics are associated with high economic losses due to contamination of grains with mycotoxins and overall reduction in seed quality (Ichinoe *et al*. 1983).

Various *Fusarium* species have been linked to FHB, but *F. graminearum* is emerging as the major causal agent in many parts of the world (O'Donnell *et al*. 2000). Crown rot can also be caused by a range of *Fusarium* species, but in Australia this disease is most commonly associated with *F. pseudograminearum* (Aoki & O'Donnell 1999). *Fusarium graminearum* and *F. pseudograminearum* are morphologically very similar, and were previously designated Group 1 and Group 2 within *F. graminearum* (Francis & Burgess 1977). Use of DNA sequence (Aoki & O'Donnell 1999), RFLP (Benyon, Burgess & Sharp 2000) and isozyme (Laday *et al*. 2000) data indicated that these two fungi were phylogenetically distinct and should be regarded as separate species.

Recently, O'Donnell *et al*. (2000) and Ward *et al*. (2002) used six gene genealogies to show that the *F. graminearum* clade (hereafter referred to as the Fg clade) is composed of eight worldwide lineages (one first found in New Zealand). Furthermore, these lineages were biogeographically structured, suggesting

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limited gene flow among these populations. The Fg clade lineages have been formally described as phylogenetically distinct species (O'Donnell *et al*. 2004). In this paper, we use the Fg clade species nomenclature of O'Donnell *et al*. 2004, with equivalent *F. graminearum* lineage given at first mention.

FHB is common in wheat in New Zealand, especially in North Island crops where the disease is primarily associated with *F. graminearum* (Sayer & Lauren 1991, Cromey *et al*. 2002). The disease is less common in South Island crops, where it is most often associated with *F. culmorum* and *F. avenaceum* (Cromey, Parkes & Fraser 2001). Braithwaite *et al*. (1998) commonly observed *F. avenaceum* and *F. graminearum* associated with foot rot of cereals. However, *F. graminearum* isolates were not classified as Groups 1 and 2, and may have been *F. pseudograminearum*, although this species had not yet been recorded in New Zealand.

Strains of both *F. graminearum* and *F. pseudograminearum* have been shown to produce trichothecene mycotoxins (Abramson, Clear & Smith 1993, O'Donnell *et al*. 2000). Three major strain-specific chemotypes have been identified within the B-trichothecene lineage of with *Fusarium*: nivalenol (NIV chemotype), deoxynivalenol and 3-acetyldeoxynivalenol (3ADON chemotype), and deoxynivalenol and 15-acetyldeoxynivalenol (15ADON chemotype) (Ward *et al*. 2002). DON chemotypes are most commonly associated with *F. graminearum*, although some isolates produce NIV (Abramson *et al*. 1993, O'Donnell *et al*. 2000, Gilbert *et al*. 2001). In New Zealand, however, Lauren *et al*. (1992) found that NIV chemotypes were as common as DON chemotypes.

Less information is available on mycotoxin production by strains of *F. pseudograminearum*, probably because this species is not normally associated with cereal grains used for food or feed. However, Ward *et al*. (2002) found that three isolates of *F. pseudograminearum* produced 3ADON, while a fourth isolate was of unknown chemotype.

Information on Fg clade species in New Zealand could also provide information on the origin of the New Zealand population, as well as potential pathways of entry for fungal plant pathogens. Such knowledge will assist in preventing the inadvertent intercontinental introduction of genetically unique foreign populations of pathogenic organisms associated with world trade (O'Donnell *et al*. 2000). Outcrossing between Fg clade species has resulted in novel pathogens (O'Donnell *et al*. 2000). Further hybridisation between Fg clade species is possible, given the wide host range of this clade.

In this study we examined 34 New Zealand isolates of *F. graminearum*/*F. pseudograminearum* (the two species were not differentiated prior to the commencement of the study) from wheat, maize and barley grains and stem bases. Seven *F. culmorum* isolates from stem bases or grains were also examined. Phylogenies were constructed using both AFLP and gene genealogy strategies to determine the evolutionary relationships of these species in New Zealand. Correlation of phylogenetic data with that of O'Donnell *et al*. (2000) has allowed us to place our results within a worldwide context, offering insight into the origin and entry of *F. graminearum* isolates into New Zealand. Species-mycotoxin relationships were also investigated providing general support for maintenance of chemotype polymorphisms in a subset of Fg clade species.

MATERIALS AND METHODS

Media and culturing conditions

Fusarium species from wheat, barley or maize were isolated by surface-sterilising cereal grains or stem bases in 1% sodium hypochlorite for 10 min, rinsing in sterile water, and plating onto Potato Dextrose agar (PDA) amended with antibiotics (120 mg ml^{-1} streptomycin, 50 mg ml^{-1} aureomycin and 50 mg ml^{-1} chloramphenicol). Plates were incubated for 4–8 d at 25 °C under conditions of alternating light and dark (12 h photoperiod). Single spore isolates of selected *Fusarium* colonies were obtained using the method of Burgess *et al*. (1994).

Morphological identification

Species identification was carried out after culturing isolates on PDA and carnation-leaf agar (CLA) using the methods and criteria of Burgess *et al*. (1994). Plates for species identification were incubated for 7 days in an alternating temperature regime, 25 ° light/20 ° dark, with a 12 h photoperiod. Colony diameters were assessed for selected isolates on PDA after dark incubation for 3 d at both 25 ° and 30 °.

Perithecium production

Isolates were screened for perithecium production on hay agar (2 g chopped meadow hay, 15 g agar, 1 l distilled water). Culture plates were incubated on a light bench (40 W cool white fluorescent tubes mounted 25 cm above bench height, 12 h photoperiod, no temperature regulation) for up to 10 wk with regular examination for development of perithecia.

Isolates used for molecular analysis

A range of isolates identified as *Fusarium graminearum*, *F. pseudograminearum*, or *F. culmorum* using morphological characters were selected for molecular analysis (Table 1). They included isolates from different regions (the North or South Islands of New Zealand), different hosts (maize, wheat or barley) and different host tissues (grains or stem bases). Maize isolates were from two samples of maize kernels of hybrid 36R10; harvested on 10 May 2000 from a Te Awamutu (North Island) property, or harvested on 20 June 2000 from a Taumaranui (North Island) property. Cultures of each

Table 1 Fungal strains used in this study, their source, and their perithecial production, growth rate, and mycotoxin production.

Isolate	ICMP accession no.	Host	Tissue	Location[a]	Perithecium production	Growth rate 25 °C (mm d^{-1})	Growth rate 30 °C (mm d^{-1})	NIV	DON 3ADON	DON 15ADON
F. graminearum										
NZ62	15504	Maize	Grain	Te Awamutu, NI	+	9.2	7.7	+++	n.d.	+
NZ61	15503	Maize	Grain	Te Awamutu, NI	+			n.d.	n.d.	n.d.
NZ66	15507	Wheat	Grain	Kairanga, NI	+			n.d.	n.d.	+++
NZ53	15495	Maize	Grain	Te Awamutu, NI	+	11.0	7.5	n.d.	n.d.	+++
NZ54	15496	Maize	Grain	Te Awamutu, NI	+			+++	n.d.	n.d.
NZ52	15494	Maize	Grain	Te Awamutu, NI	+			+++	n.d.	n.d.
NZ65	15506	Wheat	Grain	Marton, NI	+	10.8	7.7	n.d.	n.d.	+++
NZ64	15505	Wheat	Grain	Marton, NI	+			n.d.	n.d.	n.d.
NZ33	15483	Wheat	Grain	Kairanga, NI	+			+++	n.d.	+
NZ31	15482	Wheat	Grain	Kairanga, NI	+			n.d.	n.d.	+++
NZ12	15481	Wheat	Grain	Kairanga, NI	+			+++	n.d.	+
NZ35	15484	Wheat	Grain	Kairanga, NI	+	10.8	7.7	n.d.	n.d.	+++
NZ51	15493	Maize	Grain	Te Awamutu, NI	+			+++	n.d.	n.d.
NZ49	15491	Maize	Grain	Te Awamutu, NI	+			+++	n.d.	+
NZ70	15508	Wheat	Healthy stem	Marton, NI	+	10.8	7.3	n.d.	n.d.	+++
F. cortaderiae										
NZ43	15485	Maize	Grain	Taumaruanui, NI	+	11.2	6.0	+++	n.d.	+
NZ44	15486	Maize	Grain	Taumaruanui, NI	+	10.5	6.2	+++	n.d.	+
NZ45	15487	Maize	Grain	Taumaruanui, NI	+			+++	n.d.	n.d.
NZ46	15488	Maize	Grain	Taumaruanui, NI	+			+++	n.d.	n.d.
NZ47	15489	Maize	Grain	Taumaruanui, NI	+			+++	n.d.	n.d.
NZ48	15490	Maize	Grain	Taumaruanui, NI	+			+++	n.d.	n.d.
NZ50	15492	Maize	Grain	Te Awamutu, NI	+			+++	n.d.	n.d.
NZ55	15497	Maize	Grain	Taumaruanui, NI	+			++	n.d.	n.d.
NZ56	15498	Maize	Grain	Taumaruanui, NI	+	9.3	3.3	+++	n.d.	+
NZ57	15499	Maize	Grain	Taumaruanui, NI	+			++	n.d.	n.d.
NZ58	15500	Maize	Grain	Taumaruanui, NI	+			+++	n.d.	+
NZ59	15501	Maize	Grain	Te Awamutu, NI	+	10.8	6.7	+++	n.d.	+
NZ60	15502	Maize	Grain	Te Awamutu, NI	+	12.0	7.3	+++	n.d.	+
F. culmorum										
NZ6	15476	Wheat	Healthy stem	Dorie, SI						
NZ3	15474	Wheat	Stem lesion	Temuka, SI						
NZ4	15475	Barley	Healthy stem	Dunearn, SI						
NZ7	15477	Wheat	Healthy stem	Marton, NI						
NZ34	15480	Wheat	Grain	Kairanga, NI						
NZ23	15478	Wheat	Healthy stem	Irwell, SI						
NZ24	15479	Wheat	Stem lesion	Eiffelton, SI						
F. pseudograminearum										
NZ69	15513	Wheat	Stem lesion	Morven, SI	−	8.8	2.3	n.d.	+++	n.d.
NZ68	15512	Wheat	Stem lesion	Darfield, SI	−	10.0	4.8	+	+++	n.d.
NZ5	15509	Barley	Healthy stem	St Andrews, SI	−	10.2	3.5	+	+++	n.d.
NZ67	15514	Wheat	Grain	Kairanga, NI	−	8.7	6.3	+++	n.d.	+
NZ38	15511	Wheat	Grain	Kairanga, NI	−	8.8	6.3	+++	n.d.	+
NZ32	15510	Wheat	Stem lesion	Marton, NI	−			++	n.d.	n.d.

[a] NI, North Island; and SI, South Island.

isolate have been deposited in the ICMP collection of fungus cultures (Landcare Research, Private Bag 92170, Auckland, NZ; Table 1).

DNA preparation

Single spore isolates were used to inoculate 25 ml of Potato Dextrose Broth (PDB) which was then incubated for 7 d at 22 ° in an orbital shaker. Mycelium was harvested from liquid cultures by filtration onto filter paper (Whatman grade 114). After rinsing with sterile water, the mycelium was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. A slurry was formed by the addition, and mixing, of 10 ml CTAB buffer (2% hexadecyltriethylammonium bromide, 1.4 M NaCl, 20 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 0.001% mercaptoethanol), which was then transferred to a 50 ml centrifuge tube and incubated at 65 ° for 1.5 h. An equal volume of chloroform:iso-amyl alcohol (24:1) was added, mixed and centrifuged at 3500 rpm for 15 min. The aqueous phase was transferred to a 50 ml falcon tube and total DNA precipitated by the addition of one volume of 100% ethanol. After incubation at room temperature for

10 min, DNA was spooled from solution and placed in wash solution I (76% ethanol, 0.2 M sodium acetate) for 20 min at room temperature. Subsequently, spooled DNA was placed in wash solution II (76% ethanol, 0.01 M ammonium acetate) for 1 min before being placed in an Eppendorf tube and air dried. DNA was dissolved in 300–400 µl of 10 mM Tris HCl, 1 mM EDTA pH 8.0 at 4 ° overnight before further manipulation.

AFLP analyses

AFLP methodology was essentially as described by Barrett & Kidwell (1998) and Pickering *et al.* (1995) with minor modifications. Templates were prepared by digesting 500 ng of sample DNA with *Pst*I and *Mse*I for 3 h. Pst and Mse adaptors (Pickering *et al.* 1995) were then ligated to restricted genomic fragments for another 3 h at 37 °.

Two selective rounds of PCR were performed: a pre-amplification and a ^{32}P labelled PCR. Pre-amplification was carried out using Mse + 1 and Pst + 1 primers; either Mse + G/Pst + A or Mse + G/Pst + G primer pairs. For the ^{32}P labeled PCR, a combination of Mse + 3 primers in concert with Pst + 1 primers were used.

Samples were electrophoresed in a denaturing 6% 29:1 acrylamide/bis-acrylamide gel for 2 h and 10 min at 70 W. The gels were then dried on to filter paper (Whatman 3MM). For visualisation, dried gels were exposed to autoradiographic film for 1–2 d before developing using standard procedures.

DNA Sequencing

Phosphate permase (*Pho*) sequences were amplified from total DNA with primers PHO1 and PHO6 (O'Donnell *et al.* 2000). Reactions contained 0.2 µM of each primer, 40 µM dNTP's and 0.6 units Taq DNA polymerase (Roche) in a final volume of 25 µl. Thermal cycling conditions were 40 times, 94 ° for 30 s; 50 ° for 30 s; 72 ° for 1 min. PCR products were sequenced directly using Big Dye v3.0 (Applied Biosystems, Foster City, CA) and primer PHO1. Sequences were collated and edited using SEQUENCHER TM 3.0 sequence analysis software package (Gene Codes Corporation, Ann Arbor, MI). Phosphate permase sequences obtained from external sources were treated in a similar manner to allow comparative analyses.

Phylogenetic analysis

AFLP data. AFLP bands were scored as either 1 (presence of a band) or 0 (absence of a band). In total, 155 polymorphic bands were scored for the 41 isolates used in this study. Scores were used to generate a binary matrix for all isolates and phylograms constructed using the software package TREECON for Windows version 1.3b (van de Peer & Wachter 1994). The Nei & Li method was used to estimate genetic distance (Nei & Li 1979) and neighbour-joining methods were used to infer tree topology (Saitou & Nei 1987). Relative support for individual nodes was obtained with bootstrap analysis using 1000 pseudo-replicates (Felsenstein 1985). The tree was rooted to the *F. pseudograminearum* clade.

Sequence data. *Pho* sequences were aligned using CLUSTALW (Thompson, Higgins & Gibson 1994) and a phylogram constructed from these alignments using TREECON (van de Peer & Wachter 1994). The Jukes and Cantor model was used to estimate genetic distance between sequences (Jukes & Cantor 1969), and tree topology was inferred using neighbor joining methods (Saitou & Nei 1987). Support for individual nodes was obtain by calculating bootstrap values based on the analysis of 1000 pseudo-replicates (Felsenstein 1985). The tree was rooted to the *F. pseudograminearum* clade.

Mycotoxin analyses

Discs (1 cm diam) from 4 d old *Fusarium* cultures on PDA were used to inoculate 50 ml of MYRO liquid medium, $(NH_4)_2HPO_4$ (1 g), KH_2PO_4 (3 g), $MgSO_4.7H_2O$ (0.2 g), NaCl (5 g), sucrose (40 g), glycerol (10 g), and ultra-pure water to 1 l (Lauren, Sayer & di Menna 1992), in 250 ml conical flasks. The flasks were incubated on a shaker at 23 ° for 14 d. The total cultures were extracted with ethyl acetate (2 × 50 ml) and the collected extract evaporated to dryness. An aliquot of the dried residue was hydrolysed to produce parent alcohols prior to analysis by HPLC with UV detection at both 245 nm and 254 nm for NIV and DON. The detection limit was 0.2 µg flask^{-1} for NIV and DON. Further aliquots of selected samples which produced DON were analysed without the hydrolysis step and using LC-MS (liquid chromatography – mass spectrometry) to determine whether the dominant form of DON-chemotype was 3ADON or 15ADON.

RESULTS

Isolation of Fusarium *species*

A range of *Fusarium* spp. were isolated from cereal grains and stem bases (Table 1). These included *F. culmorum*, *F. avenaceum*, *F. crookwellense*, *F. poae*, *F. oxysporum*, *F. heterosporum*, *F. equiseti* as well as *F. graminearum*/*F. pseudograminearum*. All maize isolates were from grains, while isolates from wheat and barley were from stem bases as well as grains.

An attempt was made to separate isolates based on measuring the region of maximum width of conidia. However, variability within isolates was too great for differences between isolates to be meaningful.

Single spore cultures of the 34 isolates identified as *F. graminearum*/*F. pseudograminearum* were tested

for perithecial production. Of these, 28 isolates of the Fg clade produced perithecia, while the six isolates of *F. pseudograminearum* did not (Table 1). All 20 isolates from maize grains were perithecium producers, while seven of nine isolates from wheat grains and an isolate from a wheat stem produced perithecia. These 28 isolates were all from the North Island of New Zealand, and were putatively assigned to the homothallic species *F. graminearum* (previously group 2). Where 3-septate ascospores were measurable they were 20–24 μm long, agreeing with the measurement for *Gibberella zeae* ascospores given by Aoki & O'Donnell (1999).

Six isolates identified as *F. graminearum*/ *F. pseudograminearum* did not produce perithecia, and were putatively assigned to the heterothallic species *F. pseudograminearum*. Three of these isolates were from the North Island, two being isolated from wheat grains and one from a wheat stem. The other three isolates were from the South Island and were isolated from wheat or barley stems.

The mean growth rates on PDA at 25 ° or 30 ° differed for the perithecium-producers and non-producers. The mean of ten perithecium producers at 25 ° was 10.7 mm day^{-1} compared with a mean for five isolates without perithecia of 9.3 mm day^{-1}. The difference at 30 ° was greater (6.7 mm day^{-1} and 4.7 mm day^{-1} respectively). At 25 °, there was some overlap amongst the individual isolates. At 30 ° there was also some overlap, with one perithecium-producing isolate having a particularly slow growth rate.

The 28 Fg clade isolates, six *F. pseudograminearum* isolates and seven *F. culmorum* isolates from New Zealand wheat or barley stems or grains, were selected for molecular phylogenetic analysis.

AFLP analyses

Isolates of the Fg clade, *Fusarium pseudograminearum* and *F. culmorum* were subjected to phylogenetic analysis using AFLPs. Eight separate Pst + 1/ Mse + 3 primer combinations were used to generate unique amplification profiles, from which 155 polymorphic markers were scored for their presence or absence. Although showing conserved banding patterns, profiles for each species were qualitatively distinguishable by eye. The phylogram constructed from AFLP data (Fig. 1) supported the species groupings assigned on the basis of morphology and qualitative observation of AFLP profiles. Division of *F. pseudograminearum* and Fg clade species into monophyletic groups was supported by high bootstrap values. In contrast, although all *F. culmorum* isolates formed a monophyletic group, bootstrap analysis only provided moderate relative support for this division.

Fg clade isolates formed two reciprocally monophyletic groups, suggesting that significant genotypic differences exist within the New Zealand population of *F. graminearum*. Within the context of AFLP analysis we refer to these lineages as Group A (later confirmed as *F. graminearum*, = *F. graminearum* lineage 7) and B (later confirmed as *F. cortaderiae* (= *F. gramineraum* lineage 8)). In the case of group B (*F. cortaderiae*) this division was supported by a bootstrap value of 100 %. For Group A (*F. graminearum*), a bootstrap value of 69 % was obtained in support of the monophyletic assignment. Although this value is much lower than for *F. cortaderiae* isolates, on viewing the tree topology, it seems likely that the long branch length of NZ70 is largely responsible for lowering the statistical confidence in designating *F. graminearum* as monophyletic. NZ70 is considered an outlier in *F. graminearum* and differs from other *F. graminearum* members' tissue origin, being isolated from stem tissue rather than grain.

F. pseudograminearum isolates formed two reciprocally monophyletic lineages. In this instance both groupings were statistically supported by bootstrap values over 97 %. This genotypic division was associated with geographical division into North and South Island isolates. Furthermore, branching of South Island *F. pseudograminearum* isolates could be correlated with differences in host origin (wheat or barley, Fig. 1), and also demonstrated bootstrap values over 90 %.

Gene genealogy analysis

To further substantiate the existence of two Fg clade species in New Zealand, as well as to place our results in a worldwide context, we adopted the same gene genealogy strategy as that utilised by O'Donnell *et al.* (2000). Phosphate permase (*Pho*) sequence from isolates in this study were combined with the *Pho* sequence data for Fg clade, *Fusarium culmorum* and *F. pseudograminearum* isolates from O'Donnell *et al.* (2000). A phylogenetic analysis was performed for all 76 isolates using 644 bp of aligned Pho sequence (Fig. 2). As with the AFLP-based phylogram, the sequence based phylogeny supported morphological separation of Fg clade, *F. culmorum* and *F. pseudograminearum*. The seven Fg clade species described by O'Donnell *et al.* (2000, 2004) were conserved in the *Pho* analysis. Concordant with the AFLP phylogeny, *F. graminearum* and *F. cortaderiae* isolates were once again resolved as phylogenetically distinct. Using the *Pho* sequence, Gram 47 clustered with *F. austroamericanum* (= *F. graminearum* Lineage 1) isolates, not *F. cortaderiae* as seen in the AFLP based phylogram.

The six *F. pseudograminearum* isolates grouped with the four isolates used in the study of O'Donnell *et al.* (2000), however the phylogram suggested the existence of three *F. pseudograminearum* lineages. The isolates of O'Donnell *et al.* (2000) formed one distinct lineage, and two lineages of New Zealand isolates were also resolved. Tree topology for New Zealand isolates differed from that derived from AFLPs in that isolate NZ67, from a North Island wheat grain, joined isolates

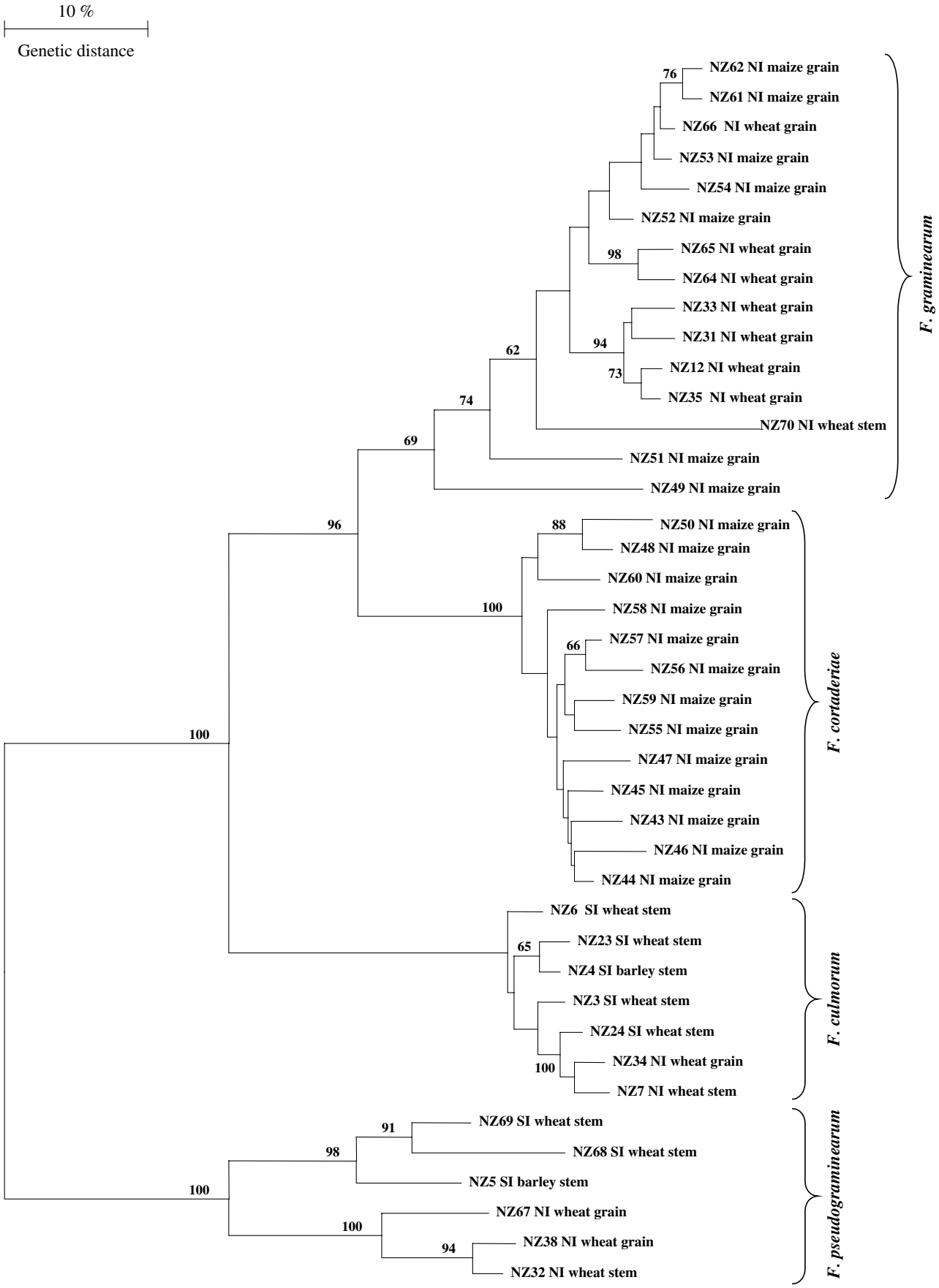

Fig. 1. Phylogram of total AFLP results for *Fusarium graminearum*, *F. cortaderiae*, *F. pseudograminearum* and *F. culmorum*. Tree topology was inferred using Neighbour Joining methods, bootstrap values >55% are shown above nodes and were calculated from 1000 pseudo-replicates. Origins of isolates are indicated after the isolate number (NI, North Island; and SI, South Island).

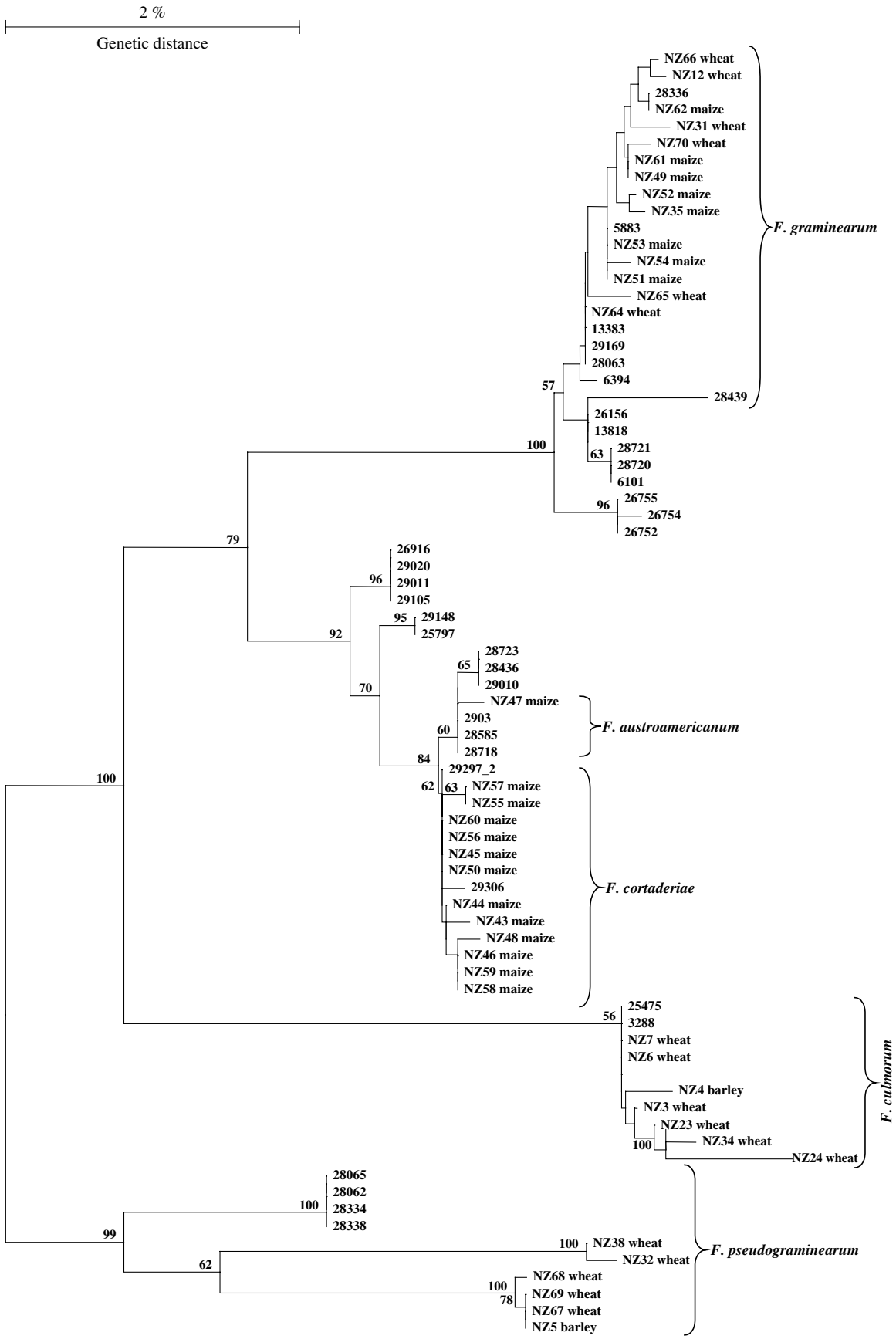

Fig. 2. Phylogram of *Fusarium* isolates from this study (isolate number and host crop) and O'Donnell *et al*. (2000) generated using sequence from the phosphate permase gene. The Jukes and Cantor model was used to estimate genetic distance, tree topology was inferred using Neighbour Joining methods, and bootstrap values $>55\%$ are shown above nodes calculated from 1000 pseudoreplicates. Relevant Fg clade species as described by O'Donnell *et al*. (2004) are indicated.

5, 68, and 69, which were from South Island cereal stems. For both analylses statistical support for respective monophyletic groupings was high, however it should be noted that AFLP based analyses sample heterogeneity over the entire genome whereas the *Pho* sequence analysis samples one locus.

Potential for gene flow amongst NZ Fg clade species

Species designations for each Fg clade isolate were correlated with available information on the region of isolation and host origin as a means to assess the potential for gene flow between species (Table 1, Fig. 1). Within our data set we identified one instance of geographical as well as host co-localisation. Both *Fusarium graminearum* and *F. cortaderiae* were isolated from maize grains at the North Island (N.I.) Te Awamutu site. Out of ten isolates, seven were *F. graminearum* and three were *F. cortaderiae*. Conversely, all ten isolates derived from maize ex grain at the Tauramanui site were *F. cortaderiae*. Isolates derived from wheat were also shown to be homogeneous, consisting solely of *F. graminearum*. In summary, these correlations show that at least two Fg clade species can co-exist in similar niches making gene flow a formal possibility. The isolation of only one species from wheat and from the Tauramanui site is harder to interpret. This data may suggest that, although co-localisation does occur, specific niche presences exist for the two species. However, a larger sampling size may well demonstrate co-localisation as was seen at the Te Awamutu site.

Mycotoxin analyses

The 15 isolates of *Fusarium graminearum*, the 13 isolates of *F. cortaderiae*, and the six isolates of *F. pseudograminearum* were assayed for their production of the trichothecene mycotoxins NIV and/or DON (either 3ADON or 15ADON). All *F. cortaderiae* isolates were NIV producers (Table 1), which agrees with the mycotoxin production by two isolates of this species analysed by Ward *et al.* (2002). Six of the isolates also produced small quantities of 15ADON, while none produced detectable levels of 3ADON. Seven of the *F. graminearum* isolates were NIV producers (four of these also produced small quantities of 15ADON), while six were DON (all 15ADON) producers. Two *F. graminearum* isolates did not produce detectable levels of either mycotoxin. When the results of *F. graminearum* mycotoxin analyses are viewed in the context of host origin, both chemotypes were associated with maize and wheat. Specifically, of the *F. graminearum* maize isolates, five were NIV producers, one was a DON producer, whereas of the eight *F. graminerum* isolates from wheat, two were NIV producers and five were DON producers. These results suggest a lack of mycotoxin correlation with *F. graminearum* isolates and their host origin. Of the six *F. pseudograminearum* isolates, three produced DON (all 3ADON) while the other three produced NIV (two of them also producing small quantities of 15ADON). The three DON producers were all isolated from South Island cereal stems, while the three NIV producers were from North Island cereals (two from grains and one from a stem lesion).

DISCUSSION

In this study we utilised two different molecular phylogenetic approaches, AFLPs and gene genealogies, to gain insight into the evolutionary relationships between members of the Fg clade and *Fusarium pseudograminearum* in New Zealand. Furthermore, we place our results in a worldwide context by adopting the same framework for gene genealogy analysis as described by O'Donnell *et al.* (2000). Analyses of mycotoxin chemotypes and subsequent correlation with biogeographic, host and species information is discussed in the context of between-species gene flow and biosecurity.

Phylogenetic analysis was carried out on 28 Fg clade isolates, six *F. pseudograminearum* isolates and seven *F. culmorum* isolates which were assigned to species groupings on the basis of morphological and physiological criteria. Phylogenies reconstructed both from AFLP and phosphate permase sequence data indicate the presence of two distinct Fg clade species, *F. graminearum* and *F. cortaderiae* (O'Donnell *et al.* 2004), in New Zealand.

Members of the Fg clade occur worldwide, and comprise nine biogeographically structured phylogenetically distinct species, indicating a long evolutionary history of reproductive isolation (O'Donnell *et al.* 2004). The ability to place the New Zealand species of the Fg clade within the context of the worldwide phylogeny provides avenues to gaining insight into the origin and possible route of entry of these pathogens into New Zealand.

A recently identified species (*F. cortaderiae*), although first detected in New Zealand, is thought to be of South American origin (O'Donnell *et al.* 2004). This species was isolated from pampas grass (*Cortaderia jubata*), suggesting it may have arrived in New Zealand on imported plants. Pampas grass occurs widely in New Zealand as a shelter and ornamental plant and as a weed, particularly in forestry areas, but originates from South America. The airborne ascospores of *F. cortaderiae* could assist in the spread of this species from hosts such as pampas grass to cereal crops.

F. graminearum, the other Fg clade species found in the present study, is presently cosmopolitan in its distribution (O'Donnell *et al.* 2004). Cereal (wheat, barley, maize) imports to New Zealand are primarily from Europe and North America, as well as Australia. O'Donnell *et al.* (2000) recorded *F. graminearum*

commonly from Europe and USA. O'Donnell (pers. comm.) had previously only detected this species from cereals in New Zealand. It is likely, then, that it arrived on cereal grain imports to New Zealand.

One isolate (NZ47) clustered with *F. austroamericanum* using the *Pho* sequence, but with *F. cortaderiae* in the AFLP based phylogram. However, there was no indication that this isolate was an outlier in the AFLP analysis. Further gene genealogies will need to be constructed to discern between the possibilities that NZ47 is either more accurately grouped with *F. austroamericanum*, or is the result of hybridisation. Otherwise, two distinct analyses gave the same two clear groups of isolates, corresponding to *F. graminearum* and *F. cortaderiae*. There was no evidence for consistent groups of isolates below the level of species; subspecific grouping of isolates differed between the two analyses.

In the past, fungi have been considered recalcitrant to inter or intra-specific genetic exchange. However, the last decade has seen increasing numbers of reports documenting specific hybridisation events amongst natural populations (Olsen & Stenlid 2002). The identification of a hybrid strain between two Fg clade species (O'Donnell *et al*. 2000) and demonstration of laboratory outcrossing amongst lineages (Bowden & Leslie 1999) indicates that novel lineages could develop given the appropriate geographical co-localisation. In the present study we have documented co-localisation of *F. graminearum* and *F. cortaderiae* in a New Zealand maize crop. Out of the ten isolates from the Te Awamutu site, seven were *F. graminearum* and three were *F. cortaderiae*. Although this is a single instance, such a finding indicates the potential for co-localisation of these species as well as the potential for genetic outcrossing. No clear hybrids between these species were detected in the present study. Given the percentage of the genome sampled, however, this is not unexpected. An extended phylogenetic analysis of the co-localised species isolated in this study may uncover evidence for hybridisation.

Presence of perithecia, molecular phylogenetic analysis, mycotoxin chemotype and, to a lesser extent, growth rate and host tissue associations indicate that two Fg clade species and *F. pseudograminearum* exist on cereals in New Zealand. To our knowledge this is the first clear documentation of *F. pseudograminearum* in New Zealand. Francis & Burgess (1977) and Burgess *et al*. (1994) suggest that *F. graminearum* and *F. psuedograminearum* cannot be reliably differentiated on the basis of morphology of macroconidia. We were also unable to confidently discern consistent conidial shape differences between isolates of *F. graminearum* and *F. pseudograminearum*, although Aoki & O'Donnell (1999) found small differences in conidia of the two species using different media and culture conditions. Presence or absence of perithecia was therefore used to initially classify isolates into the two groups. On average, the putative Fg clade isolates grew faster than the putative *F. pseudograminearum* isolates, as previously recorded by Burgess *et al*. (1994) and Aoki & O'Donnell (1999). However, there was considerable overlap between individual isolates. Therefore, growth rate was not a reliable indicator of species in itself, although it did support the species separation based on perithecia. *F. pseudograminearum* is most commonly associated with crown rot of cereals, while *F. graminearum* is most commonly associated with head blight (Francis & Burgess 1977). Likewise, in the present study, almost all the isolates assigned to the Fg clade came from cereal grains, while most isolates assigned to *F. pseudograminearum* were from cereal stems. Phylogenetic analysis confirmed the separation of the two groups of isolates, and comparison with *Pho* permease sequence data for known isolates of the Fg clade and *F. pseudograminearum* indicated that the putative designations were appropriate (O'Donnell *et al*. 2000).

Analysis of a limited number of isolates of *F. pseudograminearum* suggests some phylogenetic diversity within this species. Previous reports suggest that *F. pseudograminearum* isolates produce the mycotoxin 3ADON rather than NIV (O'Donnell *et al*. 2000). Of six isolates in the present study assigned to *F. pseudograminearum*, three were 3ADON producers and three were NIV producers. The three 3ADON producing isolates were obtained from cereal stems in South Island crops, and formed one of the two clusters of isolates indicated by AFLP analysis. These isolates would appear to be classic *F. pseudograminearum* isolates based on mycotoxin chemotype and host tissue association. The three NIV producers (two of which also produced small quantities of 15ADON) formed the other cluster, and were isolated from North Island cereal crops, two isolates being from grains and one from a stem lesion. The AFLP data therefore suggest two distinct populations separated by geography and mycotoxin chemotype. However, using the phosphate permase gene sequence, one of the North Island isolates (isolated from a grain) clustered with the South Island isolates. Morphological comparison of New Zealand *F. pseudograminearum* isolates with typical strains has confirmed their identification (Alison Bentley, pers. comm.). Further work with a greater number of isolates is being carried out to determine the population structure of *F. pseudograminearum* in New Zealand, and whether New Zealand isolates may include additional undescribed species closely related to *F. pseudograminearum* (A. Bentley, unpublished).

Biosecurity (plant quarantine) has become an increasingly important consideration throughout the world. Reflecting this concern, Ward *et al*. (2002) suggest that 'disease control and plant quarantine programmes need to exercise increased vigilance to ensure that international trade in agricultural commodities does not result in the global transposition of strains with novel toxigenic potential'. Indeed,

Benyon *et al*. (2000) using RFLP and Ouellet & Seifert (1993) using RAPD-PCR found that isolates of *F. graminearum* could not be grouped according to host or geographic origin. This finding suggests that these genetically similar organisms may have been dispersed from a common point of origin by the airborne dissemination of spores over time, or more recently by the movement of seed stock or contaminated agricultural implements. In this study we show the presence of two distinct Fg clade species in New Zealand which are not likely to face substantial geographical/host barriers to hybridisation. Conversely, we did not detect several Asian or New World Fg clade endemics. Cereal grain imports to New Zealand from Asia, South America, and Africa are much more closely controlled by quarantine measures (NZ Seed for Sowing Standard) than imports from Australia, North America and Europe. This may have prevented entry to New Zealand of the common species from Asia and South America.

Because some of the Fg clade species are reported to have a wide host range, including non-cereals, it will be important to characterise New Zealand isolates from a range of hosts. Hybrids formed via genetic outcrossing of introduced species with resident species could have novel host range, mycotoxin and pathogenicity characteristics, and quarantine measures to limit the entry or spread of further species is warranted.

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