# An *Epichloë* endophyte associated with the Afromontane grass *Festuca simensis*

Stuart D. CARD[1]*, Michael J. CHRISTENSEN[1†], Nicholas W. ELLISON[1†], Marty J. FAVILLE[1], David E. HUME[1], Richard D. JOHNSON[1], Wade J. MACE[1], Mary NAMAGANDA[2], Jaspreet Singh SIDHU[1], Wei ZHANG[1] and Alan V. STEWART[3]

[1]*AgResearch Group – Bioeconomy Science Institute, Grasslands Research Centre, Palmerston North, New Zealand*

[2]*Department of Plant Science, School of Biosciences, College of Natural Sciences, Makerere University Herbarium, Kampala, Uganda*

[3]*PGG Wrightson Seeds Ltd., Kimihia Research Centre, Lincoln, New Zealand*

[†]*Retired*

*Corresponding author: stuart.card@agresearch.co.nz

## Abstract

Filamentous fungi of the genus *Epichloë* form symbiotic associations with Pooideae grasses (family Poaceae) with some estimated at over 40 million years old, dating back to the origin of this grass subfamily. Here we provide a report on an *Epichloë* species associating with *Festuca simensis*, a tetraploid grass species of highland tropical African origin. The endophyte of *F. simensis* most resembled *Epichloë uncinata*, a fungus generally associated with *Festuca pratensis* (meadow fescue), with respect to its genetics, plant colonisation patterns and secondary metabolite profile. However, the endophyte of *F. simensis* was slower to emerge from dissected plant tissue and contained an additional copy of the β-tubulin gene, compared with *E. uncinata*. *Festuca simensis* potentially originated from a cross between a western Mediterranean diploid grass similar to *Festuca arundinacea* subsp. *fenas* as the maternal parent and a Eurasian Lolium sect. *Lolium*-type paternal parent, followed by genome duplication. The grass species likely colonised the Afromontane regions that cover the mountains of Africa and the southern Arabian Peninsula during the Pliocene epoch (5.3 to 2.6 million years ago). This paper reviews the preliminary knowledge gained about this intriguing *Epichloë*-grass symbiosis.

**Keywords:** co-evolution, loline alkaloids, plant-microbe interactions, Poaceae, symbiosis

## Introduction

The Poaceae (formally Gramineae) is the fifth largest flowering plant family and contains approximately 12,000 extant species, present in around 800 genera of annual or perennial herbs that are generally known as grasses (Piperno & Sues 2005; Soreng et al. 2022). As well as being the most ecologically dominant, the Poaceae is also the most economically important plant family as it contains many species suitable for human consumption [including barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), millet (*Pennisetum glaucum* (L.) R.Br.), rice (*Oryza sativa* L.), sugarcane (*Saccharum* spp.) and wheat (*Triticum aestivum* L.)], as forages [including bentgrasses (*Agrostis*), bluegrass (*Poa*), bromegrasses (*Bromus*), orchard grass (*Dactylis*), timothy (*Phleum*), ryegrasses (*Lolium*) and fescues (*Festuca*)], building materials (i.e. bamboo), aesthetics for recreational areas such as parks, lawns and sports grounds [including bluegrass, Bermuda grass (*Cynodon*), fescues and perennial ryegrass (*Lolium perenne* L.)] and species suitable as biofuels [e.g. maize, silvergrass (*Miscanthus* spp.), sweet sorghum (*Sorghum bicolor* (L.) Moench), sugarcane and switchgrass (*Panicum virgatum* L.)].

The grasses can be grouped within two major lineages of undefined taxonomic rank according to their photosynthetic pathways, with those using the primitive C3 photosynthetic pathway adapted to cool temperate zones, grouped within the BOP clade (collectively known as cool-season grasses) and other ancestral and recently evolved lineages, and those that have evolved a C4 photosynthesis pathway within the PACMAD clade (collectively known as warm season grasses). The main evolutionary line of the cool-

season grasses group is split according to two distinct physiological adaptations, 1) those types more suited to temperate environments and 2) those more suited to the winter rainfall regime of the Mediterranean-type zones, with the latter species generally forming an annual habit (Renvoize & Clayton 1992). The BOP clade can be further split into three subfamilies, Bambusoideae, Oryzoideae and Pooideae, from which its name refers, with the Pooideae being the largest (4126 species in 219 genera in 15 tribes) (Soreng et al. 2022).

A characteristic unique to certain grass species within the Pooideae is their coevolution over approximately 40 million years with endophytic fungi belonging to the genus *Epichloë* (family Clavicipitaceae). This ancient grass-fungal symbiosis therefore appears to have existed during early grass evolution dating back to the very origin of the Pooideae (Schardl et al. 2008). As a result of this co-evolution over many millennia, *Epichloë* species are largely naturally restricted, through genetic compatibility, to a host genus or closely related grass genera within a tribe (Schardl 2010). These naturally restricted symbioses occur in many tribes of the Pooideae, including Brachyelytreae, Brachypodieae, Bromeae, Meliceae, Poeae, Stipeae and Triticeae (White 1987; Christensen 1993; Meijer & Leuchtmann 1999; Schardl & Leuchtmann 1999; Gentile et al. 2005; Moon et al. 2007; Charlton et al. 2012; Card et al. 2014; Leuchtmann & Schardl 2022). The most utilised grass-endophyte associations reside within the genera *Festuca* and *Lolium* where many strains of *Epichloë* have been exploited, and commercialised, for agricultural benefit, particularly in the New World countries of New Zealand, Argentina, Australia, Brazil, Chile, Uruguay and the USA (Card et al. 2024).

In New Zealand, selected *Epichloë* strains are a key element of the agricultural landscape as they confer bioprotective traits to their host grasses, particularly pest deterrence via the production of several alkaloidal secondary metabolites (Bastías et al. 2024). In natural ecosystems *Epichloë*-grass symbioses are prevalent across the continents of Eurasia and the Americas with fewer records of *Epichloë* forming associations with grasses outside of these global regions (Caradus et al. 2021). Here we provide a first report on an *Epichloë* endophyte associating with *Festuca simensis* Hochst. ex A. Rich. (not to be confused with *Festuca sinensis* Keng ex. E.B. Alexeev, a species of grass endemic to China), a tetraploid grass species of highland tropical African origin genetically similar and evolutionary close to *Festuca* subgen. *Schedonorus* and *Lolium* (Namaganda et al. 2006; Inda et al. 2014; Minaya et al. 2015).

## Materials and Methods

### Plant germplasm

Seed from four accessions of *Festuca simensis*, originally collected from highland tropical locations in Uganda, were sourced from the Margot Forde Genebank, New Zealand's national gene bank of grassland plants. Seed was sown into potting mix within small plastic pots, watered as required, and held within a non-heated glasshouse at AgResearch, Palmerston North, New Zealand.

### Association with an *Epichloë* endophyte

*Microscopy observations and isolation onto media*

Plants from the four seed accessions were assessed for the presence of filamentous *Epichloë* endophytes using a modified microscopy method of Christensen et al. (2002). In summary, a leaf together with its sheath was removed from each plant, dissected into 2-3 cm strips and mounted in 0.1% (w/v) aniline blue before being examined with a compound microscope under 10× and 40× magnification. Further observations with bright field microscopy and transmission electron microscopy (TEM) (as described in Voisey et al. 2016) were conducted to investigate fungal colonisation of inner and outer leaf sheaths, leaf blades, and vascular bundles.

On identification of an *Epichloë* endophyte within plant tissue, fungi were isolated into axenic culture following a modified method of Christensen et al. (2002). In summary, sections of leaf sheath (4-5 cm) were surface disinfected by dipping in 97% ethanol, followed by a 3 min wash in 0.1% (v/v) sodium hypochlorite plus Tween-80 (1 drop in 1L), before rinsing twice in sterile tap water and drying. Sections of leaf sheath were further cut into 1-2 mm longitudinal pieces and plated onto potato dextrose agar (PDA) amended with the antibiotic chloramphenicol (0.1g/L) to inhibit bacterial growth. Plant tissue pieces were observed daily, using a dissecting microscope, for fungal growth. Once cultured, axenic cultures of endophytes were grown on sterilised wheat grain and stored in 20% glycerol at -80 °C for long-term storage.

*Secondary metabolite analysis*

Several plants from each accession were sampled and analysed for the four recognised groups of alkaloidal secondary metabolites produced by *Epichloë*-grass associations identified to date: indole-diterpenes (e.g., lolitrems and epoxyjanthitrems), ergot alkaloids (e.g., ergovaline), lolines, and peramine, using established extraction and analysis protocols (Bastías et al. 2018; Lukito et al. 2019). Loline alkaloids were measured using gas chromatography (modified from Bastías et

al. 2018). In summary, 50 mg samples of lyophilised grass tissue were ground with a bead ruptor (FastPrep FP120, Savant Instruments Inc., USA) with 3 × 3-mm stainless steel beads in a 2 mL vial (10 sec at 5 m/sec). Samples were extracted for 1 hour with 50 µL of 40% methanol/5% ammonia and 1 mL 1,2-dichloroethane containing 54.8 ng mL−1 4-phenylmorpholine as an internal standard. After centrifuging for 5 mins at 5,000 G the supernatant was transferred to glass gas chromatography vials via a 10 µm filter for analysis, that was conducted using a gas chromatography-flame ionization detector (GC2010 Plus, Shimadzu Corp., Japan) fitted with a ZB-5 capillary column (30 m × 0.32 mm × 0.25 µm film; Phenomenex, USA). The detection limit using this technique is 25 µg $g^{-1}$ dry weight.

*Genetic analysis*

1) Sequencing of the 1-α translation elongation factor (*tefA*) and β-tubulin (*tubB*) genes

DNA was extracted from the mycelia of an unknown endophyte strain sample from *F. simensis* accession 1 using the Quick-DNA™ Fungal/Bacterial MiniPrep kit (Zymo Research Corp., USA) according to the manufacturer's instructions. Sequencing of the *tefA* and *tubB* genes was performed as described by Moon et al. (2004) using primers as published for *tefA* and modified *tubB* primers, RJ251-F and RJ252-R (Table 1). These samples were sent out to Massey Genome Centre for sequencing using BigDye Terminator v3.1. Sequence alignment and phylogeny analysis were performed using 'Phylogeny.fr' (Dereeper et al. 2008). PCRs were performed in 50 µL reactions using 10-100 ng of endophyte genomic DNA. The reagents in Table 2 were set up on ice and mixed well before being transferred to a thermocycler (Biorad iCycler, Biorad Hercule, USA). PCR conditions were used as shown in Table 3.

**Table 1** Primer pairs used for the amplification of tefA and tubB regions using PCR.

| Primer name | Primer sequence |
|---|---|
| tef1-exon1d-1 | GGG TAA GGA CGA AAA GAC TCA |
| tef1-exon5u-1 | CGG CAG CGA TAA TCA GGA TAG |
| β-tubulin forward primer (RJ251-F) | TCG GCC TCA CGA CGC ACA AC |
| β-tubulin reverse primer (RJ252-R) | CCC ATA CAT TAC ACC TTT CTG GCG |

**Table 2** PCR reagents and their final concentrations.

| Reagents | Stock concentration | Final concentration | Volume (µL) |
|---|---|---|---|
| PCR mix without $MgCl_2$ | 10 × | 1 × | 5.0 |
| $MgCl_2$ | 50 mM | 1.5 mM | 1.5 |
| Template DNA | n/a | 10-100 ng/µL | 5.0 |
| Forward primer | 10 µM | 0.2 µM | 1.0 |
| Reverse primer | 10 µM | 0.2 µM | 1.0 |
| Distilled water | n/a | n/a | 35.85 |
| dNTPs mix | 25 mM of each | 200 µM of each | 0.40 |
| Taq polymerase | 5 U/µL | 1.25 U | 0.25 |

PCR buffer composition (10 ×): 200 mM Tris-HCl (pH 8.4), 500 mM KCl

2) Simple sequence repeat analysis

Simple sequence repeat (SSR) analysis was conducted as described by Card et al. (2014) on unknown endophyte strain samples from two *F. simensis* accessions alongside 19 comparator strains of *E. festucae*, *E. coenophiala*, *E. uncinata, E. siegelii* and *E. typhina* sourced from different *Festuca, Elymus* or *Lolium* plant hosts (Table 4). Briefly, vegetative plants with endophyte-infected tillers were sampled for DNA isolation by excising 100 mg of basal tiller tissue. Total genomic (plant + endophyte) DNA was isolated from

**Table 3** Different PCR steps represented by different time intervals, temperatures and cycles.

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Pre-incubation | 95°C | 4 min | 1 × |
| Amplification | 95°C | 30 sec | |
| | 56°C | 30 sec | |
| | 72°C | 1 min | 45 × |
| Elongation | 72°C | 5 min | 1 × |
| Cooling | 4°C | ∞ | Hold |

**Table 4** Unknown *Epichloë* strain samples (AR1511 and AR1512) from two *F. simensis* accessions (accessions 1 and 2) alongside 19 comparator strains of *E. festucae*, *E. coenophiala*, *E. uncinata*, *E. siegelii* and *E. typhina* sourced from different *Festuca*, *Elymus* or *Lolium* plant hosts.

| Fungal code | Fungal species | Original host species |
|---|---|---|
| AR501 | *Epichloë coenophiala* | *Festuca arundinaceae* |
| AR525 | *E. coenophiala* | *F. arundinaceae* |
| AR584 | *E. coenophiala* | *F. arundinaceae* |
| AR601 | *E. coenophiala* | *F. arundinaceae* |
| AR1002 | *Epichloë uncinata* | *Festuca pratensis* |
| AR1003 | *E. uncinata* | *F. pratensis* |
| AR1004 | *E. uncinata* | *F. pratensis* |
| AR1006 | *E. uncinata* | *F. pratensis* |
| AR1007 | *E. uncinata* | *F. pratensis* |
| AR1015 | *E. uncinata* | *F. pratensis* |
| AR1016 | *E. uncinata* | *F. pratensis* |
| AR1018 | *E. uncinata* | *F. pratensis* |
| AR1019 | *Epichloë siegelii* | *F. pratensis* |
| AR1511 | Unknown | *Festuca simensis* |
| AR1512 | Unknown | *F. simensis* |
| AR1968 | *Epichloë festucae* | *Festuca gigantea* |
| AR1969 | *E. festucae* | *F. gigantea* |
| AR1970 | *E. festucae* | *F. gigantea* |
| FG1 | *E. festucae* | *F. gigantea* |
| AR3018 | *Epichloë bromicola* | *Elymus dahuricus* |
| E8 | *Epichloë typhina* | *Lolium perenne* |

tiller tissue using the FastDNA kit (MP Biomedicals, USA) following the manufacturer's instructions. Data for 23 SSR markers were generated using the method described by Card et al. (2014). The SSR primer pairs used were: B10 and B11 (Moon et al. 1999), ans015, ans016, ans017, ans019, ans025, ans030, ans031, ans033, ans036, ans047, ans049, ans054, ans056 and egs027 (Kirkby et al. 2011; Card et al. 2014), alongside ans024, ans032, ans035, ans044, egs002, egs004 and egs0010 (Table 5). Each SSR fragment was scored as present (1) or absent (0) and the resulting binary matrix was used to estimate pairwise Jaccard's coefficient of similarity (Sneath & Sokal 1973) amongst strains, using NTSYSPC v2.20x (Rohlf 2008). A dendrogram based on Jaccard coefficient data was constructed using the unweighted pair group method with arithmetic averages (UPGMA; Sneath & Sokal 1973) with the same software.

**Table 5** Primer pairs used for the amplification of SSR markers not previously described (*cf* Moon et al. 1999; Kirkby et al. 2011; Card et al. 2014).

| SSR name | Primer sequence |
|---|---|
| ans024 | (F) CTGGACTACCTCCTCCACAATC |
| | (R) GCCCGCATTAAAGAAATAAGG |
| ans032 | (F) GATTCACCTCATCTACCATCTACC |
| | (R) GGTCGAGTTTATATCTCCGATG |
| ans035 | (F) CGGTGCTCTTTACCTTTTGACT |
| | (R) CATATAAGAGGCCCATCTCACC |
| ans044 | (F) TCCAGAAGTAGTCGGGATCATGT |
| | (R) ATGAGAGGGGAATTGAAGACAA |
| egs002 | (F) TAAGGCGAGGTCAGTTATTAGCTT |
| | (R) CTCTTTTGGGACCTACCTAGTCAA |
| egs004 | (F) TATACAGACTACGAACACCCTCCC |
| | (R) GGATGTTACTGAATGAAAACTCCC |
| egs0010 | (F) ATTCCTTGTCCTGTTGTCTCAAAC |
| | (R) ATGGTTGCTCGAAAAGACTTACTC |

## Results

### Plant germplasm

Plants grown from the seed of the four accessions of *F. simensis* (accession 1 [n=2], accession 2

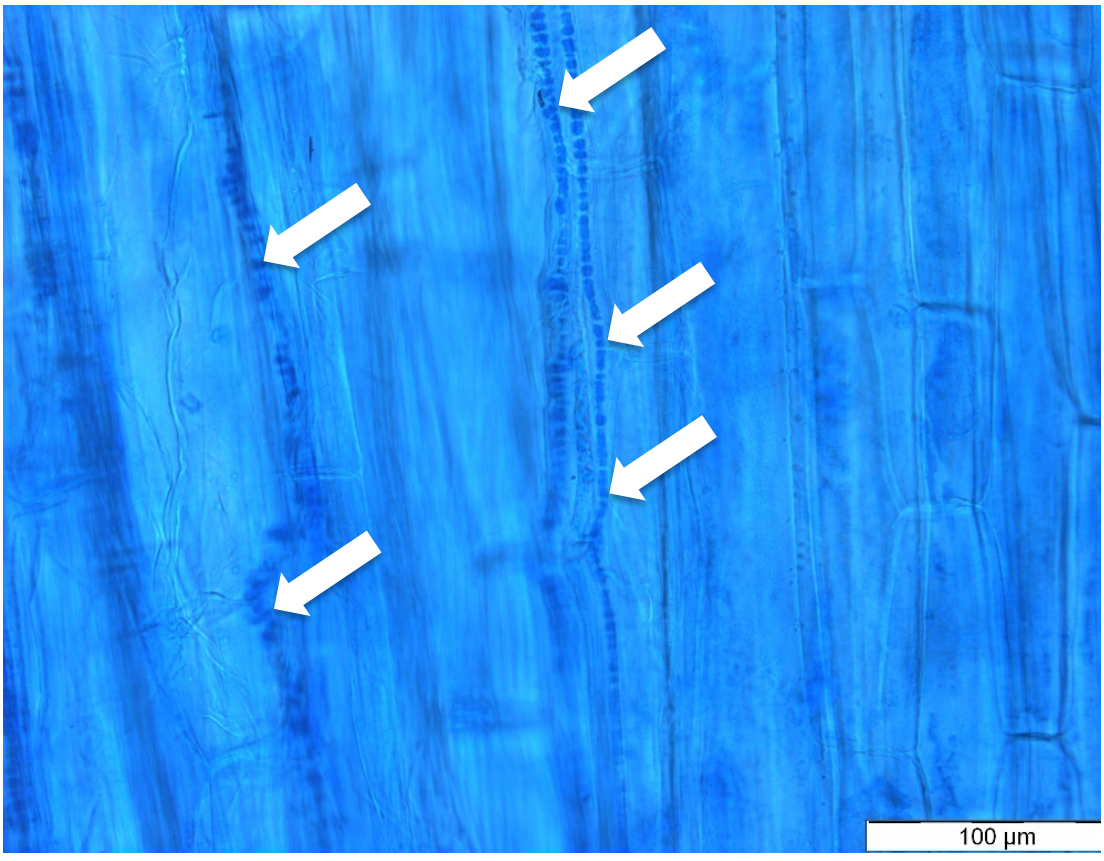


**Figure 1** Endophytic hyphae of *Epichloë* sp. colonising the intercellular spaces of plant cells, growing perpendicular to the leaf axis within the leaf sheath of *Festuca simensis*.

[n=1], accession 3 [n=5] and accession 4 [n=5]) were completely symptomless (no signs of disease) throughout the study.

**Association with an endophytic *Epichloë* sp.**

*Microscopy observations and isolation onto media*

Preliminary microscopy observations of at least one plant per accession revealed the presence of septate filamentous fungal hyphae growing perpendicular to the leaf axis. Hyphae colonised the intercellular spaces of plant cells within the leaf sheath, but no branching was observed (Figure 1).

These fungi were subsequently assigned a unique AR (AgResearch) code, with five endophytes, AR1508-AR1512, designated (one endophyte per accession) with the exception of accession 4 that yielded two endophytes, AR1509 and AR1510. Further observations with TEM showed that hyphae of AR1511, although sparse in number, could be easily seen within the inner and outer leaf sheaths, measuring approximately 3.6 and 3.8 µm in width respectively, with greater colonisation around the vascular bundles (Figure 2). No hyphae were observed colonising the leaf blades of *F. simensis* and no epiphyllous hyphae were observed.

After 28–35 days incubation, filamentous fungi characteristic of *Epichloë* were observed emerging from either end of the excised plant tissue from all four of the *F. simensis* accessions examined. No conidia were observed from any of the strains.

*Secondary metabolite analysis*

Endophyte-infected plants from all grass accessions produced no quantifiable amounts of indole-diterpenes, ergot alkaloids or peramine. However, these endophyte-infected plants did produce loline alkaloids with the dominant compound being *N*-formylloline (Table 6). The results from accession 4 also gave a wider dispersion in the concentration of loline alkaloids compared to the other accessions (575-5733 µg $g^{-1}$ for *N*-formylloline, 0-256 µg $g^{-1}$ for *N*-acetylloline and

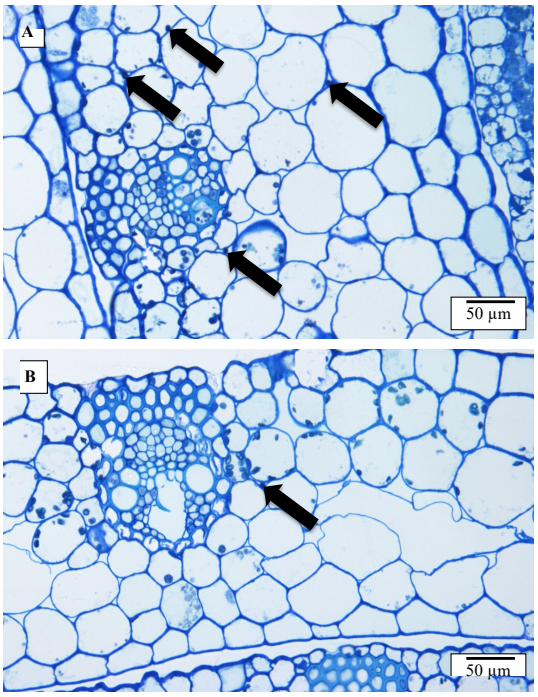


**Figure 2** Endophytic hyphae of *Epichloë* sp. strain AR1511 colonising the intercellular spaces of the inner (A) and outer (B) leaf sheaths of *Festuca simensis*. Vascular bundles are groups of smaller plant cells.

45-316 µg $g^{-1}$ for *N*-acetylnorloline) as reflected in the coefficient of variation (Table 6).

*Genetic analysis*

1) Sequencing of the *tefA* and *tubB* genes

PCR amplification yielded two copies each of the *tefA* and *tubB* genes from the endophyte designated AR1511 isolated from the *F. simensis* accession (Figure 3 and 4). Phylogenetic reconstruction showed that allele 1 and allele 2 nested with known *Epichloë* species, namely those of *E. bromicola* and *E. poae*, respectively, indicating AR1511 belongs to *E. uncinata* (*E. bromicola* × *E. poae*).

2) SSR analysis

A total of 183 polymorphic PCR products were amplified from 23 SSR loci across the panel of 20 plant-endophyte associations. UPGMA clustering based on Jaccard coefficient data identified two major groups comprising *E. festucae*/ *E. coenophiala*/ *E. siegelii* in group 1 and *E. uncinata*/ *E. bromicola* strains in group 2 (Figure 5). The composition of the group 1 is concordant with *E. festucae* being one of the progenitors of *E. coenophiala*. The two *F. simensis*-derived strains, AR1511 and AR1512, genetically identical by this SSR panel, were most closely associated with the *E. uncinata*/ *E. bromicola* group 2 (Figure 5). The strain E8, originally isolated from *L. perenne*, is the only *E. typhina* strain examined and forms a singleton branch distinct from the two other groups. The position of AR1019, the sole *E. siegelii* strain, alongside *E. festucae* in the group 1 may reflect its evolutionary history as a hybrid of *E. bromicola* × *E. festucae*.

## Discussion

Here we provide a report on an *Epichloë* species from central Africa associating with *F. simensis*, a tetraploid grass species of highland tropical African origin. *Festuca simensis* potentially originated from a maternal parent which would have colonised the Afromontane regions that cover the mountains of Africa and the southern Arabian Peninsula during the Pliocene epoch (5.3 to 2.6 million years ago). The endophyte of *F. simensis* most resembled *E. uncinata*, a fungus generally associated with *F. pratensis*, with respect to its genetics, plant colonisation patterns and secondary metabolite profile. However, the endophyte of *F. simensis* was slower to emerge from dissected plant tissue, had a significantly slower growth rate in culture and contained an additional copy of the β-tubulin gene, compared with *E. uncinata*.

The most robust method for the identification and taxonomic classification of *Epichloë* endophytes is via molecular phylogenetic analysis of DNA sequence comparisons using the housekeeping genes, such

**Table 6** Mean concentration of loline alkaloids produced by selected endophyte-infected accessions of *Festuca simensis*. The number of plants (n) and the coefficient of variation as %, = std deviation/mean is provided (CV%).

| Grass accession | | Loline alkaloid concentration µg $g^{-1}$ (CV%) | | |
|---|---|---|---|---|
| | n | N-formylloline | N-acetylloline | N-acetylnorloline |
| Accession 1 | 2 | 1488 (16%) | 75 (49%) | 82 (20%) |
| Accession 2 | 1 | 841 | 17 | 44 |
| Accession 3 | 5 | 3730 (22%) | 379 (12%) | 321 (10%) |
| Accession 4 | 5 | 2338 (88%) | 87 (120%) | 119 (95%) |

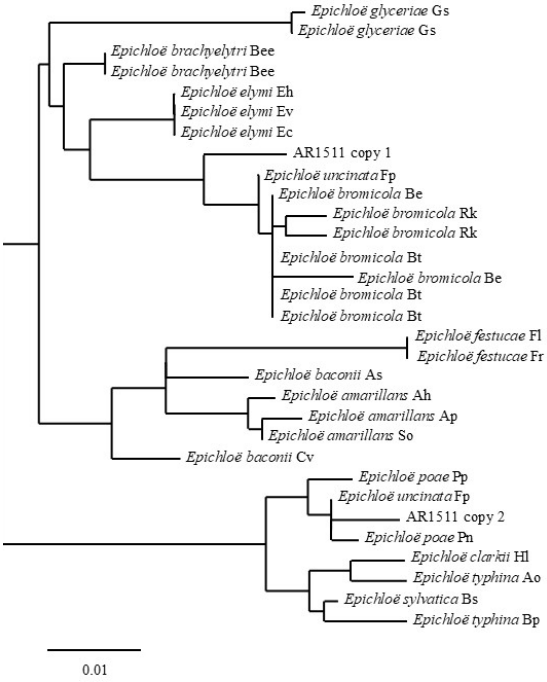


**Figure 3** Gene phylogeny of *Epichloë* species derived from maximum likelihood analysis of the *tefA* gene and copies of the hybrid endophyte designated AR1511 isolated from *Festuca simensis.* Letters after each endophyte species refer to host abbreviations as follows: Ah = *Agrostis hyemalis*, Ao = *Anthoxanthum odoratum*, Ap = *Agrostis perennans*, As = *Agrostis stolonifera*, Be = *Bromus erectus*, Bee = *Brachyelytrum erectum*, Bp = *Brachypodium pinnatum*, Bs = *Brachypodium sylvaticum*, Bt = *Bromus tomentellus*, Cv = *Calamagrostis villosa*, Ec = *Elymus canadensis*, Eh = *Elymus hystrix*, Ev = *Elymus virginicus*, Fl = *Festuca longifolia*, Fp = *Festuca pratensis,* Fr = *Festuca rubra* subsp. *rubra*, Gs = *Glyceria striata*, Hl = *Holcus lanatus*, Pn = *Poa nemoralis*, Pp = *Poa pratensis*, Rk = *Roegneria kamoji*, So = *Sphenopholis obtusata.*

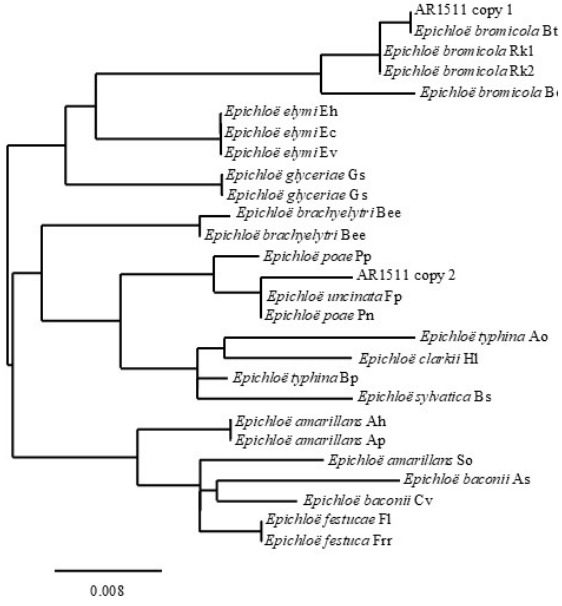


**Figure 4** Gene phylogeny of *Epichloë* species derived from maximum likelihood analysis of the *tubB* gene and copies of the hybrid endophyte designated AR1511 isolated from *Festuca simensis.* Letters after each endophyte species refer to host abbreviations as listed for Figure 3.

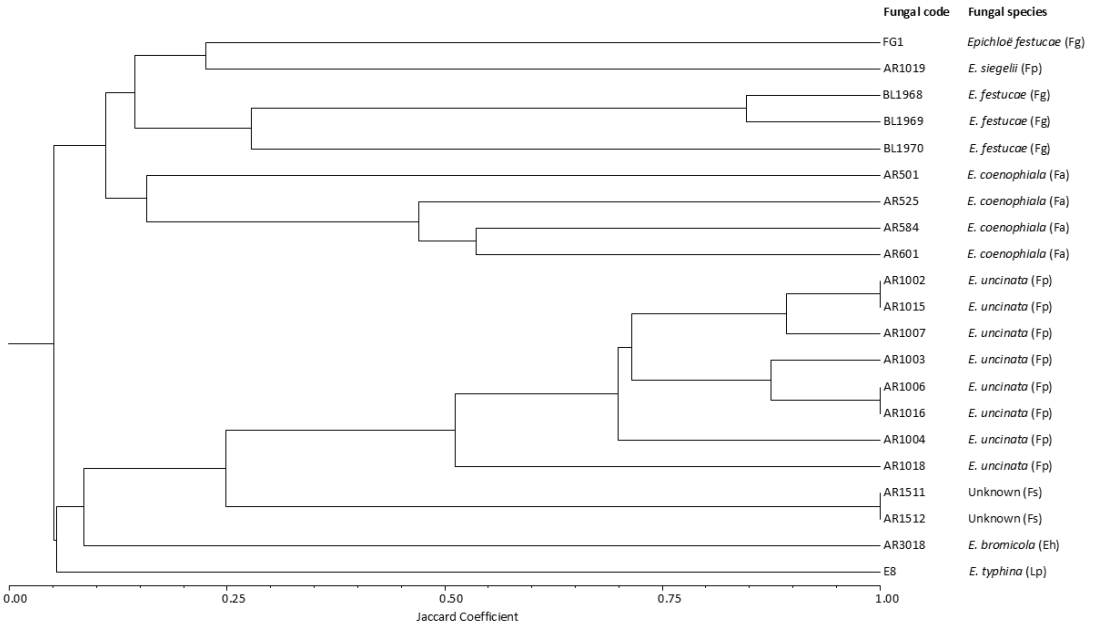


**Figure 5** Dendrogram of genetic similarity amongst selected *Epichloë* endophytes associated with *Festuca gigantea* (Fg), *F. pratensis* (Fp), *F. arundinacea* (Fa), *F. simensis* (Fs), *Elymus dahuricus* (Eh) and *Lolium perenne* (Lp), assessed by the unweighted pair group method of arithmetic averages. Genetic similarity is indicated by branch lengths between pairs of endophytes. Endophyte identity is indicated by AR code, species and original host plant species.

as those encoding *tubB*, *tefA* and *actG* (Craven et al. 2001). While sexual *Epichloë* species are haploid, an intriguing feature of this genus is the prevalence of interspecific hybrids among the asexual species that have ancestries involving two or more distinct *Epichloë* lineages, which contributes to the genetic diversity of these otherwise clonal species (Moon et al. 2004; Schardl 2010; Thünen et al. 2022). *Epichloë* has the greatest number of interspecific hybrids of any fungal genus (Campbell et al. 2017). PCR amplification yielded two copies each of the *tefA* and *tubB* genes from the endophyte isolated from *F. simensis*. Sexual *Epichloë* species contain a single copy of the *tubB* gene while some asexual species contain multiple copies with each copy aligning closely to that of a different sexual ancestral species. Previously characterised strains of *E. uncinata* (*E. bromicola* × *E. poae*) identified in grasses derived from Europe have lost one of the *tubB* alleles with only one copy present (Ekanayake et al. 2013). We therefore suggest that the endophytes of *F. simensis* pre-date those of *E. uncinata* from *F. pratensis*, with whole genome sequencing required to confirm this.

There is substantial variation between and within different *Epichloë* species, particularly with respect to their *in-planta* colonisation patterns, genetics and chemotypical diversity (Bastías et al. 2024). The endophytes of *F. simensis* were large in diameter and had characteristics similar to several known *Epichloë* species. For example, the colonisation patterns of AR1511 within *F. simensis* were similar to that recorded for some strains of *E. coenophiala* (=*Neotyphodium coenophialum*) within *F. arundinacea* in that hyphae were only observed colonising the leaf sheaths of tillers and not the leaf blades (Hinton & Bacon 1985; Christensen et al. 1998). Leaf blade colonisation patterns can differ with plant age and host and *Epichloë* genotype. It has been suggested that the ligular zone located between the leaf sheath and blade acts as a physical barrier to the advancement of endophytic hyphae and restrict some fungal genotypes from colonising the blade tissues (Christensen et al. 2002). Due to the unique way *Epichloë* colonise their host grass plants, via intercalary hyphal extension, as grass leaves age, hyphae can increase in diameter, and therefore biomass, but not in frequency (Christensen & Voisey 2006).

Isolation of endophytes AR1508-12 onto PDA took 28–35 days, significantly longer than many other *Epichloë* species and was reminiscent of *E. occultans* (=*Neotyphodium occultans*), recorded from annual *Lolium* grasses including, *L. canariense, L. multiflorum*, *L. persicum, L. remotum, L. rigidum, L. temulentum* and *L. subulatum*, perhaps indicating a very old association with its host grass (Moon et al. 2000). However, the

mycelium of AR1508-12 was not restricted to the lower leaf sheath of their hosts as with *E. occultans* and was instead observed throughout the leaf sheaths of their hosts.

The greatest similarity with respect to secondary metabolite profiles of AR1508-12, however, is with *E. siegelii* and *E. uncinata*, fungi that form associations with *F. pratensis*. There are four main recognised groups of alkaloids produced by *Epichloë*-grass symbioses; indole-diterpenes (e.g., lolitrems and epoxyjanthitrems), ergot alkaloids (e.g., ergovaline), 1-aminopyrrolizidines (e.g., the lolines, and pyrrolopyrazines (e.g., peramine); with different *Epichloë* species capable of producing different types, combinations, and concentrations of these compounds

(Leuchtmann et al. 2000). AR1508-12 did not produce indole-diterpenes, ergot alkaloids or peramine but did produce loline alkaloids. Lolines are a group of compounds with a saturated pyrrolizidine core structure, which kill, deter, or slow development of invertebrate herbivores, including insect pests, and which exhibit no substantial mammalian toxicity (Bush et al. 1997). Associations of AR1508-12 with *F. simensis* produced lower loline-alkaloid concentrations than those recorded for *E. uncinata*-*F. pratensis* associations and were closer to those recorded from *E. coenophiala*-*F. arundinacea* associations (Bush et al. 1993).

Two asexual *Epichloë* species have been identified associating with *F. pratensis*, namely *E. siegelii* (=*Neotyphodium siegelii*), a hybrid species of *E. bromicola* × *E. festucae*), *and E. uncinata*, a hybrid *E. bromicola* × *E. poae.* The SSR analysis clearly showed that the endophytes associated with *F. simensis* group with several strains of *E. uncinata* and a strain of *E. bromicola.* The endophytes of *F. simensis* did not group with the *E. festucae*/*E. coenophiala* clade.

*Festuca* is a genus of evergreen or herbaceous perennial tufted grasses, commonly called fescues, with a cosmopolitan distribution occurring on every continent except Antarctica. Taxonomically, *Festuca* represents a major evolutionary line within the tribe Poae of the subfamily Pooideae and is closely related to *Lolium* (commonly known as ryegrass) forming a polyploid series ranging from diploid (2n = 2× = 14) to tetradecaploid (2n = 14× = 98) (Clayton & Renvoize 1986; Martínez-Sagarra et al. 2021). The taxonomy of *Festuca* is problematic as the genus lacks monophyly and many species are known to form hybrids with *Lolium* and *Vulpia*. However, *Festuca*, together with the genera *Lolium* and *Microcytosis*, represent a monophyletic group and can be collectively separated into a broad leaf clade, the *Schedonorus*-*Lolium* complex and the fine leaf clade, *Festuca* subgenus *Festuca* (Minaya et al. 2017; Ansari et al. 2022). The fine-leaved clade of the subgenus *Festuca* largely includes turfgrasses within the '*F. ovina*' and '*F. rubra*' complexes while the broad-leaved clade includes the forage species *F. arundinacea* (= *Lolium arundinaceum* = *Schedonorus arundinaceus*; tall fescue) and *F. pratensis* (= *L. pratense*; meadow fescue) (Cheng et al. 2016; Minaya et al. 2017).

Eight species of *Festuca* have been described in Uganda; six are narrow-leaved (*Festuca abyssinica*, *F. chodatiana*, *F. claytonii*, *F. elgonensis*, *F. pilgeri* and *F. richardii*) and two are broad-leaved (*F. africana* and *F. simensis*) (Namaganda et al. 2006; Minaya et al. 2017; Mairal et al. 2021). *Festuca simensis* is a perennial, allotetraploid grass documented over west-central tropical, northeast tropical and east tropical Africa including from Uganda, Cameroon, Ethiopia, Kenya, Sudan and Zaire, mainly occurring below 3,000 m (Clayton & Renvoize 1986; Clayton et al. 2025). *Festuca simensis* is more genetically similar to grass species within the *Schedonorus*-*Lolium* complex than to the fine-leaved fescues within the subgenus *Festuca* and likely originated from a cross between a Eurasian diploid *Lolium*-type paternal parent and a diploid ancestor of a maternal parent similar to the tetraploid grass *F. arundinacea* subsp. *fenas* (Lag.) Arcang., followed by genome duplication (Namaganda et al. 2006; Minaya et al. 2015; Minaya et al. 2017).

*Festuca simensis* plausibly first colonised the Afromontane regions that cover the mountains of Africa and the southern Arabian Peninsula during the Pliocene epoch (5.3 to 2.6 million years ago) (Inda et al. 2014; Minaya et al. 2015). *Festuca simensis* has the closest phylogenetic relationship with *F. gigantea* (syn. *L. giganteum*; giant fescue), an allohexaploid species within the *Schedonorus*-*Lolium* complex (Namaganda et al. 2006; Moreno-Aguilar et al. 2022). *F. pratensis* is a putative diploid subgenome donor of allohexaploid *F. gigantea* with Ansari et al. (2022) further suggesting *F. simensis* could be the potential donor of the remaining two sub-genomes.

The Poaceae is likely to have originated in the Gondwana region of Pangea during the Early–mid Cretaceous period and had dispersed to all continents by the Late Cretaceous (Piperno & Sues 2005; Bouchenak-Khelladi et al. 2010). The Pooideae, like the entire grass family, has a complex evolutionary history involving a high frequency of polyploidisation and polymorphism with stem and crown genetic divergence ages of approximately 73.7 and 68.7 million years, respectively (Schubert et al. 2019). The evolution of *Epichloë* has largely followed the evolution of their grass hosts with early cladogenesis events in the Pooideae corresponding to early cladogenesis events in *Epichloë* (Schardl et al. 2008).

*Epichloë* species produce a range of secondary metabolites that can have negative effects on herbivores, including both invertebrates and vertebrates. Lolitrem B (an indole-diterpene) and ergovaline (an ergot alkaloid), for example, are responsible for neurotoxicoses such as ryegrass staggers and fescue toxicosis, respectively, and continue to be problematic in several agricultural systems around the world (Gallagher et al. 1984; Bacon 1995). However, in many New World countries, including New Zealand, 'animal-safe' grass-endophyte associations that confer bio-protective properties for increased pasture persistence and productivity have been developed and commercialised (Johnson & Caradus 2019; Caradus et al. 2021). These 'animal-safe' grass-endophyte associations (also termed novel endophyte-grass associations) are purposefully developed for pastoral agriculture by incorporating selected *Epichloë* strains with elite grass cultivars. These novel endophyte-grass associations confer beneficial traits that protect the host from abiotic stresses (e.g. drought) and biotic stresses (e.g. herbivory from invertebrates) while having minimal impact on the health and welfare of ruminant livestock (Bastías et al. 2024; Card et al. 2024). Loline producing endophytes are of great interest from an agricultural viewpoint as they exhibit potent insecticidal and invertebrate feeding-deterrent properties with no known toxic effects on mammals, including ruminant livestock. Further applied research failed when many grass seedlings inoculated with AR1508-12 resulted in endophyte-free plants as newly developed host tillers escaped endophyte colonisation (de Bonth, unpublished). Coupled with the exceptionally slow rate of growth of AR1508-12 in culture, which restricts transfer of endophytes from their original host grasses to elite grass cultivars (Bastías et al. 2024), and the relatively low concentrations of lolines in their original hosts prohibited the feasibility of using these endophytes within an agricultural context. Nevertheless, the endophytes of *F. simensis* offer great scientific interest especially with respect to the coevolution of these fascinating grass endophyte symbioses.

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