Research article

Epichloë is not associated with the pollen of infected ryegrass plants

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Abstract

Observations taken over decades of seed production, and examination of anther tissues from *Epichloë* infected grasses suggest that asexual *Epichloë* endophytes are vertically transmitted through seed and are not horizontally transferred by pollen to new host plants. However, no study has quantified the potential for *Epichloë* transmission *via* pollen. We studied the incidence of *Epichloë* DNA detection in pooled pollen samples collected from subsets of 1680 *Lolium perenne* plants undergoing seed multiplications in a containment glasshouse. Most plants were infected with one of several *Epichloë* strains, representing different species of *Epichloë*. These data were compared with the incidence of *Epichloë* DNA in anthers, pseudostem and seed with known infection status. Sensitive qualitative and quantification assays, high resolution melting analysis and digital droplet PCR were employed to detect and/or quantify either *Epichloë* or *Lolium* DNA within plant-host tissues. *Epichloë* DNA sequences were not detected in any pollen samples tested. *Epichloë* is therefore unlikely to be spread between plants *via* pollen. In countries such as New Zealand, where genetically modified organisms are regulated, risks of potential spread into the environment associated with maternal compared to paternal transmission may affect regulatory decisions regarding the use of such *Epichloë*-grass symbioses outside of physical containment facilities.

Keywords: digital droplet PCR, environmental spread, fungal quantification, horizontal transfer, *Lolium perenne*

Introduction

Grass plants are primarily pollinated through wind-blown pollen, and produce thousands of tiny (25 – 60 µm) pollen grains in their male reproductive structures (anthers), which are released during dehiscence. Of the pollen released, a relatively small proportion (5 – 20%) of the airborne particles will reach another plant depending on a number of factors affecting the efficiency and aerodynamics of pollination (Cresswell et al. 2010). Nonetheless this seems to be sufficient to ensure the continuation of grass species and their associated microorganisms, e.g. the species-specific pollen-associated microbiomes comprising bacteria and fungi that have been described for timothy grass (*Phleum pratense* L.) (Obersteiner et al. 2016), and for other grass species wind borne pollen-associated viruses (Fetters & Ashman 2023). It could be assumed that all endophytic microorganisms intimately associated with floral tissues of their grass hosts may be pollen transmitted. However, the Pooideae-infecting endosymbiotic *Epichloë* fungi that reside in the intercellular spaces and appear physically connected to host cell walls in foliar tissues, do not appear to be transmitted horizontally *via* pollen. This is supported by years of monitoring in commercial breeding programmes, plus a few reports of microscopic examinations suggesting that the endophytic hyphae are not associated with pollen (Majewska-Sawka & Nakashima 2004; Liu et al. 2017). No enumeration of this phenomenon has been assessed due to insufficient technology to aid in the measurements until now.

Traditional methods for isolating fungi from tissues and conducting biological assays have practical limitations in terms of time efficiency, sensitivity, and statistical robustness and reproducibility. Quantitative polymerase chain reaction (qPCR) is highly sensitive, relatively easy to perform and reproducible at scale. Endpoint analyses can aid in distinguishing sequence variability between strains, such as in the high resolution melting (HRM) technique (Er & Chang 2012). The development of the molecular technique digital droplet PCR (ddPCR™) additionally offers statistically robust

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calibration curve-independent absolute quantification of DNA, enabled through the partitioning of a single PCR reaction into thousands of droplet reactions and using established statistical models (Taylor et al. 2017; Whale et al. 2017). ddPCR is increasingly being utilised to quantify target nucleic acids in microbes, including detection of plant pathogens (del Pilar Martínez-Diz et al. 2020; Ren et al. 2022; Amoia et al. 2023). The strengths of ddPCR, i.e. high sensitivity and precision quantification at low input copy number in complex samples (such as fungi in plants), make the method ideally suited to investigating the transfer of endophytic DNA between plants.

Epichloë presence significantly enhances the resilience of the grass against various stresses, thereby improving its persistence and overall quality as forage (Johnson et al. 2013). However, while these natural associations are beneficial, they can also pose risks, particularly when toxic alkaloids are synthesised by endophyte strains that are potentially harmful to grazing animals. To mitigate this, efforts are underway to create solutions for New Zealand farming by using genetic technologies to produce *Epichloë* strains that are animal safe, whilst retaining the resilience against insect predation (Johnson et al. 2021). Meanwhile, New Zealand's geographical isolation in the south Pacific Ocean has produced unique biodiversity of flora and fauna and is strongly protected by various environmental standards (www.epa.govt.nz). Consequently, organisms new to New Zealand, or those created through genetic technologies, must undergo rigorous assessment to understand their mechanisms of propagation, and potential to initiate establishment of unwanted populations in the environment.

Gene-edited *Epichloë* endophytes, tailored to enhance traits in non-modified ryegrass hosts, require outdoor testing to assess the efficacy of the strain modifications with respect to New Zealand pasture challenges (e.g., damage by pest insects). In New Zealand, these symbioses are considered to be genetically modified organisms and thus far have been propagated indoors under containment restrictions. Specialised pollen filters enable open flowering in containment, together with auditable controls to prevent accidental spread from containment enclosures. This report presents the evaluation of ryegrass (pollen) and *Epichloë* DNA in samples collected from pollen clouds of plants undergoing seed production in containment. The study aims to assess the potential for release of ryegrass symbioses comprising genetically modified *Epichloë* endophytes into the environment *via* a pollen vector.

Materials and Methods

Biological materials

Strains of *Epichloë* species and subspecies, *Epichloë* sp. LpTG-3 (AR37), *Epichloë festucae* var. *lolii* (AR1 and AR5) and *Epichloë hybrida* (AR6), including gene-edited strain variants as described in Miller et al. (2022) and unmodified wild type strains were used in this study. Perennial ryegrass (*Lolium perenne* L), cultivar Reason, had been infected with the strains in the period 2021 to 2023 using standard procedures (Latch & Christensen 1985). Uninfected (E-) plants were included as control plants. All ryegrass-endophyte associations were healthy and without abnormal host or endophyte phenotypes. Plants were maintained as mature plants in proprietary potting mixture (Daltons potting mix, Hamilton, New Zealand) in PB3/4 (0.6 L) bags and grown in ambient glasshouse conditions (Grasslands Research Centre, Palmerston North, New Zealand). The glasshouse is rated at Physical Containment Level 2 (PC2+) under Australia/New Zealand Plant Standards (AS/NZS2243.3) with rooms fitted with pollen trapping filters to enable open air pollination without environmental release. For seed multiplication, a mature plant was divided into ten replicates and potted into fresh potting mix in PB3/4 bags and grown for at least eight weeks. At this checkpoint (designated Set A, Table 1), at least one tiller per plant was harvested and subjected to immunoblot assay and analytical chemistry to check infection status and for the expected alkaloid chemical profile as previously described (Simpson et al. 2012; Miller et al. 2022).

Seed multiplications in a containment glasshouse

Multiple rounds of seed production were conducted in containment over the 2021 – 2023 period under the approval code EPA (ERMA) APP203832 (GMD102663), BSO/DFO approved risk assessment 2021-11 for *Epichloë* species organism. Generally, in July the appropriately (un)infected plants were subjected to vernalisation in a controlled growth room for 11 weeks at a constant 7°C, 8 hour daylength at 150 – 200 lumens. Thereafter, conditions were adjusted to a 14°C, 10 hour daylength at 150 – 200 lumens for 7 days. Plants were then divided into two PB2 (~1.2 L) planter bags and placed in their final position, on a randomised grid for open flowering. For example, in 2023 there were 1680 plants (140 host genotype associations), representing clones of associations including 13 strains from four *Epichloë* species and E- plants which were spread over 12 benches. Rapid flowering inductive conditions were used, i.e., 23 hour daylength achieved through supplemented light (Grolux® SHP lamps, Sylvania, Global) in addition to

Table 1 Overview of collections and methods used to detect *Epichloë* in cultivar Reason host plant tissues over three plant generations

Set	Generation	Location	Tissue type	Year sampled	Method(s)
A	1	NZL	Tillers	2021 – 2024	HRM, ddPCR, Blot, Chem
B	1	NZL	Pollen & Anthers	2023	ddPCR
C	2	NZL	Seed	2021 – 2024	HRM, ddPCR, Chem
D	2	NZL	Tillers	2023	HRM, ddPCR, Blot
E	2	AUS	Tillers	2023	HRM, ddPCR, Blot
F	3	AUS	Seed	2024	HRM, ddPCR
G	3	NZL	Tillers	2024	Blot

Abbreviations: NZL, New Zealand; AUS, Australia; Blot, immunoblot assay; Chem, analytical chemistry (*Epichloë* alkaloids); ddPCR, digital droplet PCR assay; HRM, high resolution melting assay

ambient glasshouse settings. Supplemental plant feed, (Thrive Fruit and Flower, Yates New Zealand, New Zealand) was applied according to the manufacturer's recommendations at four and seven weeks post transplanting. Plants were grown for nine weeks during which time they flowered (November) and were wind pollinated, aided by airflow created by glasshouse fans. Pollen and anthers were sampled during this period (Set B, Table 1) as described below. Following pollination (December), flower heads of plants were enclosed in custom designed nylon netting sleeve bags to contain and collect seed over the next four weeks. Thereafter matured seedheads were harvested (in January) and dried in an oven (Model BD260, Binder GmbH, Germany) at 28°C for one week. Generation 2 seeds (Set C, Table 1) were threshed and debris cleaned away (South Dakota seed blower tubes & cups, Seedburo Equipment Company LLC, USA).

Seed multiplications in the field (Australia)

To allow assessment of *Epichloë* incidence across three generations of plants we monitored the endophyte status of plants in field multiplications of second-generation seed from Set C. In some Australian states, gene-edited organisms generated *via* site directed nuclease type 1 (SDN-1) methods that do not use external DNA repair templates are not considered to be genetically modified, and therefore are not regulated, and can be grown outdoors without containment measures. Seeds (23 lines, including E-) from plants infected with SDN-1 gene-edited *Epichloë* strains expressing the expected molecular and chemical alkaloid profiles were collected from plants in containment in New Zealand and later used in 2023 for seed multiplications. The multiplications were achieved through individual spaced plants that were grown in multiple, duplicate field plots at Ballarat, (Victoria Australia, DLF Seeds). Prior to flowering identity checks were performed to ensure all pollen donors were from the 23 lines. For this test, vegetative tillers were collected (Oct 2023, AUS Set E, Table 1), freeze dried and sent to Grasslands for HRM and ddPCR. The tests occasionally identified volunteer plants (i.e., weeds) from the environment, and these were removed before flowering commenced.

Third generation seeds were collected in pools of multiple plants from Dec 2023 – Jan 2024. Subsamples (Set F Table 1) of seed from each line were tested as described above to confirm sample identity and genetic homogeneity before bulking all seed from each line.

***Epichloë* detection in seeds**

Seeds (96 for each of 24 lines) were sown in soil and grown under ambient glasshouse conditions (March 2024, NZL Set D and Set G, Table 1). From six weeks, the plants were subjected to immunoblot detection of *Epichloë* as previously described (Miller et al. 2022).

Pollen and anther collection

Pollen was collected when available from mid to late November 2023 (Set B, Table 1) using a small vacuum apparatus connected to a layer of filters (Figure 1). The vacuum apparatus (Bestway airbed AC/DC pump 12V battery powered, Bestway Global Holding Inc., China) was modified by installing pollen trap filters. To facilitate installation the vacuum was turned on and filter layers inserted beginning with a disc of miracloth (22 – 25 µm, MilliporeSigma, Merck KGaA, USA) followed by three 50 mL cell strainer filters, two with 40 µm and one with 70 µm pore sizes. Strainers were modified by trimming off the side walls to fit all three filters within the vacuum port. Finally, a 100 µm pore mesh bag was placed over the vacuum port outlet to act as a prefilter to prevent larger debris from entering the filtration unit.

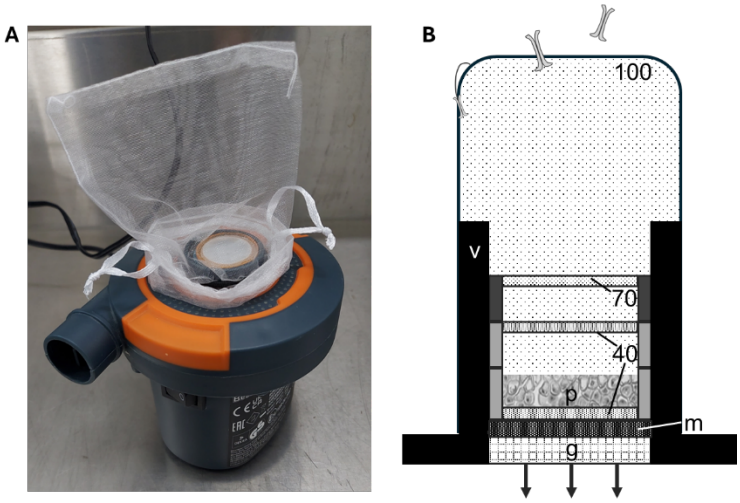

Figure 1 A. Pollen collection apparatus with filters installed. B. Schematic layout and orientation of filters (miracloth m, 40, 70 and 100 μm pore size) in the apparatus. Arrows indicate the path of the vacuum in the vacuum port (v) through the grate (g). Pollen (p) accumulates between the two 40 μm filters. Note that the bottom 40 μM filter was underlaid with miracloth (22 – 25 μm) to prevent pollen escaping from the apparatus.

An overview of pollen collection is given in Table 2. We avoided moving or disturbing plants to minimize pollen loss. Consequently, only plants within one arm's reach were sampled (1 – 2 plants deep on accessible sides). To collect pollen, the stems below flowering heads were gently tapped to release the pollen, and the cloud was sucked directly into the pollen collector. For each collection date, harvesting began from bench 1 to 12 and the number of tubes of pollen collected each day was according to the availability of pollen, for example bench 9 – 12 were delayed in pollen production and were harvested from the second collection (Table 2). The amount of pollen collected into each tube was determined as when the pollen mass became visible at the top of the 40 µm filter. For disassembly, the vacuum remained on until the two outer filters had been cleared of debris using the air outlet port of a second vacuum unit (without filters) to carefully blow away debris that had bound to the outside of the apparatus. During this process the filter stack was held in place with the thumb of the other hand. When cleaned, the prefilter mesh bag was removed and smaller debris cleared from the 70 µm filter by blowing and both vacuums were then turned off. The pollen between the two 40 µm filters was dispensed onto weigh paper, checked for non-pollen material and transferred to microtubes for storage at -20°C until required for genomic DNA extraction (~2 weeks). Pollen samples 4 and 7 were contaminated with non-pollen plant tissue and have been included as expected *Epichloë* positive samples.

To compare the incidence of *Epichloë* in anthers, 20 – 30 anthers plus filaments were harvested from 16 individual plants, 12 E+ and 4 E- plants (Set D, Table 1) and stored as described for pollen above.

Detection of endophyte by molecular techniques

Extraction of genomic DNA

For Sets A, C, D, E (Table 1), genomic DNA was extracted from (a single) seed or two 2 mm tiller stem segments by a commercial facility (Slip-Stream Automation, Palmerston North NZ) using their standard plate-based extraction method and diluted 1:10 with nuclease-free TE (10 mM Tris-Cl, 1 M EDTA, pH 8.0) (Ghanizadeh et al. 2024). These DNA samples were used as templates for HRM analysis and ddPCR assays. For Set B, genomic DNA for ddPCR was extracted using a slight modification of the Qiagen DNeasy Plant Pro Kit (Qiagen NV, Germany) method. Pollen grains (15 ± 4 mg) or anthers (15 – 20 anthers) were collected at the bottom of a microfuge tube by centrifugation at 10,621 *g* (1 min). Samples were disrupted using metal beads (2 × 3 mm diameter) in the proprietary lysis buffer and a Bead Ruptor 24 bead mill homogeniser, (Omni international Inc., USA) set to four 20 second cycles at 4 m/s with a 10 second dwell time. The lysate was further processed according to the manufacturer's instructions, and the resulting DNA was quantified using a Qubit spectrophotometer (Thermo Fisher Scientific).

High-resolution melting (HRM) assay

HRM assays were performed at Slipstream Automation

Table 2 Overview of pollen sampling from *L. perenne* plants

Date	No. of plants	Tube	Bench number											
Nov 2023		No.	1	2	3	4	5	6	7	8	9	10	11	12
16	518	1	x	x	x	x	x							
	238	2						x	x	x				
	580	3, 4*	x	x	x	x	x	x						
	228	5									x	x		
20	580	6	x	x	x	x	x	x						
	580	7*							x	x	x	x	x	x
25	870	8	x	x	x	x	x	x	x	x	x			
	290	9										x	x	x
27	290	10	x	x	x									
	290	11				x	x	x						
	290	12							x	x	x			
	290	13										x	x	x
30	290	14	x	x	x									
	290	15				x	x	x						
	290	16							x	x	x			
	290	17										x	x	x
	290	18										x	x	x

Date of collection (Nov. 2023) with continuous line separating each collection date. The maximum possible number of pollen donor plants (No. of plants) that were accessible for pollen extraction into tubes (Tube No.). The 'x's denote benches where plant pollen was collected. Most benches had two long sides accessible, excepting benches 1, 6, 7 and 12 having one. *Tubes 4 and 7 were noted to have debris associated with the sample.

as described previously (Ghanizadeh et al. 2024) employing *Epichloë* strain-specific primer pairs designed to identify strains of *Epichloë* using the LightCycler® 480 II system (Roche NZ, New Zealand). The HRM dissociation curve obtained in response to increasing temperature enabled both the presence and identity of the endophyte strain to be confirmed (Johnson & Voisey 2017). The cycle threshold value (Ct) was used as a proxy for *Epichloë* DNA concentration and the volume was adjusted to fit within the range for efficient PCR amplification using ddPCR.

Digital droplet PCR (ddPCR)

Specific primers and probes were designed against conserved *Epichloë* DNA sequences within *E. festucae* GenBank accession CP031385.1) and from the Leafy gene of *L. perenne* (GenBank accession NC_067245.1). To enhance the specificity of the multiplex assays, primers and probes incorporated LNAs, locked nucleic acids (indicated by a plus (+) sign in the sequences below); a methylene bridge which locks the ribose ring (between 2'-oxygen and the 4'-carbon) conformation for optimal binding to complementary nucleic acids. These primers and probes were developed for use with *Epichloë* species: EfM3.027630_F (5′-GTG +CTT +GAT AAC +GAC ATT GC-3′), and EfM3.027630_R (5′-GAT +GTG AGA ACC GGG +AGA AG-3′), that amplify a 127 bp PCR fragment, and the EfM3.027630 locked probe (/56-FAM/TGG+CGCTCC+GTTTCACCC/3IABkFQ/). The probe included a 5′-attached FAM fluorophore, which emits green fluorescence when excited and it is used to detect the presence of target sequence after probe cleavage and a 3′-attached Iowa Black® FQ dark quencher to quench the fluorescence from FAM until the probe is cleaved during PCR, allowing signal detection only when the target is present. The primers Leafy_F (5′-GGACTACCTGTTCCATCT-3′) and Leafy_R (5′-CTTGGTTGGAGACTTGTG-3′), that amplify a 94 bp PCR fragment, and Leafy_Probe (/5HEX/AGTCCATGG/ZEN/CCAAGCTGCAT/3IABkFQ/) were developed for *L. perenne* species. This latter primer pair served as a calibrator for plant tissue. The probe included a 5′ HEX fluorophore (yellow-green fluorescence), and quenchers (internal = ZEN and 3′ 3IABkFQ) for improved sensitivity and specificity.

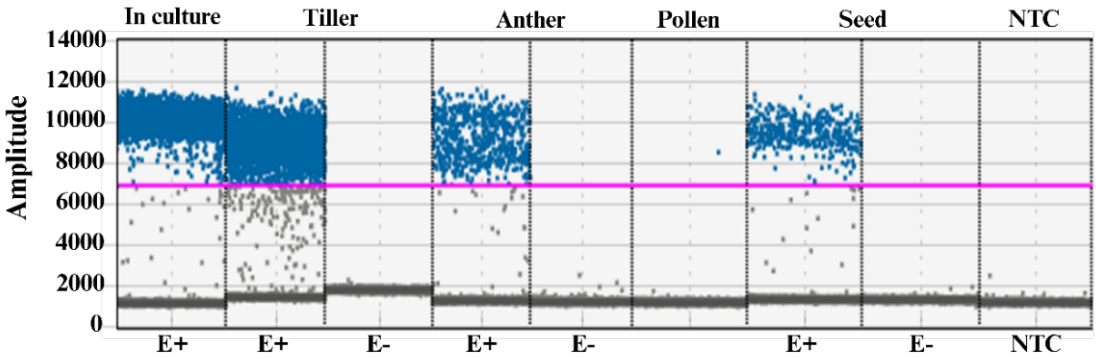

Figure 2 ***Epichloë*** **presence in representative paternal plant tissues using ddPCR.** A one-dimensional droplet digital PCR (ddPCR) plot showing fluorescence amplitude of PCR amplification in representative examples of fungal genomes derived from cultured cells, and infected (E+) and uninfected (E-) ryegrass host plant tissues: tiller, anther, pollen, and seed tissues. A threshold is marked by the continuous 'pink' line crossing the Amplitude axis at around 6,800 separates PCR amplification positive droplets (above) from negative droplets (below). No Template Control (NTC) is included as a reference blank.

Primers and probes were obtained from Integrated DNA Technologies (Republic of Singapore).

The study used Bio-Rad's QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories, USA). The ddPCR reactions were prepared following manufacturer instructions for the ddPCR supermix for probes (Cat#1863023), typically using 100 – 150 ng of DNA in 3 µL of template DNA, and 10 – 40 ng of DNA that was processed as part of the HRM procedure. Droplet generation was conducted using a Bio-Rad Auto DG system. Thermocycling parameters were as directed, including an optimised annealing/extension temperature of 60°C for 60 s (ramp rate of 2°C/s). Post-PCR, the plate was analysed in the Bio-Rad QX200™ droplet reader device, with fluorescence of each droplet automatically counted. Data analysis was performed using the Bio-Rad QuantaSoft analysis software version 2.1.0.

The threshold amplitude set at 6,800 for ddPCR analysis was determined using the definetherain program (Jones et al. 2014), accessible through a web browser (https://definetherain.org.uk/) (Figure 2).

To assess the reproducibility and evaluate the dynamic range of the assay and assay limits, DNA extracted from the pure fungal culture (*E. festucae*, AR37) was spiked with DNA isolated from uninfected *L. perenne* plants (from Set A) and used as templates for a dilution series (5-fold for seven dilution points), beginning at 1 ng and 100 ng plant, respectively. For high and low concentration samples three and six replicated measurements were used to generate a calibration curve. Several nucleotide-free water replicates were included in all experiments, all returning 0 target copies/20 µL reaction. The limit of the blank (LoB) for the *Epichloë* targets was determined considering the background of the host plants. Using the DNA derived from 123 E- tissues using the formula LoB = $mean_{blank}$ + 1.645(SD_{blank}) (Armbruster & Pry 2008). This calculated 2.6 targets per reaction and agreed with the 95th percentile value. The limit of detection (LoD) was calculated using LoD = LoB + 1.645 (SD low concentration sample) as 20 copies/reaction (i.e., 1 copy / µL) using low concentration standards that were used to generate the calibration curve to calculate the SD. The limit of quantification (LoQ) was taken as the value where the coefficient of the variable (CV) moved below 30% on the calibration curve (32 copies/reaction, i.e.,1.6 copies / µL).

Results

We sought evidence for an association between ryegrass pollen and endosymbiotic *Epichloë* using high throughput genetic methods (ddPCR and HRM) to detect and quantify significantly present *Epichloë* DNA. We chose to use ddPCR for quantification because it is considered as the current gold standard for when looking for low concentration DNA (Table 3). For each reaction, a minimum of 10,000 droplets was considered an acceptable outcome, with 19,900 droplets obtained on average per sample. Analysis of the ddPCR output from 18 pollen-derived DNA samples did not detect fluorescence associated with *Epichloë* DNA in the 115 observations derived from 16 "clean" DNA samples (where no plant debris contamination was apparent at the time of collection). As the sample size did not meet the minimum threshold required for reliable inference, statistical analysis was not conducted. There were two samples (Sample 4 & 7) that were noticeably contaminated with debris before preparation of DNA, and these observations yielded detectable amounts of

Table 3 Average concentration of *Epichloë* .and *L. perenne* ddPCR amplicons in DNA from *L. perenne* pollen (Set B)

DNA sample	No. of ddPCR reactions	Total number of positive droplets (plant and endophyte)	Total number of droplets counted	Average copies DNA / µL (ddPCR)	
Sample	n			*Epichloë*	*Lolium*
1	5	60,507	91,481	<LoD	1,180 ± 430
2	7	101,218	140,447	<LoD	1,630 ± 300
3	7	105,635	144,293	<LoD	1,690 ± 280
4*	7	102,049	137,678	1.64 ± 0.31	1,730 ± 270
5	9	143,126	179,873	<LoD	2,050 ± 320
6	7	110,219	140,009	<LoD	1,990 ± 310
7*	7	105,763	139,828	<LoQ	1,810 ± 280
8	7	107,098	135,482	<LoD	2,000 ± 280
9	7	113,555	140,594	<LoD	2,100 ± 350
10	7	106,505	140,101	<LoD	1,830 ± 250
11	7	107,693	144,210	<LoD	1,780 ± 330
12	7	112,029	140,112	<LoD	2,050 ± 270
13	9	130,931	171,588	<LoD	1,830 ± 310
14	7	92,524	140,992	<LoD	1,370 ± 230
15	7	112,084	138,736	<LoD	2,110 ± 270
16	7	108,142	139,795	<LoD	1,910 ± 280
17	7	113,767	144,664	<LoD	1,980 ± 280
18	7	105,862	143,378	<LoD	1,710 ± 250

DNA derived from 18 pollen isolations that were subjected to several technical repetitions (n) of ddPCR. The corresponding LoD and LoQ estimates for the *Epichloë* ssp. values are 1.0 and 1.6. *Pollen samples 4 and 7 were contaminated with non-pollen plant tissue.

Table 4 Summary of ddPCR quantification of four different plant tissues expressed as the average DNA concentration in copies/µL ± standard deviation of *Epichloë* and *Lolium* PCR products. (Sets B – D)

Tissue	Set, status	No. of plants	No. of reaction	*Epichloë (E)*	*Lolium (L)*	Ratio L/E
Anther	B, E-	10	10	<LoD	1,325 ± 260	2650 ± 520
Anther	B, E+	7	25	12 ± 29	1,454 ± 465	286 ± 225
Pollen	B, E+/E-	18	121	<LoD	1,814 ± 369	3510 ± 870
Seed	C, E-	35	35	<LoD	124 ± 82	248 ± 164
Seed	C, E+	3	3	112 ± 87	143 ± 97	1.4 ± 0.24
Tiller	D NZL, E-	12	18	<LoD	1,698 ± 511	3390 ± 840
Tiller	D NZL, E+	10	18	1,431 ± 1,963	1,234 ± 602	2.6 ± 2.1

E+ and E- denote *Epichloë* infected or free samples. The LoD for the *Epichloë* copies/µL values are 1.0 and LoQ =1.6. L/E Ratios were calculated for each association by dividing copies/***µL*** for *Lolium* by that of *Epichloë* and substituting values with <LoD or <LoQ for LoD/2 or LoQ/2 respectively and the average ratio given in the table. Error is SD.

Epichloë DNA as expected, and with four from seven sample 4 replicate observations above background but at very low levels (Table 3).

We also compared ddPCR results of the pollen with DNA samples from other available tissues of known infection status, i.e., anthers, seed and vegetative tillers (Table 4). High standard deviation values suggest that the concentration of *Epichloë vs*. *Lolium* can vary considerably for any given infected tissue. To approximate the relative amounts of *Lolium* to

Table 5 Incidence of *Epichloë* .in subsets of *L. perenne* cv. Reason generation 2 plants (Sep 2023) and generation 3 seedlings grown from seed multiplications (Jan 2024)

	Set D, E	**Sets C, F**	**Sets C, F**	**Set G**
	G2	**G3**	**G3**	**G3**
	Tillers	Seed	Seed	Tiller
Strain	HRM & ddPCR	HRM	ddPCR*	IB
Infected	1,380 (100%)	1,281 (89%)	207 (100%)	942 (95%)
AR1	45	50	11	63
AR37	909	775	118	562
AR5	238	246	40	137
AR6	188	210	38	182
Uninfected	74 (100%)	92 (100%)	14 (100%)	80 (100%)
Total	**1,454**	**1,373**	**359**	**942**

*ddPCR was used to spot check the results in a subset of the G3 samples used for HRM analysis. Abbreviations: G2 and G3 are generation 2 and 3; IB, Tiller immunoblot detection assay

Epichloë cells, we estimated the range of *Lolium*/*Epichloë* (L/E) concentration ratios for each tissue. For samples where *Epichloë* DNA concentrations were too low to be measured, a substituted value of half the LoD or LoQ were used to enable the L/E calculation. Very high relative L/E ratios in the E- samples and pollen are in keeping with non-detectable levels of endophyte. In contrast, lower L/E values in seeds and tillers suggest highest levels of endophyte are found in these tissues. In contrast, 1 – 2 orders of magnitude less *Epichloë* was observed in anther tissue, which suggests diminishing association of *Epichloë* with anthers.

Examining infection across generations

To investigate the stability of endophyte associations, as part of the seed multiplication process, the infection status of the plants in Sets C – E were examined using HRM and ddPCR (Table 5). HRM data were generated *via* a high throughput semi-automated process whereby genomic DNA, purified from a seed or tiller segment, was subjected to qPCR and high-resolution melting. Through this method, *Epichloë* strain identification was enabled by using strain specific primer pairs to amplify target DNA and distinct end point high resolution melting analysis. The same DNA was also subjected to precision testing by ddPCR, which by design, can also determine specific gene edit events in addition to strain detection. The results of both tests agreed, with all uninfected *L. perenne* plants in generation 1 remaining E- across multiple generations, and for infected plants, >89% remained so (Table 5 from set E to F).

Similarly, the seed from third-generation plants was checked for infection status, and viability, through tissue print immunoblotting of 96 seedlings for each of the 23 lines from Set D NZL and Set G. The results were as expected, with >95% infection confirmed in endophyte-infected plants and 0% of E- plants testing positive on the immunoblots.

Discussion

With respect to *Epichloë* and grass species, Sampson, 1933 states "It is not impossible that pollen might carry fragments of mycelium, since this is sometimes present in anthers which produce what seems to be normal pollen, but it is highly improbable that this method of transmission occurs frequently, and it has not yet been proved." By using the highly robust molecular detection and quantitative methods of HRM and ddPCR and taking advantage of the large numbers of available symbioses in seed multiplication pipelines across three generations, this study supports Sampson's assertions as there was no evidence of an association between asexual *Epichloë* pasture endophytes and ryegrass host pollen. The negative findings from both methods were in agreement, thus supporting the hypothesis generated through previous published and unpublished observations (Majewska-Sawka & Nakashima 2004; Liu et al. 2017). The results are likely to be similar for other asexual *Epichloë* strains given their strong reliance on host flowering for vertical transmission through seed. However, for plants infected with sexual species of *Epichloë* that can produce ascospores, further analysis should be undertaken. Considering the relative ease and much lower cost of high throughput HRM, this method would be sufficient to confirm E- status with confidence, as established in this study using ddPCR.

The methods described are also useful for evaluating the physical relationship between endosymbiont and distinct host tissues, here enumerating the diminishing association of the endophyte with developing male reproductive tissues, which agrees with previous observations (Sampson 1933; Liu et al. 2017). *Epichloë* endophyte concentrations are generally highest in the base of tillers (Christensen & Voisey 2006), and that this can be highly variable, and these are reflected in the high average copies of fungus in the tiller stems, very high SD and low L/E ratio. The low L/E ratios of endophyte to plant in seeds, indicating relatively high mycelial infection reflects both transmitted embryo-associated hyphae and hyphae fixed between the aleurone and pericarp layers (Zhang et al. 2017).

This study was constrained by the necessary regulatory controls requiring containment of pollen collections and associated equipment entirely within one pollen-proof room. This requirement along with airflow from cooling fans moving pollen, anthers and small plant remnants presented an issue with managing a cross-contamination risk during sample collection. However, considering endophyte DNA could not be detected in almost all samples, the filter system in the pollen collection apparatus seems to have been sufficient to exclude most debris. Contamination was also a risk during transfer of the pollen pool from weigh paper to tubes, and in two instances these events were directly observed (Sample 4 and 7). In future a small area in the room that is sheltered from the air borne material would facilitate a clean collection. Further evidence that the findings result from contamination, and not infection, come from observing that replicate samplings of the same benches on different days were also below the limit of detection (Table 2).

Practical implications

The mechanism of endophyte transmission in plant reproductive tissues has practical implications for the spread of endophytes beyond agricultural land and is particularly relevant for ryegrass symbioses containing gene-edited or genetically modified *Epichloë* strains. Since there does not appear to be an association of *Epichlo*ë with the pollen and considering that only a small proportion of released pollen successfully completes pollination in the first instance (Cresswell et al. 2010), it is unlikely that a self-sustaining population could be formed by this route. To date, the establishment of systemic foliar symbioses requires careful inoculation of seedling meristematic tissues with a scalpel and guided by a microscope. Horizontal transmission of viable vegetative conidia or hyphal mycelium between plants has not been observed under controlled conditions and is thought to be a rare event in nature. As *Epichloë* fungi also do not infect neighbouring plants through root systems, the highest likelihood of forming a self-sustaining population is therefore *via* the seeds.

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