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Assessing Endophyte Frequency Distributions and the Effect of *Epichloë brachyelytri* in the Chemotypic and Genotypic Diversity of *Brachyelytrum erectum*

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ASSESSING ENDOPHYTE FREQUENCY DISTRIBUTIONS AND THE EFFECT OF *EPICHLOË BRACHYELYTRI* IN THE CHEMOTYPIC AND GENOTYPIC DIVERSITY OF *BRACHYELYTRUM ERECTUM*

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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ABSTRACT OF THESIS

ASSESSING ENDOPHYTE FREQUENCY DISTRIBUTIONS AND THE EFFECT OF *EPICHLOË BRACHYELYTRI* IN THE CHEMOTYPIC AND GENOTYPIC DIVERSITY OF *BRACHYELYTRUM ERECTUM*

Seed-transmissible epichloid fungal endophytes are best known for their roles as defensive mutualists in cool-season grasses. Historically, the discovery of fungal endophytes was driven by investigations of plant toxicity to livestock, followed by extensive study of their alkaloids and protection against insects and nematodes. Epichloae can produce four classes of alkaloids: ergot alkaloids, lolines (saturated aminopyrrolizidines), indole–diterpenes, and peramine. It is increasingly evident that these hereditary symbionts have much more diverse chemical profiles both in individual populations and between them. To this end, differences in chemotypic profiles of these symbionts may translate to different evolutionary and environmental advantages across plant species, as well as influences on phenotypic measurements. In an ecological sense, the chemotypic diversity within the species may reflect frequency-dependent selection for the alkaloids, which can be metabolically expensive to produce. To date, there has yet to be extensive study on the alkaloid profiles of the fungal symbiont *Epichloë brachyelytri* in the cool-grass host *Brachyelytrum erectum*. This project aims to initiate the required genomic population analyses to comprehend endophyte frequency and chemotypic diversity among and between populations throughout the state of Kentucky.
In a previous survey of *B. erectum* in the Kentucky Palisades, 50% of the plants had fungal endophytes, with two endophyte genotypes that differed in alkaloid profiles. In the investigation, 21 populations of *B. erectum* were sampled and the genetic and chemotypic diversity analyzed by high-throughput tiller extraction and multiplex PCR. Biosynthesis genes for ergot alkaloids (e.g., chanoclavine and ergovaline), aminopyrrolizidines (e.g., lolines), and pyrrolopyrazines (e.g., peramine), as well as genes for mating type and phylogenetic barcodes, were interrogated to determine endophyte presence and diversity. Morphological measures for all samples were also assessed, and six populations were sampled for transcriptome analysis. The results of these studies will expand the understanding of how endophyte frequency distributions and genetic diversity can influence functional and phylogenetic diversity.

KEYWORDS: Endophyte, Chemotypic Diversity, Alkaloid, Frequency

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11/18/2023

Date

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1.1 ENDOPHYTIC LIFE

The endophytic lifestyle is extremely common for a highly diverse range of microorganisms, most commonly fungi or bacteria. Through antagonistic or mutualistic relationships, endophytes reside within plant tissue and usually do not cause disease symptoms. Endophytes are of particular interest due to their production of influential secondary metabolites or hormones, which can have critical effects on plant growth. Of the different classes of endophyte (described in Rodriguez et al., 2009), the fungal family Clavicipitaceae (phylum Ascomycota, order Hypocreales) contains the widest studied genera of symbiotic endophytes for their mutual association in cool season grasses (family Poaceae, subfamily Poöideae; Blankenship, 2004; Schardl et al., 2004). The fungal genus *Epichloë*, previously separated into *Epichloë* and *Neotyphodium* spp., produce a range of anti-herbivore alkaloids and can reproduce both sexually and asexually (Schardl, 2012). This dual reproductive ability, and more specifically, the nature of their interactions with flowering tillers, is also a major determinant in the type of association *Epichloë* may have with its host.

When reproducing asexually, *Epichloë* will asymptomatically colonize seed embryos and transmit vertically when seeds are dispersed, making for a more mutualistic symbiosis (Schardl et al., 2004; Schardl, 2012). In the asexual cycle, the fungus remains completely inside the host plant throughout the season, beginning with hyphal entrance into developing ovules through flowering (Liu et al., 2017; Zhang et al., 2017). No sexual spores or external fungal structures are involved in vertical transmission. When reproducing sexually, *Epichloë* produce stromata (a sheath of mycelia) surrounding immature inflorescence and carrying spermatic spores (conidia)

that mediate cross-fertilization and, perhaps in some cases, horizontal transmission (Berry et al., 2022; Blankenship, 2004; Schardl, 2012; Tadych et al., 2007). Production of these stromata is otherwise known as choke disease, and may completely suppress maturation of the tiller, thereby halting seed production. Sexual fertilization only occurs if the conidia from one mating type are transferred to the conidial stroma of the opposite mating type, which then forms perithecia that contain ascospores (Schardl et al., 2004). The sexually derived ascospores are ejected and can mediate horizontal transmission (Chung & Schardl, 1997). This fertilization process is most often carried out by *Botanophila* species of flies (Blankenship, 2004; Schardl et al., 2004). Though stromata are required for their sexual cycle, the frequency of stroma formation varies across *Epichloë* species and strains (Berry et al., 2022). This could indicate a difference in growth patterns and host transcriptome interactions specific to different *Epichloë* species. Berry et al., (2019) documented conserved transcriptional changes by a set of 135 differentially expressed fungal genes in stromatized tissues during virulent growth of sexually reproducing *Epichloë* species that suggest links to differential regulation in adapting to the external environment and stroma formation, among other associations. Though each host plant only harbors a single *Epichloë* individual, an endophyte can exhibit the sexual cycle or asexual cycle on different tillers within the same plant (Clay & Schardl, 2002; Schardl et al., 2004). The variability and apparent randomness at which an individual epichloid endophyte may cause choke disease or instead act in an inconspicuous partnership has led some to describe this relationship across a continuum of different types of mutualism, ranging from pathogenic to mutualistic (Clay & Schardl, 2002).

Over the last century, the epichloid infection and growth process has been elucidated in detail. In 1904 , Edward Freeman first accounted that hyphal entrance to the embryo occurred

long before seed maturation (cited in Schardl et al., 2004). Once entered, hyphae grow into the ovules, further proliferate in the nucellus tissues, and later colonize the embryonic axis (running the length of the embryo from the plumule to the radicle) of the developing seed (Leuchtmann, 2003). After seed germination, hyphae cease their dormancy within the intercellular spaces of the embryo and extend into meristematic cells in leaf primordia and axillary buds (Christensen et al., 2008). Endophytic growth is closely coordinated and strictly synchronized with growth of the host plants above ground tissues and usually follows distinct basal to apical hyphal concentration gradients (Schardl et al., 2004). The hyphae seem to originate from extensively branching mycelium in the basal meristems and transitions to single, thread-like hyphae in mature tissues (Schardl et al., 2004). In most other endophytic associations, infections may only consist of a handful of epidermal cells in a highly localized manner (Clay & Schardl, 2002). However, in epichloid grass systems, infections are systemic throughout all above ground organs of the hosts, with highest abundance in basal leaf meristems and leaf sheaths (Clay & Schardl, 2002; Leuchtmann, 2003). In the mature tissues, sparsely branched hyphae grow strictly intercellularly, parallel to the long axis of plant cells (Clay & Schardl, 2002; Leuchtmann, 2003). This form of intercalary growth (attaching to enlarging meristematic host cells and extending at the same rate) is rare for fungi, as typical fungal growth is thought to be hallmarked by hyphal tip extension almost exclusively (Christensen et al., 2008; Schardl et al., 2004). In contrast to many pathogenic and mycorrhizal fungi, *Epichloë* hyphae never breach the cell walls, produce haustoria, or develop arbuscules, and therefore never directly cause cell damage (except in cases of stromata production) (Clay & Schardl, 2002). Living solely in the apoplastic region, the endophyte feeds on plant extracellular substrates released into the apoplast, such as sugars and amino acids (Blankenship, 2004; Clay & Schardl, 2002; Florea, 2009).

Infections of *Epichloë* are maintained throughout the entire lifespan of the host, though occasional loss of infection in particular segments of host plants and loss of viable endophyte in previously infected seeds due to warm temperatures and high humidity have been observed (Clay & Schardl, 2002; Leuchtmann, 2003). Symbiotic endophytes, when asexually reproducing, are thought to operate under imperfect vertical transmission where the symbiont is not transmitted to all offspring and maintains an endophyte-infected population at <100% (Afkhami & Rudgers, 2008). *Epichloë* spp. are reported to undergo imperfect vertical transmission at three life-history transitions: among tillers (i.e., infected plants may produce some uninfected tillers), from tillers to seeds (i.e., the endophyte may not grow in all seeds produced by an infected tiller), and from seeds to seedlings (i.e., endophyte in seed tissue becomes inviable or unable to infect embryo) (Afkhami & Rudgers, 2008). Mathematical models of symbiosis are greatly influenced by the rate of vertical transmission, which can affect both the evolution of virulence and host-symbiont dynamics (Yamamura, 1993). Resource availability and environmental conditions may also influence endophyte expression and ecological persistence (Malinowski & Belesky, 2006). Given the range of pathogenicity that differs across species of *Epichloë,* along with physiological and ecological impacts of endophytic infection, further phylogenetic and environmental studies of symbiotic fungal endophytes have captured the interest of researchers.

1.2 ALKALOID PRESENCE AND CONTRIBUTIONS TO SYMBIOSIS

As previously mentioned, presence of *Epichloë* spp. often confers enhanced herbivory resistance for the cool-season grass hosts. These associations, based primarily on protection of the host from abiotic and biotic stressors, are unlike most other traditionally studied plant/microbe symbioses. In response to endophyte infection, numerous alkaloids (e.g., ergot

alkaloids or lolitrems) and nonalkaloid secondary metabolites (e.g., fatty acids or phenolic compounds) can be produced by either the endophytes themselves or the grass hosts (Malinowski & Belesky, 2019). Protection against herbivory, a primary biotic stressor, is associated with bioactive alkaloids produced by the endophytes that are toxic to both vertebrates and invertebrates, including aphids and nematodes. *Epichloë* species can produce four classes of alkaloids; 1-aminopyrrolizidines (including lolines), pyrrolopyrazines (including peramine), indole-diterpenes, and ergot alkaloids (Vikuk et al., 2019). All four classes are absent in grasses lacking these or related endophytes, and are reported to be produced in pure fungal culture, hence representing fungal origin of the alkaloids (Clay & Schardl, 2002). Other physiological effects such as heightened nutritional and water stress tolerance, increased biomass production, increased tillering, and root growth changes have also been studied, but are not sue to the aforementioned alkaloids (Schardl et al., 2004; Schardl, 2012). This section aims to give a brief description of the four alkaloid classes along with supporting evidence of secondary host physiological effects, both of which have been topics of research to characterize the mutualism (and cost thereof) in cool grass endophytes.

Lolines are a group of highly water soluble, saturated exo-1-aminopyrrolizidines that display broad spectrum insecticidal activity (Schardl, 2012; Clay & Schardl, 2002). Much of the loline alkaloid-biosynthetic pathway has been elucidated, with the non-subtelomeric *LOL* cluster encoded in a single locus. The single locus contains 11 genes: *lolF, lolC, lolD, lolO, lolA, lolU, lolP, lolT, lolE, lolN,* and *lolM* (Schardl, 2012; Spiering et al., 2005; Pan et al., 2014). Different lolines have distinctive substituents linked to the 1-amine (Schardl, 2012). Compared to other endophyte alkaloids, lolines act with more overt toxicity against a broader range of pests, including aphids and nematodes (Schardl et al., 2004; Schardl, 2012; Schardl et al., 2013). These

effects can occur even at low concentrations of loline, though levels can profoundly accumulate to over 2% dry weight (likely exceeding endophyte biomass) (Schardl, 2012; Schardl et al., 2004). Further investigations into accumulation of lolines uncovered particular increases in loline alkaloid production in response to mock herbivory, leaf clipping, heat, and drought (Schardl et al., 2004; Blankenship, 2004). These observations are significant in indicating alkaloid production as an inducible defense mechanism, while also remaining nontoxic to plant cells (Schardl et al., 2004). Endophytes that can produce these neurotropically active alkaloids impart a significant enhancement of competitiveness to their grass hosts. These ecological impacts leave alkaloid production as an important factor to consider in the characterization of an endophyte's diversity.

Peramine, first identified from extracts of endophyte-infected perennial ryegrass, is the primary pyrrolopyrazine alkaloid found in clavicipitaceous grass endophytes (Schardl, 2012; Clay & Schardl, 2002). Several new pyrrolopyrazine variants were discovered that indicated a surprisingly high level of secondary metabolite diversity, and methods for assessing presence of additional pyrrolopyrazines are aims for future research (Berry et al., 2019). Similar to lolines, a single locus (*PER*) is sufficient for biosynthesis of peramine (Berry et al., 2019). Additionally, peramine activity is linked to toxification or deterrence of insect feeding (Schardl et al., 2004; Clay & Schardl, 2002; Schardl, 2012; Fuchs et al., 2017). Some insects, like the major stem weevil pest in New Zealand (*Listronotus bonariensis*), are extremely sensitive to peramine (Malinowski & Belesky, 2019). Peramine and loline deterrence against aphids is important due to the consequent decrease of their vectoring of important grass pathogens like barley yellow dwarf virus (Schardl et al., 2004; Clay & Schardl, 2002; Wilkinson et al., 2000). However, lolines and peramine are not linked to any toxic symptoms in grazing mammals (Schardl et al.,

2013). Compared to other alkaloids, peramine is also more widely distributed among endophyte-infected grasses and remains relatively evenly distributed throughout the plant over the growing season (Clay & Schardl, 2002). However, peramine has also been shown to act as an inducible defense in response to locust herbivory (Fuchs et al., 2017). Because peramine and derivatives are known to be produced by many *Epichloë* species, it is important to include in preliminary detection and diversity analyses.

The last two classes associated with clavicipitaceous grass endophytes are the indole-diterpenes and ergot alkaloids. Members of both groups have potent neurotropic activities in mammals as well as insects (Schardl et al., 2004; Schardl et al., 2013). Indole-diterpenes encompass a broad range of bioactive compounds that exert their toxicity through various ion channel activation, such as calcium-activated potassium channels or glutamate-gated chloride channels (Schardl et al., 2013; Smith et al., 2000). Indole-diterpenes are primarily monitored for their tremorgenic neurotoxicity to sheep in perennial ryegrass (Clay & Schardl, 2002). Lolitrems are the most known indole-diterpene that can act against feeding vertebrates, commonly resulting in tremors or staggers (Malinowski & Belesky, 2019). Dangerous tremorgenic effects can also result from other indole-diterpenes, such as paxilline (Vikuk et al., 2019). The activity and presence of indole-diterpenes should be included in toxicity and chemotypic characterizations due to the threat posed to grazing livestock.

Ergot alkaloids are tricyclic compounds named for the ergot fungi (*Claviceps*). Ergot alkaloids are famous for historical mass human poisonings and well as agriculturally significant implications for livestock toxicosis (Schardl et al., 2013; Schardl et al., 2004). Feeding on ergot alkaloid-producing endophyte-infected grasses can result in summer slump (i.e., hyperthermia, early fetus abortion, winter coat retention) and/or fescue foot (i.e., dry gangrene of limbs)

(Malinowski & Belesky, 2019). Ergot alkaloids are also suggested to reduce some root-knot and migratory nematode densities (Malinowski & Belesky, 2019). Similar to lolines and peramine, ergot alkaloids can also represent an inducible defense response, with their levels increasing upon mock herbivory (Bazely et al., 1997; Schardl et al., 2004). Chanoclavine, a clavine-type ergot alkaloid, is an organic tricyclic compound (1,3,4,5-tetrahydrobenzo[*cd*]indole substituted at position 4 by a methylamino group and at position 5 by a 3-hydroxy-2-methylprop-1-en-1-yl group) that is the parent to other chanoclavine derivatives agroclavine, elymoclavine, festuclavine and lysergic acid amide, which in turn is linked to three amino acids in biosynthesis of the ergopeptines such as ergotamine and ergovaline (Schardl et al., 2013). After a series of toxicology analyses, chanoclavine remains unlikely to contribute to mammal toxicity and was determined to be safe for inclusion into cereal crops (Finch et al., 2019). Some endophytes express only clavine compounds, and others express the whole range of ergot-alkaloid derivatives (Finch et al., 2019). The more complex tetracyclic ergopeptines (i.e., ergotamine, ergocryptine, ergovaline, and ergobalansine) have more documented links to health effects in feeding livestock compared to chanoclavine (Schardl et al., 2013; Finch et al., 2019). Ergovaline activity is particularly observed to contribute to fescue toxicosis, especially in tall fescue (*Lolium arundinaceum)* dominant regions of the United States (Vikuk et al., 2019).

Interestingly, the specific chemical composition and concentrations vary with the endophyte-host combinations for these alkaloid classes (Clay & Schardl, 2002). The fungal genome almost entirely regulates the enzymatic pathways for alkaloid synthesis, with their profiles determined by the fungal species and strain in combination with their host species (Malinowski & Belesky, 2019). It is now well known that *Epichloë* species exhibit exceptional chemotypic diversity, with considerable variation in alkaloid profiles and concentration (Schardl

et al., 2013; Clay & Schardl, 2002). This diversity and applications to human and animal health make these four classes important to incorporate into initial chemotypic analyses.

Though feeding deterrence offers an important protection for plants from grazing animals, fungal endophytes can have secondary effects unrelated to herbivory on their host. Many of these effects can occur whether endophytes produce alkaloids or not (Clay & Schardl, 2002). As mentioned previously, avoidance of sucking insects may reduce infections of plant viruses that are vectored by those insects (Schardl et al., 2004). Protection against other plant pathogens, like increased resistance to *Rhizoctonia* seedling blight and crown rust caused by *Puccinia coronata*, is another benefit noted with endophyte infections in tall fescue (Clay & Schardl, 2002). Endophytes may also be responsible for increased drought tolerance and improved recovery following water stress by increasing cell wall elasticity and bettering turgor maintenance via altered osmotic adjustments and stomatal behaviors (Schardl et al., 2004; Clay & Schardl, 2002). Increased production of phenolic compounds and other antioxidants in endophyte-infected plants may also facilitate an increased ability of the host grass to manage oxidative stress (Malinowski & Belesky, 2019). Additional benefits found to be a result of endophyte infection include increased tolerance to low light and low soil fertility, increased biomass production, clonality, tillering, and germination success, and even the capacity to prevent soil erosion (Clay & Schardl, 2002; Schardl, 2012; Faeth & Fagan, 2002; Schardl et al., 2004). Tolerance in less fertile soil may be a result of altered fine root structure and increased root growth in endophyte infected plants that change chemical environments near the root zones and allows for better regulated nitrogen and nutrient uptake (Faeth & Fagan, 2002; Schardl et al., 2004). These physiological changes may even extend to effects in hormone production or photosynthesis.

Tall fescue endophytes were found to produce auxin, a major plant growth hormone that can influence a variety of morphological and physiological responses (Clay & Schardl, 2002). Additionally, sugar alcohols produced by the conversion of sucrose by fungal endophytes may reduce feedback inhibition during photosynthesis, thereby allowing increased growth compared to uninfected hosts (Clay & Schardl, 2002). Lastly, endophyte presence in populations may have wider ecological effects to positively influence their grass hosts. Endophyte alkaloids can alter competitive interactions via allelopathic effects, and have inhibited germination and growth of other plants in greenhouse experiments (Faeth & Fagan, 2002; Clay & Schardl, 2002). What should be mentioned, however, is the close relationship between endophyte infection and environments adverse to the specific adaption endophyte infected plants hold over non-infected hosts (e.g., E+ perennial ryegrass from dry regions showing reduced growth in wet conditions) (Malinowski & Belesky, 2006). Consequences of endophytic infections are vast and have multidirectional flows of influence, resulting in an exciting area of research both on an individual and ecological scale.

Described so far are the effects of endophyte infection that place the host as the beneficiary. It is important to consider factors that the host plant provides to the endophyte as well, as is crucial to defining a mutual symbiosis. While endophytes contribute a diverse range of protections to plants, hosts in return provide spatial structure and preservation against desiccation to the fungi. Hosts also act as a source of nutrients that pump photosynthates to their endophytic sinks. Furthermore, in the case of vertical transmission, host reproduction also disseminates the endophytes along with host seeds (Faeth & Fagan, 2002). The physiological cost of hosting an endophyte needs to be outweighed by benefits in a mutualistic relationship. Otherwise, imbalanced interactions between host and endophyte may result in antagonistic interactions,

causing disease of the host plant (Malinowski & Belesky, 2006). The cohabitation of asymptomatic endophytes and grass requires complex communication and intimate coordination of their life cycles and biochemical behaviors. This evolutionary marvel has persisted due to the selective advantage endophytic infections have on many Pooideae populations.

1.3 *BRACHYELYTRUM ERECTUM, EPICHLOË BRACHYELYTRI,* AND RECTIFYING A KNOWLEDGE GAP

One of the most intensely studied endophyte systems is that of *Lolium arundinaceum* (tall fescue) and *Epichloë coenophiala*. Many other endophytes are related to the tall fescue endophyte in cool-season grasses that share similar characteristics of systemic and pleiotropic symbiosis and bioprotection via alkaloids (Schardl & Leuchtmann, 2005). Previous work on Clavicipitaceous endophytes has mostly been applied to agronomically significant grasses (*L. arundinaceum*, *Lolium perenne* and *Lolium pratense*) for practical research into livestock toxicoses and growth ramifications attributable to endophytes (Schardl & Leuchtmann, 2005; Vikuk et al., 2019). Currently, researchers are looking towards understanding endophyte populations in native settings, including focuses on infection frequency, diversity in alkaloid profiles, and ecological impacts. Endophyte infection frequencies seem to be more variable in natural settings compared to pastoral, which suggests that endophyte expression and attributable benefits depend on environmental conditions in which the endophyte-host system was formed (Malinowski & Belesky, 2006). Projects on grass endophyte infections in native settings have been conducted in Germany, northern China and Arizona (US), which have led to increased knowledge about natural endophyte distribution as well as descriptions of new *Epichloë* species (Vikuk et al., 2019; Song et al., 2016; Saikkonen et al., 1999). As phylogenetic analyses have

advanced, characterization of new *Epichloë* species has blossomed to contain 18 haploid and 24 hybrid species (Thünen et al., 2022; Leuchtmann & Schardl, 2022; Leuchtmann et al., 2014). Mycologists and molecular biologists alike hope to continue these regional studies to elucidate more insights into the complex endophytic symbiosis and ecological impacts in native settings.

The aforementioned agricultural crops popular in endophyte studies taxonomically reside in the Poaceae subfamily Pooideae. Pooideae is the most speciose subfamily in the Poaceae and include important crops like wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and oat (*Avena sativa*), as well as pasture grasses like ryegrass (*Lolium perenne*; Zhang et al., 2022). Pooideae are renowned for their remarkable ecological dominance in temperate regions and can inhabit a large range of environments like forest understory, open grassland, temperate wetland, deserts, and even saline-alkaline areas (Zhang et al., 2022). An astounding range of habitats reflects the extensive diversification and species richness in Pooideae, particularly in transitioning to temperate and cold climates. Climate cooling was a probable trigger and driving force of Pooideae diversification throughout the Cenozoic period (Schubert et al., 2019). Grass-*Epichloë* symbioses can be found in nearly all tribes of the Pooideae, with most relationships being highly specialized to individual host species, genera, or tribes (Schardl et al., 2008). Furthermore, this high degree of specialization is associated with corresponding cladogenesis events that illustrate evolutionary codivergence between cool-season grasses and their endophytes (Schardl et al., 2008). It is suggested that this codivergence may have played a role in the habitat expansion of early Pooideae due to the mutualistic tendencies exerted by endophytic infections in response to new selection pressures (Schardl et al., 2008).

The earliest split in the phylogeny of Pooideae formed the Brachyelytreae lineage, preceding divergence of 14 other tribes (Zhang et al., 2022). Morphological, phytogeographical

and genomic evidence supports the recognition of three distinct species in *Brachyelytrum*: *B. japonicum, B. aristosum* and *B. erectum* (Saarela et al., 2003). *Brachyelytrum* can be distinguished with a combination of festucoid and bambusoid characteristics, including (among others) leaf blades that are constricted at the base, one-flowered spikelets, reduced glumes, two terminal styles, embryonic scutellar clefts, short first internodes, uniformly sized root epidermal cells and spiral phyllotaxis in the inflorescence (Saarela et al., 2003, Kellogg et al., 2013). Members of the *Brachyelytrum* genus often serve as outgroups in Pooideae and cool-season grass endophyte phylogenetic studies. If it is postulated that the symbiotic systems emerged concurrently with origin of the Pooideae, and Brachyelytreae is the sister to all other Pooideae tribes, then the endophytic relationship(s) in *Brachyelytrum* species must be among the oldest partnerships in the evolutionary history of cool-season grasses.

Brachyelytrum erectum is a native North American grass sometimes referred to as the long awned wood grass or the bearded shorthusk. *B. erectum* can serve host to *Epichloë brachyelytri*, which acts like a pleiotropic symbiont. This symbiotis is often completely asymptomatic and rarely produce choke disease (Schardl & Leuchtmann, 1999). The *B. erectum* symbioses with *E. brachyelytri* have occasionally been included in endophyte profile surveys, but have never been the focus of extensive characterization (Schardl, 2012; Schardl et al., 2013). In a multi-genome analysis of Clavicipitaceae, *E. brachyelytri* seems to have lost *LOL* genes downstream of chanoclavine (Schardl et al., 2013). As stated previously, *Epichloë* species endophytes have extensive diversity in their alkaloid profiles, mainly due to presence or absence of the alkaloid biosynthesis genes (Vikuk et al., 2019; Schardl, 2012; Schardl et al., 2013). For example, premature truncation due to a transposable element in the allele of the *ppzA* (= *perA*) gene which otherwise encodes the nonribosomal peptide synthase that catalyzes peramine

biosynthesis, was shown to result in subfunctionalization to give several novel pyrrolopyrazines (Berry et al., 2019). Diversity in secondary metabolites can also arise from gene duplication (whole-genome duplication, tandem duplication, or transposon-mediated duplication) and neofunctionalization events (e.g., *ltmE* and *ltmJ*) (Zhang et al., 2022). Gene loss events make it possible to predict synthesized alkaloids based on detection of genes encoding key pathway steps. This reasoning, paired with the knowledge gap that exists for *B. erectum*, inspired the project described in this thesis to analyze the chemotypic profile, infection frequency, and possible phenotypic consequences of *E. brachyelytri* in natural populations of *B. erectum.* This knowledge will further our understanding of endophyte dynamics in native settings and chemotypic diversity present in *Epichloë,* elucidate conceivable morphological impacts due to endophyte presence, as well as provide data for future use in transcriptional and evolutionary studies using this particular endophyte-host system.

In this thesis, surveys sampled 22 populations of *B. erectum* in diverse ecoregions in Kentucky and (for one population) Indiana. Chemotypic diversity was analyzed by high-throughput tiller extraction and multiplex PCR using methods developed by the Young group (Saha et al., 2009; Charlton et al., 2012). Biosynthesis genes for ergot alkaloids (e.g., chanoclavine and ergovaline), aminopyrrolizidines (e.g., lolines), and pyrrolopyrazines (e.g., peramine), as well as genes for mating type and phylogenetic barcodes, were interrogated to indicate endophyte presence and diversity. Morphological measures for all samples were also assessed, and six populations were sampled for transcriptome analysis. The results of these studies expand our understanding of how endophyte frequency distributions and genetic diversity can influence functional and phylogenetic diversity.

CHAPTER 2. PHENOTYPIC IMPACTS OF ENDOPHYTE INFECTION

2.1 INTRODUCTION

Enhanced growth of endophyte-infected plants has been observed in many grass-endophyte associations, independent of biotic or abiotic stresses (Clay et al., 1989; Clay & Schardl, 2002). Growth pattern differences in infected plants compared to uninfected plants include increased clonality, biomass production, and tillering, among others (Clay & Schardl, 2002; Schardl, 2012; Schardl et al., 2004). Comparing measurements of phenotypic elements of infected and uninfected hosts, particularly functional traits, could be helpful in empirical quantification of endophytic growth effects. Plant functional traits are the morphological, physiological, and phenological features that represent ecological strategies and determine how plants respond to environmental factors (Pérez-Harguindeguy et al., 2013). Closer study of plant functional traits can advance understanding of ecological and evolutionary patterns, as well as build a predictive standard for relationships between plants and a multitude of environmental factors. Applying these scientific measures towards endophytes and their hosts would be a valuable tool in further understanding effects endophyte presence may have on morphology and physiology in infected hosts, as well as ecological impacts in the population dynamics and changes in biodiversity that may occur in infected populations. Investigating endophyte presence will provide insight into the selective pressures acting on the endophyte symbioses in different populations as well. Additionally, these data would increase the available information and validity of standardized protocols for quantifying trait variation. Increasing validity in functional trait studies will build confidence in how traits can result in response to environmental factors, and how those affect or predict ecosystem properties (Funk et al., 2017). With more studies,

endophyte presence could be introduced as a trait with the power to predict functional measures and plant-ecosystem level processes.

Epichloë species are fungal endophytes of cool-season grasses. Many cool-season grasses can be infected (with an estimate of possibly infected grass species reaching around 900), though most studies have previously focused on agricultural grass species like *Lolium perenne* and *Lolium arundinaceum (=Festuca arundinacea*; Vikuk et al., 2019). It is thought that natural populations show more variable infection frequencies compared to pastoral settings (Malinowski & Belesky, 2006). Other cool-season grass-endophyte systems should be examined for further understanding of endophyte dynamics, as well as how these might change in native populations compared to agricultural settings. Members of Pooideae that can be infected include the genus *Brachyelytrum,* comprised of the three species *B. japonicum*, *B. aristosum* and *B. erectum.* The endophyte that can reside within *Brachyelytrum* (so far confirmed only in *B. erectum), Epichloë brachyelytri,* is mostly asexual and causes no symptoms. Members of *Brachyelytrum* (and their *Epichloë* counterpart) have been included in endophyte characterization and molecular phylogenetic studies (see Schardl et al., 2013; Clay & Schardl, 2002; Schardl, 2012; Leuchtmann, 2003; Schardl et al., 2008; Schardl & Leuchtmann, 2005), and the grass represents a clade that is basal to all other clades of the Poöideae. However, the chemotypic profiles, infection frequencies across multiple regions, and the native distribution of this grass-endophyte system has yet to be examined. For these reasons, *Brachyelytrum erectum,* a prominent native grass found in the eastern half of the contiguous United States, was chosen as the subject of the extensive characterization study described in this chapter. Plant functional traits to be studied as effects of endophyte infection were chosen that would be minimally destructive to measure and easy to reproduce across all sampling events and were selected from the Pérez-Harguindeguy

handbook on functional trait measurements. Traits measured were reproductive height, specific leaf area, specific stem density, and tiller count.

Polymerase chain reaction (PCR) has previously been used to determine endophyte presence as well as species diversity (Vikuk et al., 2019). PCR was used in this project (based on primers and protocols developed by Charlton et al., 2012 and Charlton et al., 2014) to determine endophyte presence and alkaloid diversity from fresh tiller samples of the grass. Based on previous growth effects found due to epichloid infections of other grass species, I hypothesized that presence of *Epichloë brachyelytri* in *B. erectum* would correspond to patterns in functional measurements. In addition, the variance in functional measurements may be observed in correspondence with geographic location.

2.2 MATERIALS AND METHODS

2.2.1 SAMPLING:

Sampling events (for genomic DNA purposes) occurred throughout the state of Kentucky beginning in July and ending in October for both 2021 and 2022. This temporal period was chosen to catch the peak growth period of *Brachyelytrum erectum*, but before the seeds had dropped. *B. erectum* samples were identified and spaced at least one meter apart to ensure diversity in plant sampling by avoiding clones of individuals. A 60-cm ring was used to provide a standard area around the target plant to catalog neighboring plants and assess ground cover. Before measurements were taken, a picture was taken above the plant centered within the 60-cm ring for a GPS location and to capture surrounding plant species data. For all but 10 populations, a passive integrated transponder (PIT) tag was placed into the ground near the base of the plant sampled. Tags were placed in case the sample site would need to be located at a later date. The

tip of the seed head was gently grasped and pulled upwards until the plant was vertical and perpendicular with the ground. Using the 100-cm ruler, this height was recorded as the reproductive height. Next, the tiller was cut at the base of the crown and placed into a plastic, labeled Ziplock bag. A total of 24 to 28 samples was taken from each population. Samples were stored in a cooler with frozen ice packs for transportation back to the University of Kentucky in Lexington, KY.

2.2.2 PROCESSING:

Sample processing included measuring leaf dry weight and area, collection of seeds, stem specific density, and lyophilization. Seeds were removed from the tiller and placed in a coin envelope labeled with sample number, date, and location. Seed envelopes were sealed with tape and stored in -20℃. The largest, intact leaf most free of herbivory or disease was removed from each tiller and pressed. After 48 hours, dry leaves were weighed and photographed under plexiglass to keep them flat, with a ruler included for scale. These photographs were analyzed with ImageJ to measure the area of the flattened leaf.

Specific leaf area was calculated by dividing the leaf area by the dry weight. During this project, it was brought to our attention that fresh leaves tend to shrink after drying. To standardize and to allow comparison with other studies, a correction factor of 1.1116 and linear regression was calculated based on twelve fresh and dry leaves from Mammoth Cave Wet Prong Trail. This correction factor accounts for the difference in fresh leaf versus dry leaf area (Figure 2.1). The correction factor was applied to all other dry leaf samples from every population.

To measure stem specific density, a 5-cm section was cut from between the first and second node from the tiller base (cut points approximated equal distances to the nodes). The stems had approximately elliptical cross-sections, so widths of the long and short axes (x and y)

of the stem section were measured with an electronic caliper at three points along the tiller piece (approximately 25%, 50%, and 75% along the internode section). Widths were averaged and stem volume was calculated using the formula $V = 50 \times \pi \times \overline{x} \times \overline{y}$, yielding a volume in mm^3. The stem sections were placed in a coin envelope and set in an Isotemp Oven at 80℃ to dry. After 48 hr, the stem sections were weighed. Specific stem density was calculated using the formula (stem dry weight/stem volume) * 1000, to yield a specific density in g/ cm^3. The remaining plant sample material was lyophilized (24 hr) in labeled paper bags for use in the DNA extraction procedure.

2.2.3 DNA EXTRACTION:

Plant material was lyophilized before DNA extraction to maximize yield. DNA extractions were completed with the use of Zymo Research Fungal/Bacterial DNA Miniprep Kit (Cat. No. D6005/Lot No. 216688) (Zymo Research, Irvine, CA) and Qiagen DNeasy® 96 Plant Kits (Cat. No. 69181/Lot No. 169029282) (Qiagen, Hilden, GE) according to manufacturer's instructions.

2.2.4 POLYMERASE CHAIN REACTION (PCR):

PCR was performed in a total volume of 25 μL containing 5 μL DNA, 1.0 U GoTaq™ DNA Polymerase (Promega Corp., Madison, WI), 1× Green GoTaq™ Reaction Buffer (Promega Corp., Madison, WI) containing 1.5 mM $MgCl_2$, 2 × 10 mM each dNTP (New England BioLabs, Ipswich, MA), and 1 μM each of the target-specific primers (Integrated DNA Technologies, Newark, NJ). The cycling parameters were 94°C for 1 min, then 35 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 1 min, followed by 72°C for 7 min. The product was stored at 4°C. Amplicons were analyzed by gel electrophoresis with a 2% agarose gel (EZ BioResearch, St. Louis, MO) in 0.5 × Tris-Boric-EDTA (TBE) buffer (National Diagnostics, Atlanta, GA). DNA

fragments were visualized with GelGreen® (Biotium, Fremont, CA) by UV or blue-light transillumination.

2.2.5 STATISTICAL ANALYSES

Statistical analyses were performed in R with the guidance of Dr. Christopher Searcy and Dr. Michelle Afkhami of the University of Miami. Individual populations were analyzed by running an ANOVA on a fixed effect model that used the morphological parameter (e.g., tiller count) as the outcome variable and endophyte status as the predictor variable. For the morphological parameters of all combined samples, an ANOVA was run on log-transformed data in a fixed effect model with parameters set as in the first analysis, with population serving as an additional random effect parameter. The *p*-values were output and used as indicators of significance.

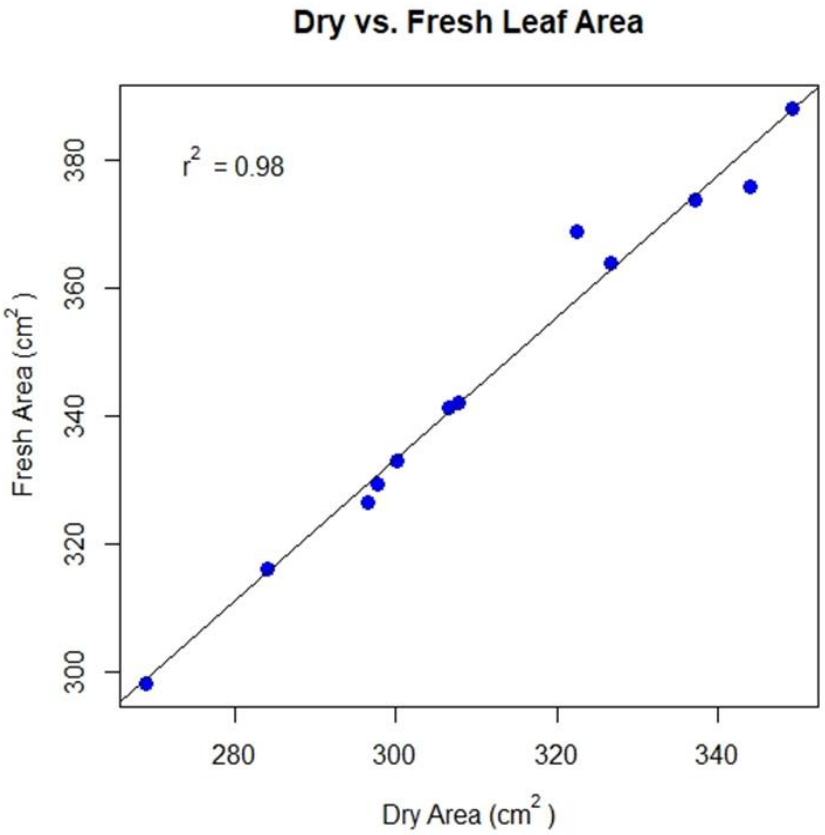

Figure 2.1. Regression and correction factor calculated for fresh and dry leaves from twelve Wet Prong leaf samples graphed linearly. A strong r^2 was calculated at 0.98. The average ratio (1.1116) was applied as the correction factor to the dry areas for all leaves in all populations for specific leaf area measurements.

2.3 RESULTS

Table 2.1 Site Abbreviation Key

Abbrev.	Location Name	GPS	Abbrev.	Location Name	GPS
BCFIFT	Berea College Forest Indian Fort Trail	37.559°N, 84.235°W	MACAE	Mammoth Cave Echo River Trail	37.179°N, 86.108°W
BCFEP	Berea College Forest East Pinnacle	37.558°N, 84.229°W	MACAMS	Mammoth Cave Maple Springs	37.209°N, 86.133°W
BFJYL	Bernheim Forest Jackson Yoe Loop	37.903°N, 85.640°W	MACAWPT	Mammoth Cave Wet Prong Trail	37.243°N, 86.191°W
BFPF	Bernheim Forest Poplar Flat	37.913°N, 85.652°W	MAYWN	Maywoods North	37.484°N, 84.435°W
CCWMA	Cane Creek Wildlife Management Area	37.048°N, 84.288°W	RFCT	Robinson Forest Cell Tower	37.472°N, 83.161°W
CKWMA	Central Kentucky Wildlife Management Area	37.608°N, 84.195°W	RFWR	Robinson Forest Wood Road	37.469°N, 83.135°W
CUGAWT	Cumberland Gap Wilderness Trail	36.609°N, 83.678°W	RRGSCC	Red River Gorge Swift Camp Creek Trail	37.804°N, 83.590°W
CUGATP	Cumberland Gap Tristate Point	36.606°N, 83.667°W	RRGWB	Red River Gorge Whittleton Branch Trail	37.786°N, 83.664°W
FC	Floracliff	37.902°N, 84.362°W	TDC	Tom Dorman Chase	37.766°N, 84.623°W
LCW	Lilley Cornett Woods	37.079°N, 83.003°W	TDJBR	Tom Dorman Jesse Brims Road	37.767°N, 84.629°W
LH	Laure Hare Indiana	39.171°N, 86.144°W	TDR27	Tom Dorman Route 27	37.767°N, 84.619°W
MACAC	Mammoth Cave Campground	37.184°N, 86.100°W			

Table 2.1. Abbreviation key for sample locations.

Table 2.2 Endophyte Status by Population							
Location	**E+**	**E−**	**Infection Frequency**	**Location**	**E+**	**E−**	**Infection Frequency**
BCFIFT	25	0	100%	MACAC	24	0	100%
BCFEP	21	3	87.5%	MACAE	20	3	86.9%
BFJYL	6	18	25%	MACAMS	24	0	100%
BFPF	25	0	100%	MACAWPT	24	0	100%
CCWMA	24	0	100%	MAYWN	11	13	45.8%
CKWMA	8	16	33.3%	RFCT	25	3	89.2%
CUGAWT	10	14	41.7%	RFWR	1	23	4.2%
CUGATP	4	0	100%	RRGSCC	12	12	50%
FC	24	0	100%	RRGWB	8	16	33.3%
LCW	26	0	100%	TDJBR	15	13	53.5%
LH	17	31	35.4%	TDR27	14	8	63.6%

Table 2.2. Endophyte status by population. Abbreviation index provided in Table 2.1.

Twenty-two populations were sampled and analyzed for the plant morphological traits and endophyte infection (this chapter) and endophyte genotype (see Chapter 3). The total number of samples collected ranged from 23 to 28 plants. From an additional population at Cumberland Gap (designated CUGATP) only four plants were sampled. Ten populations were 100% infected and the other 13 were “mixed populations”, having both endophyte-infected and non-infected plants (Table 2.2). Average infection frequency was 72.1% across all populations and 48.8% across the mixed populations, respectively. Robinson Forest Wood Road (RFWR) contained only one infected plant, compared to the 25 infected plantss collected at the neighboring site, Robinson Forest Cell Tower (RFCT) (Table 2.2). Of the four populations sampled in a transect through the Mammoth Cave National Park (MACAC, MACAE, MACAMS, MACAWPT), only

the Echo River site (MACAE) contained uninfected plants (Table 2.2). The two Kentucky Palisades identified on the Tom Dorman trail (TDJBR and TDR27) both had similar infection frequencies at 53.5% and 63.6%, respectively (Table 2.2). Four phenotypic measurements were taken for the populations: reproductive height, specific leaf area, specific stem density, and tiller count. Specific stem density and tiller count were only taken for the last ten sampling events. Samples from Berea College Indian Fort Trail (BCFIFT) had the highest median reproductive height, whereas MACAE samples had the lowest (Figure 2.2A). BCFIFT also contained the tallest reproductive tiller (105 cm), whereas the shortest tiller (20 cm) resided in the Bernheim Forest Poplar Flat (BFPF) location (Figure 2.2A). MACAE samples had the highest median specific leaf area, whereas BCFIFT samples had the lowest (Figure 2.2B). Mammoth Cave Campground (MACAC) samples had the highest median stem specific densities, whereas samples from Central Kentucky Wildlife Management Area (CKWMA) had the lowest (Figure 2.2C). CKWMA and Floracliff (FC) plants displayed an especially large range in the numbers of tillers per crown compared to the eight other populations where tiller number was also counted (Figure 2.1D).

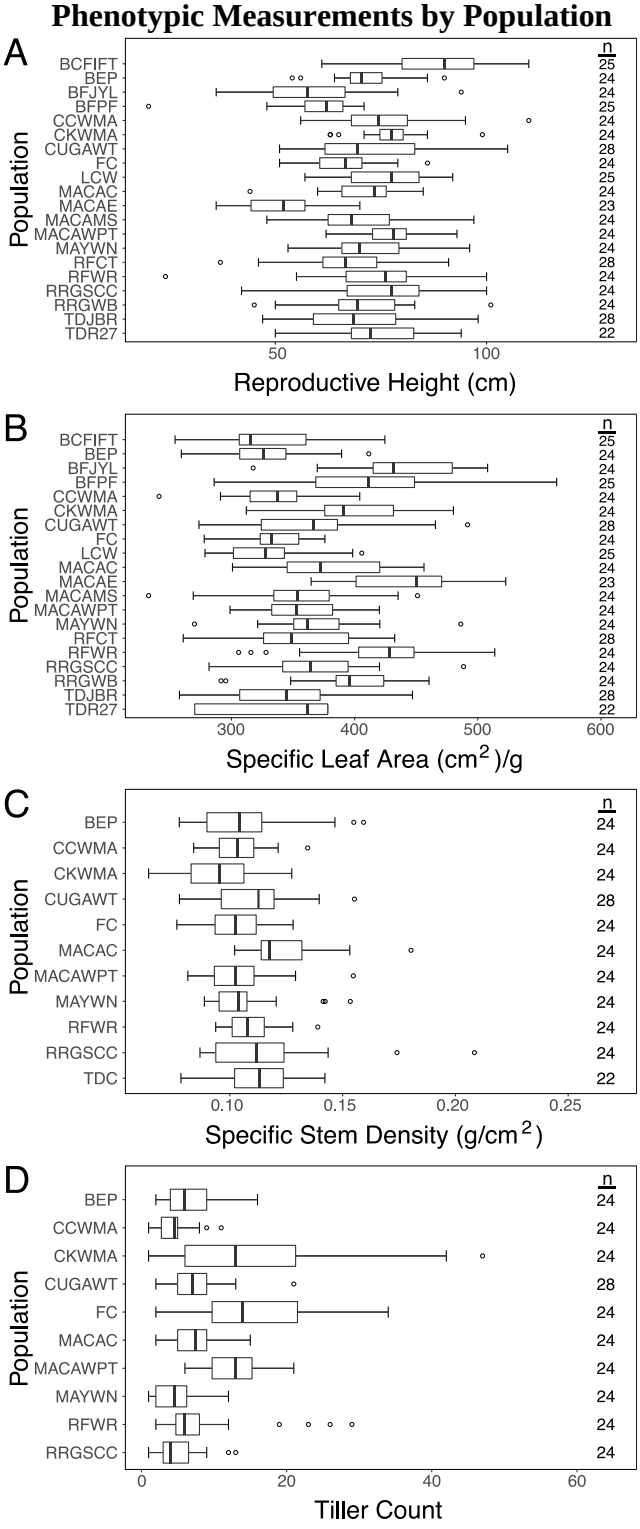

Figure 2.2. Phenotypic measurements for each population sampled. Specific stem density and tiller count were only measured in the last 10 populations sampled. (A) Reproductive Height, (B) Specific Leaf Area, (C) Specific Stem Density, and (D) Tiller Count. Line indicates median, boxes represent data within 75% of median, whiskers at 95% of median, and dots represent outliers.

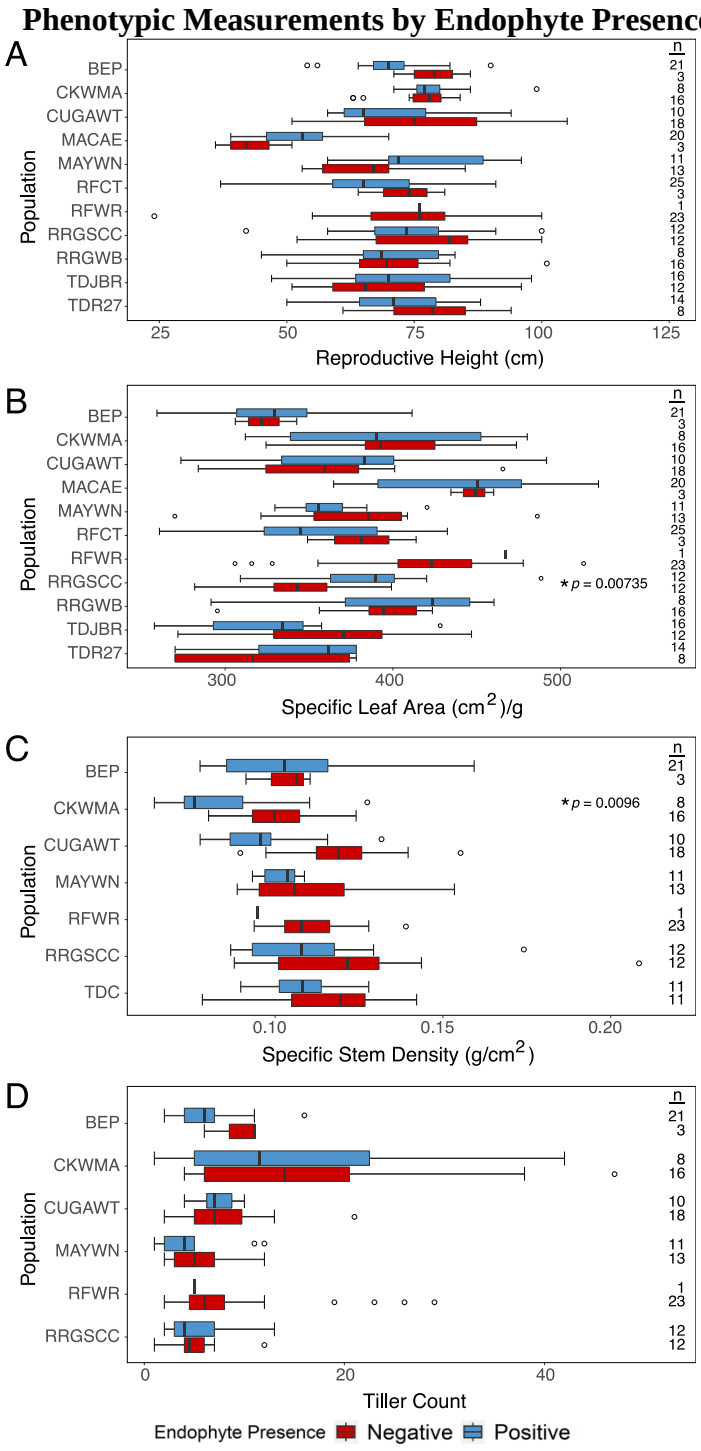

Figure 2.3. Phenotypic measurements for populations with both endophyte negative and endophyte positive samples, separated by endophyte status. (A) Reproductive Height, (B) Specific Leaf Area, (C) Specific Stem Density, (D) Tiller Count. * = statistical significance, color indicates endophyte status (red = E-, blue = E+). Line indicates median, boxes represent data within 75% of median, whiskers at 95% of median, and dots represent outliers.

Phenotypic measurements were then analyzed with endophyte presence. For simplicity, populations that were 100% infected were omitted from Figure 2.3 and Figure 2.4. Reproductive height for most populations trended higher in endophyte negative samples compared to positive but were not statistically significant. This did not include MACAE, Maywoods North (MAYWN) and Tom Dorman Jesse Brims Road (TDJBR) where average reproductive heights trended higher in positive samples (Figure 2.3A). Average specific leaf areas trended lower for endophyte negative samples in a majority of the populations except for CKWMA, MAYWN, TDJBR, and Robinson Forest Cell Tower (RFCT) sites (Figure 2.3B). The difference in lower specific leaf area of negative samples compared to positive was statistically significant in the Red River Gorge Swift Camp Creek (RRGSCC) population with a *p*-value of 0.00735 (Figure 2.3B). Specific stem density findings more consistently followed a trend, with each population measured showing a higher average specific stem density for endophyte negative samples compared to infected samples (Figure 2.3C). CKWMA samples were statistically significant for higher specific stem density in negative samples, with a *p*-value of 0.0096. Similar results were found for tiller count analysis. Average tiller counts for each population trended lower in positive samples compared to non-infected plants, but the differences were not statistically significant (Figure 2.3D). CKWMA exhibited a larger range and highest medians in tiller counts for both infected and non-infected plants compared to the other populations (Figure 2.3D).

Seeing the trend in specific stem density, the group sampled an additional population to further investigate that parameter. Stems of 22 plants from this Tom Dorman chase (TDC) population, which was 130 m due north of TDR27, were measured, dried and weighed before endophyte status was determined, and I included the results in the analysis (Figs. 2.2, 2.3).

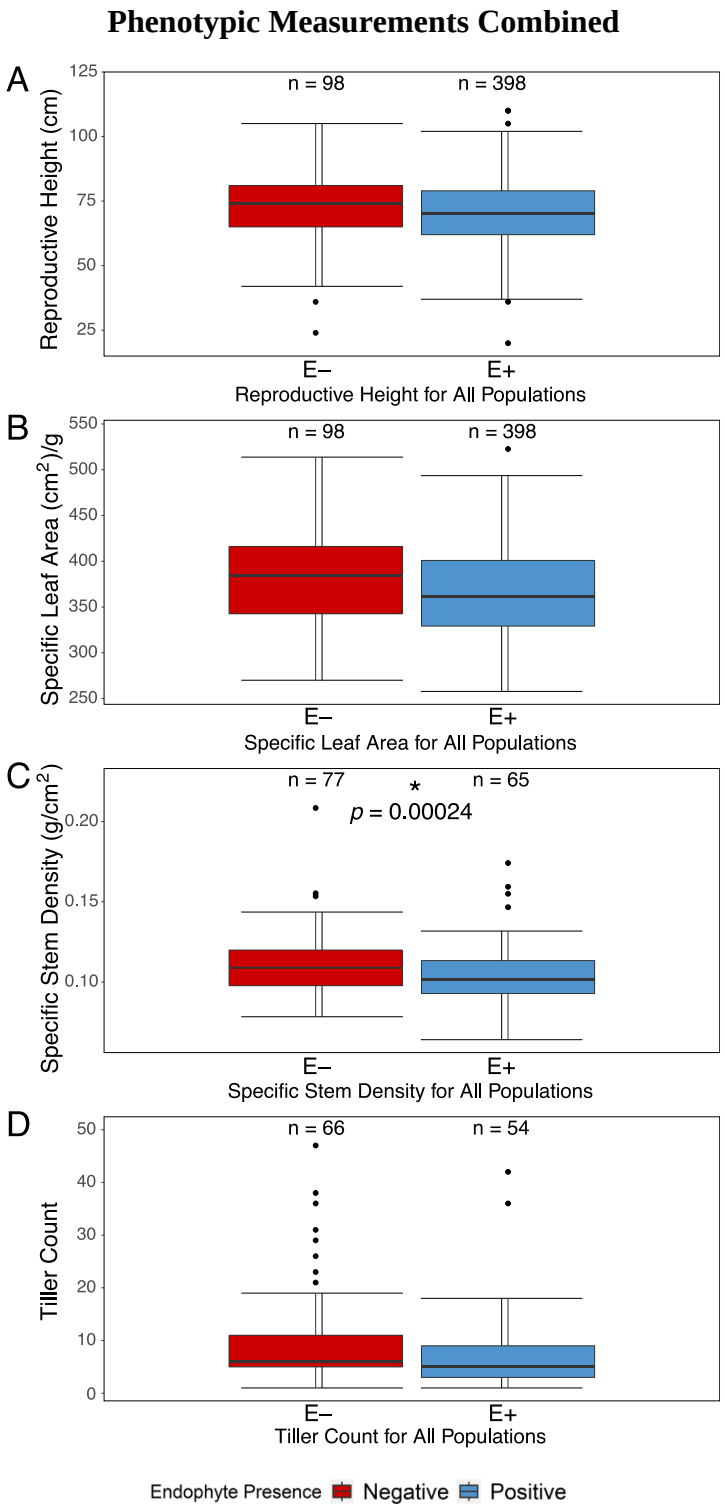

Figure 2.4. Phenotypic measurements for all samples combined and separated by endophyte status. (A) Reproductive Height, (B) Specific Leaf Area, (C) Specific Stem Density, (D) Tiller Count. * = statistical significance, color indicates endophyte status (red = E-, blue = E+). Line indicates median, boxes represent data within 75% of median, whiskers at 95% of median, and dots represent outliers.

In combined analyses of all populations, median stem specific density was greater in endophyte negative samples with a statistically significant *p*-value of 0.00024 (Figure 2.4C). Also, reproductive height and specific leaf area trended higher in endophyte-negative samples compared to positive samples, though not significantly (Figure 2.4A, B).

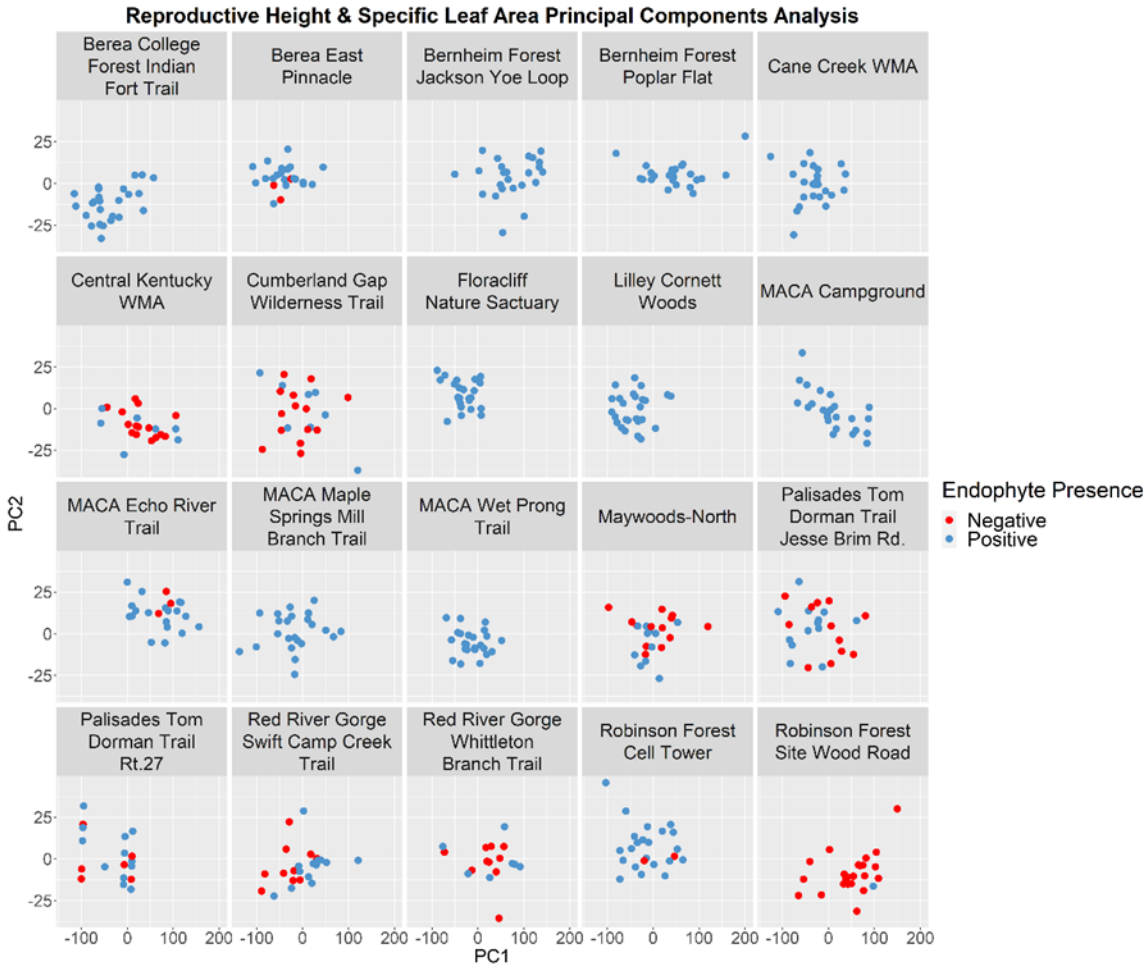

Figure 2.5. Principal component analysis on all population samples for reproductive height and specific leaf area. Color indicates endophyte status (red = E-, blue = E+).

Principal component analysis was performed on samples from every population for reproductive height and specific leaf area. Both Berea College Forest East Pinnacle (BCFEP) and MACAE had endophyte negative samples that grouped together (Figure 2.5). Comparatively, RRGSCC had a larger number of negative samples that grouped generally to the left of the positive samples from that population (Figure 2.5). For other populations such as Cumberland Gap Wilderness Trail (CUGAWT), MAYWN, and both Tom Dorman populations TDJBR and TDR27 plants did not group distinctly based on endophyte presence for the two measurements (Figure 2.5). The nine other populations were entirely endophyte-positive, and therefore did not group based on endophyte presence in this analysis (Figure 2.5).

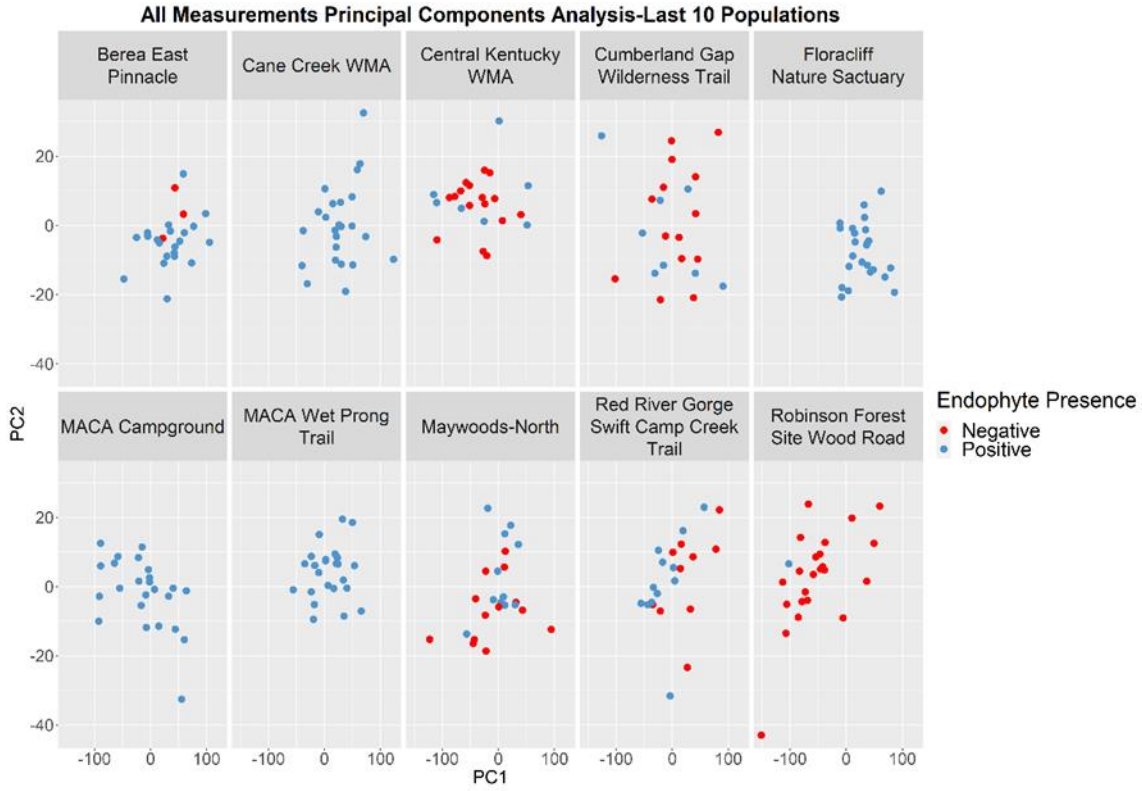

Figure 2.6. Principal component analysis for all measurements (reproductive height, specific leaf area, specific stem density, and tiller count) that were collected for ten populations. Color indicates endophyte status (red = E-, blue = E+).

Principal component analysis was then performed for all four measurements (reproductive height, specific leaf area, specific stem density, and tiller count) that were collected for ten populations. Similar to the principal component analysis that was performed with only reproductive height and specific leaf area, RRGSCC samples appeared to group based on endophyte status with negative samples trending to the right of positive ones with slightly higher PC1 values (Figure 2.6). Other populations with mixed endophyte status (BCFEP, CKWMA, CUGAWT, MAYWN and RFWR) did not appear to have a substantial grouping based on endophyte status (Figure 2.6).

Principal component analysis for reproductive height and specific leaf area was also conducted by population rather than endophyte status to see direct comparisons of individual collection sites (Figure 2.7). Some notable clustering of populations were observed when variance was graphed by population sites. For example, BCIFT vs. BFJYL populations were well separated horizontally, with BFJYL lying mostly to the right of BCIFT. Compared to MACAE, however, BCIFT has more negative PC2 measurements leaving the population lower and to the left of MACAE measurements. RFWR samples are more consistent when in comparison to other populations, with measurements typically lying to the right and/or below another sites', except for CKWMA where they were more aligned. Many comparisons did not show clustering, including LCW vs. Mammoth Cave Wet Prong Trail (MACAWPT), CUGAWT vs. RFCT, and RRGSCC vs. TDR27.

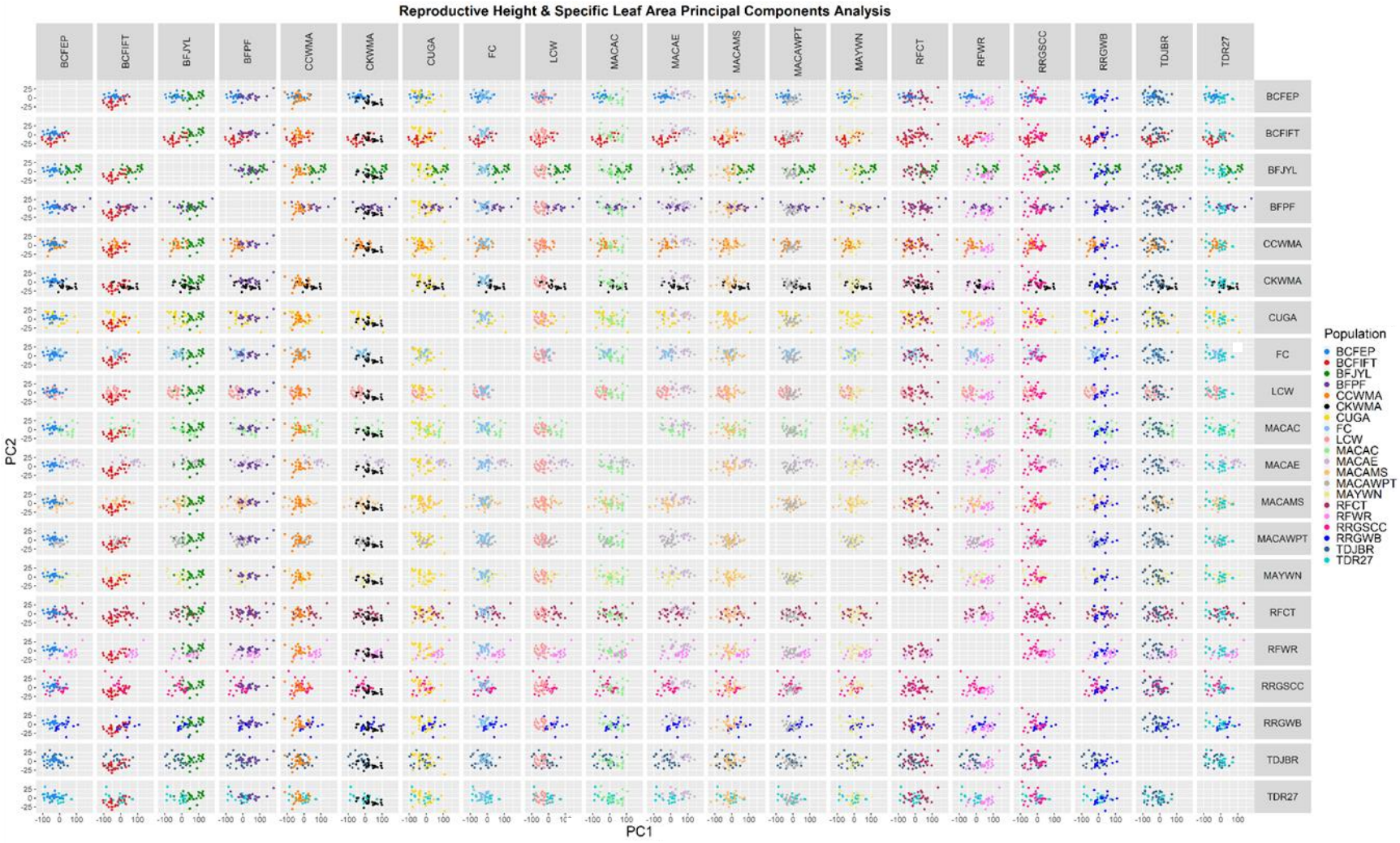

Figure 2.7. Principal component analysis using reproductive height and specific leaf area based on population.

2.4 DISCUSSION

Endophyte-infected grasses have often been reported to show improved physiological responses to adverse environmental conditions, as well as enhanced growth and competitive ability (Schardl et al., 2004; Malinowski & Belesky, 2019). In this chapter, endophyte frequencies in natural populations of *Brachyelytrum erectum* and phenotypic effects of endophytic infection were explored. Native populations were sampled to broaden the understanding of endophyte-host relationships outside of managed pastoral settings. Twenty-two populations were sampled. Reproductive height, specific leaf area, specific stem density, and tiller count were measured as phenotypes to compare with endophyte status as determined using established PCR methods (Charlton et al., 2012; Charlton et al., 2014). Preliminary samplings led to an estimation that populations of *B. erectum* maintain around a 50% infection frequency (Nagabhyru, P. & Schardl, C., unpublished). Upon more in-depth surveys, average infection frequency was closer to 70% because many populations (10 of 22) were 100% infected (Table 2.2). Looking at only the locations with both endophyte positive and negative plants, the average infection frequency was around 49%, closer to the original 50% prediction. It may be that unless otherwise unknown environmental factors encourage a 100% infection rate in a population, regions of *B. erectum* more usually hover at a 50% infection with *E. brachyelytri* (Figure 2.8). In studies of *L. perenne*, infection rates differed slightly between regions, appearing lower (8 to 28%) in Germany, and higher (70%) in New Zealand and Australia (Vikuk et al., 2019). The average infection rate considering all populations of *B. erecutm* more closely mirrors that of *L. perenne* in New Zealand and Australia. Regional discrepancies in infection rates could play a role in the range of infection found throughout the populations of *B. erectum* as well.

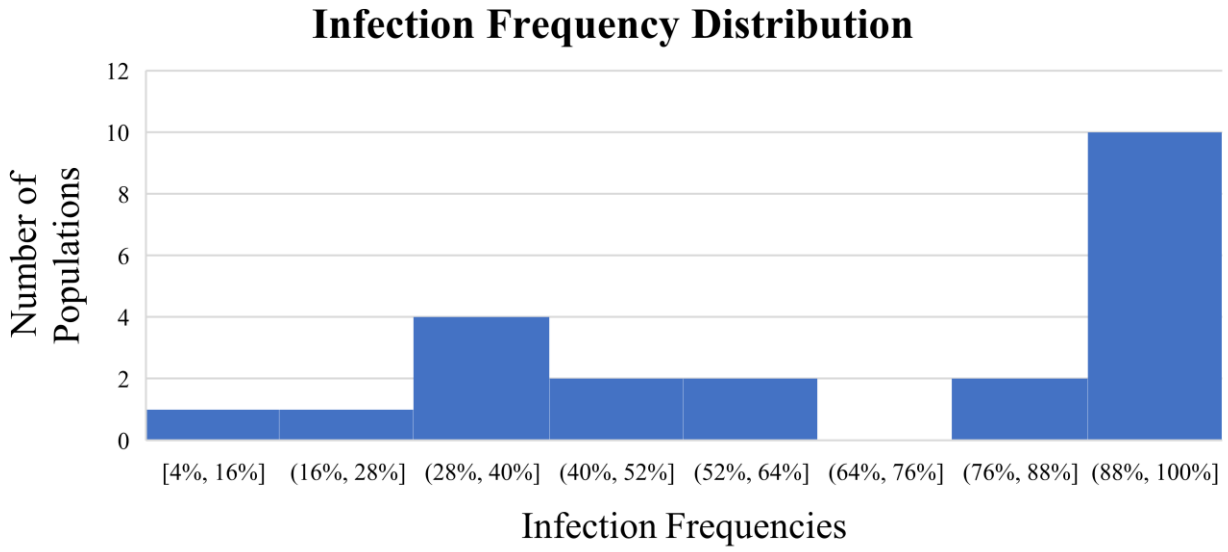

Figure 2.8 Infection Frequency Distribution. Infection frequency histogram of all 22 populations using 10 bins, displaying a bimodal distribution.

Environmental stresses can trigger the restriction of full plant development, resulting in physical growth alterations. Following colonization of plants by an endophyte, various plant growth parameters and stress cues are subject to significant influence by the endophyte's production of various compounds (like invertases, glucanases, proteinases, amines, carboxylic acids, growth factors, lipids, phenolics, and polyols), auxin-like metabolites, carbohydrates (mannitol and trehalose), and anti-herbivore alkaloids (Schardl et al., 2004; Lam et al., 1994; McNear et al., 2015; Moy et al., 2002; Reddy et al., 1996; Thrower & Lewis, 1973; Torres et al., 2012). In turn, a plant's growth and physical characteristics can be altered due to endophyte presence, especially in conjunction with environmental and biotic stressors (Malinowski & Belesky, 2019; Torres et al., 2012). The goal of this project was to analyze specific plant parameters and compare the measurements against a plant's endophyte status to see if any phenotype was influenced by endophyte presence.

Phenotypic measurements that were included in the investigation were reproductive height, specific leaf area, tiller count, and specific stem density. Two populations were statistically significant for differences in two measurements due to endophyte presence (Figure 2.2). The RRGSCC site had significant differences in the specific leaf area of endophyte positive and negative samples (p=0.00735), with negative samples having higher specific leaf area (Figure 2.2B). Specific leaf area, used to describe the amount of leaf area for light capture per unit of biomass, is a function of the 'leaf economics spectrum' of leaf allocation patterns and physiological function (Torrez et al., 2013). Environmental and biotic factors should provide strong selection towards optimal allocation of resources to build tissues for light capture (Markesteijn, 2010; Torrez et al., 2013). Species with a high specific leaf area score at the quicker-return end of the leaf economic spectrum (fast-growing species with cheap tissue investment and rapid returns on that investment) compared to species with lower specific leaf area at the conservative end (slow-growing species with expensive tissue investment and slower returns) (Torrez et al., 2013; Zhao et al., 2016). Though RRGSCC displayed a significant trend of lower specific leaf area in negative samples (Figure 2.3B), average specific leaf area of negative samples from all populations combined trended higher than in positive samples (though this difference was not significant) (Figure 2.4B). Light availability (which is an important elicitor of leaf growth) across our populations was variable and not measured, which would have also affected average specific leaf area of a population. These observations suggest two possibilities: 1) endophyte presence can, but will not always, impart a significant effect on specific leaf area when looking at individual populations, and 2) because a lower specific leaf area can cause a decrease on the leaf economics spectrum, the benefits of hosting an endophyte must outweigh the disruption to a plant's resource allocation strategy.

Using another phenotype, the CKWMA population exhibited a statistically significant lower specific stem density of endophyte-positive plants, with a *p*-value of 0.0096 (Figure 2.3C). More generally, combined analysis of seven populations indicated lower stem specific density in endophyte-positive plants with a high level of significance (*p*-value of 0.00024) (Figure 2.4C). Stem specific density is used as a core functional trait because of its importance in the stability, defense, architecture, hydraulics, and growth potential of plants (Pérez-Harguindeguy et al., 2013). Residing in most or all above-ground tissues and growing intercellularly, it is reasonable to expect an endophyte-related effect in dry matter, volume, and density in above-ground plant parts. Under salt stress, Chen et al. (2021) found that wild barley (*Hordeum brevisubulatum*) showed an increase in stem thickness when infected with *Epichloë bromicola.* Using 'thickness' as a component of stem volume utilized in the specific stem density calculations, an increase in stem thickness without a corresponding increase in biomass would result in a decrease in stem specific density. The significant findings of stem specific density for endophyte-infected plants in the CKWMA site, as well as the overall significance when all populations were combined, perhaps reflect those previous findings in wild barley of lower density during infection. Stem specific density illustrates a growth-survival tradeoff; in that low density (with larger vessels) contributes to fast growth due to increased hydraulic capacity and ease of volume production, whereas high density (with smaller vessels) increases survival due to security of biomechanical structure and hydraulic flow, and heightened resistance against pathogens, herbivores, or physical damage (Pérez-Harguindeguy et al., 2013). Considering endophyte presence together with stem specific density, it may be that a host can afford to lower stem density (and reap the benefits of faster growth) while also relying on the endophyte to serve as an alternative form of resistance against pathogens, herbivores, and other abiotic stressors.

Two parameters, reproductive height and tiller count, did not result in any statistically significant differences in relation to endophyte presence. In this case, *E. brachyelytri* did not significantly influence average reproductive height or tiller number in *B. erectum.* Though a significant effect was not observed in my study, an increase in tiller number in endophyte-infected samples has been noted before both in *Hordeum borgdanii* and *Elymus cylindricus* (Malinowski & Belesky, 2006). Additional samplings with higher n-values could elucidate more information on endophytic effects on reproductive height and tiller count, as we only counted tillers in ten of the 22 *B. erectum* populations sampled. Alternatively, these findings could be an indication that *E. brachyelytri* does not affect this plant species in terms of tiller number.

Detailed research on the ecology of endophyte-infected populations of native grasses is scarce (Malinowski & Belesky, 2006). In this thesis, investigating infection frequency and phenotypic effects as a result of infection helps further describe endophyte behavior in native populations, as well as characterize biological effects of *E. brachyelytri* on *B. erectum.* Ideally, this work can be extended to analyze morphological parameters not included in this project, as well as inspire additional studies on other native populations of cool-season grasses and their endophytes. Moreover, consideration should be given to endophytic effects as a part of future research on wide-scale plant evolutionary theory.

CHAPTER 3. ANALYZING CHEMOTYPIC DIVERSITY OF *EPICHLOË BRACHYELYTRI* IN POPULATIONS OF *BRACHYELYTRUM ERECTUM*

3.1 INTRODUCTION

Among the symbioses that encompass grasses (family Poaceae) and the fungal family Clavicipitaceae, a continuum of mutualistic to antagonistic interactions is particularly evident for the fungal genus *Epichloë* (Schardl et al., 2004). Although these systemic epichloid infections may vary in their host interactions, there is still considerable evidence for the selective advantage and increased competitive ability of endophyte-infected grasses (Malinowski & Belesky, 2019). The improved performance of infected hosts (especially under environmental stress) and a broad range of host interactions can largely be attributed to the production of alkaloids and nonalkaloid secondary metabolites (Malinowski & Belesky, 2019; Schardl et al., 2013; Christensen et al., 2008). *Epichloë* fungi are extraordinarily diverse in their alkaloid profiles, both between and within species (Schardl et al., 2013; Vikuk et al., 2019; Malinowski & Belesky, 2000). Alkaloid diversity occurs at two levels; the first being the presence or complete absence of one or more of the four known classes of epichloid alkaloids (most commonly associated with absence of a functional gene for the first pathway determinant step), and the second being diversified structures within each alkaloid class (Schardl et al., 2013; Vikuk et al., 2019). The structure and position of alkaloid loci suggest that these fungi are under selection for alkaloid diversification, and that this diversification serves to enhance overall fitness of the endophyte-grass symbiosis (Schardl et al., 2013).

The best-known alkaloids produced by *Epichloë* are ergot alkaloids, lolines, pyrrolopyrazines (such as peramine), and indole-diterpenes for their risks posed to vertebrates and invertebrates feeding on endophyte-infected plants (Malinowski & Belesky, 2019).

Assessing infection rates alone is insufficient to estimate livestock risk in agricultural settings, so previous work by Vikuk et al., (2019) conducted work on the alkaloid capability in infected grass species in German grasslands and using PCR to identify *Epichloë* species diversity, showed that alkaloid profiles differ between grass species and that some alkaloids can be toxic to livestock. Predicting alkaloid production is possible due to detecting the presence or absence of key pathway steps for alkaloid biosynthesis genes, thereby linking genetic diversity to actual chemotype (Schardl et al., 2013). Similarly, this project aims to evaluate *Brachyelytrum erectum* infected with *Epichloë brachyelytri* to better understand the alkaloid capabilities and diversity present in natural populations, as well as how this diversity may be spread across a geographic range. Alkaloid presence and mating types were evaluated by using established PCR methods (Charlton et al., 2012; Charlton et al., 2014) to screen lyophilized *B. erectum* samples as well as cultures grown from fresh tissue.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLING:

Sampling events (for genomic DNA purposes) occurred throughout the state of Kentucky beginning in July and ending in October for both 2021 and 2022. This temporal period was chosen to catch the peak growth period of *Brachyelytrum erectum,* but before the seeds had dropped. *B. erectum* samples were identified and spaced at least one meter apart to ensure diversity in plant sampling by avoiding clones of individuals. A 60-cm ring was used to provide a standard area around the target plant to catalog neighboring plants and assess ground cover. Before measurements were taken, a picture was taken above the plant centered within the 60-cm ring for a GPS location and to capture surrounding plant species. For all but 10 populations, a passive integrated transponder (PIT) tag was placed into the ground near the base of the plant

sampled. Tags were placed in case the sample site would need to be located at a later date. Functional trait measurements were taken as described in Chapter 2. The tiller was cut at the base of the crown and placed into a plastic, labeled Ziplock bag. A total of 24 to 28 samples were taken from each population. Samples were stored in a cooler with ice packs for transportation back to the University of Kentucky in Lexington, KY.

3.2.2 PROCESSING:

Seeds were removed from the tiller and placed in a coin envelope labeled with sample number, date, and location. Seed envelopes were sealed with tape and stored in -20℃. After processing functional trait measurements (described in chapter 2), the remaining plant sample material was lyophilized in labeled paper bags (24 hrs) for use in the DNA extraction procedure.

3.2.3 CULTURING FROM FRESH TISSUE:

Epichloë brachyelytri was cultured from fresh tiller samples of *B. erecutm.* Tillers were sterilized in 95% ethanol (Decon Laboratories Inc., King of Prussia, PA) for 1 minute, rinsed 3 times with deionized water (DI), then 50% commercial bleach (The Clorox Company, Oakland, CA) for 3 minutes and rinsed 3 times with DI water. Tiller pieces were plated onto potato dextrose agar (PDA) (Becton, Dickinson, and Company, Sparks, MD) plates with penicillin (Sigma-Aldrich, St. Louis, MO) and streptomycin (Sigma-Aldrich, St. Louis, MO) (1g/1mL). Plates were wrapped with parafilm and stored in a 25°C incubator. Growth began around 10 days after plating. Cultures were subcultured onto PDA.

3.2.4 DNA EXTRACTION:

Plant material was lyophilized before DNA extraction to maximize yield. DNA extractions were completed with the use of Zymo Research Fungal/Bacterial DNA Miniprep Kit (Cat. No. D6005/Lot No. 216688) (Zymo Research, Irvine, CA) and Qiagen DNeasy® 96 Plant Kits (Cat. No. 69181/Lot No. 169029282) (Qiagen, Hilden, GE) according to manufacturer's instructions.

3.2.5 POLYMERASE CHAIN REACTION (PCR):

PCR was performed in a total volume of 25 μL containing 5 μL DNA, 1.0 U GoTaq™ DNA Polymerase (Promega Corp., Madison, WI), 1× Green GoTaq™ Reaction Buffer (Promega Corp., Madison, WI) containing 1.5 mM $MgCl_2$, 2 × 10 mM dNTP (New England BioLabs, Ipswich, MA), and 1 μM target-specific primers (Integrated DNA Technologies, Newark, NJ) (Table 3.1). The cycling parameters were 94°C for 1 min, then 35 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 1 min, followed by 72°C for 7 min. The product was stored at 4°C. Amplicons were analyzed by gel electrophoresis with a 2% agarose gel (EZ BioResearch, St. Louis, MO) in 0.5 × Tris-borate-EDTA (TBE) buffer (National Diagnostics, Atlanta, GA). DNA fragments were visualized with GelGreen® (Biotium, Fremont, CA) by UV transillumination.

3.2.6 SOIL SURVEYS:

Soil types were analyzed using the Web Soil Survey application published by the United States Department of Agriculture Natural Resources Conservation Service (Staff, 2019). GPS coordinates that were acquired during time of sampling were input to the Web Soil Survey and an AOI (Area of Interest) was drawn to roughly trace the area of sampling. Information was then gathered from the ‘Soil Map’ tab on the application. Genotype analysis was limited because GPS coordinates were not recorded for every sample in a population in most cases. Images of mapped

samples were superimposed on the soil map area with coordinates that were captured for a population (example in Figure 3.3).

Table 3.1 Primer Table			
Gene	**Primer name**	**5' Primer sequence 3'**	**Band size (bp)**
tefA	tef1-exon1d-1	GGGTAAGGACGAAAAGACTCA	860
	tef1-exon6u-1	CGGCAGCGATAATCAGGATAG	
perA	per T2-F	TCTTCAGGCATCGCAGGAAC	600
	per T2-R	TCGGCCACCTCCAGCCTGATG	
lolC	lolC-3a	GGTCTAGTATTACGTTGCCAGGG	442
	lolC-5b	TCTAAACTTGACGCAGTTCGGC	
dmaW	dmaW-F4	GTGTACTTTACTGTGTTCGGCATG	282
	dmaW-6R	GTGGAGATACACACTTAAATATGGC	
mtA	mtAC-F	CAATGGTGGTCACCTGAGAAG	785
	mtAC-R	CGGTCTCATTCTTCCAGAGAGAGG	
perAred	per red F2	GAGATCAGTTCGCAGTTGTCAG	587
	per red R	CTAGCCTCCAGATCTTGTGAAAG	
mtB	mtBA-F	TCTACCGCAAGGAACGACACAATACCG	213
	mtBA-R	GCTTTTCCAGCAAGGCTTGCTTGACTC	
Table 3.1. Locus-specific primers used in multiplex PCR for alkaloid and mating type characterization.			

3.3 RESULTS

Table 3.2 Gene Presence by Population										
Site	**E+**	**E−**	**Culture**	**tefA**	**perA**	**perAred**	**dmaW**	**lolC**	**matA**	**matB**
BCFEP	21	3	2	23	23	23	23	23	22	1
BCFIFT	25	0	10	35	35	35	35	35	-	35
BFJYL	6	18	2	8	8	8	8	4	4	4
BFPF	25	0	4	29	29	29	29	29	-	29
CCWMA	24	0	27	51	51	51	51	37	-	51
CKWMA	8	16	N/A	8	8	8	8	8	-	8
CUGATP	5	0	N/A	5	5	5	5	5	5	-
CUGAWT	8	15	N/A	8	8	8	8	8	3	5
FC	20	0	4	24	24	24	24	-	15	9
LCW	26	0	2	28	28	28	28	28	1	27
LH	17	31	N/A	17	17	17	17	17	14	3
MACAC	24	0	N/A	24	24	24	24	24	-	24
MACAE	20	3	N/A	20	20	20	20	20	-	20
MACAMS	24	0	N/A	24	24	24	24	24	-	24
MACAWPT	24	0	N/A	24	24	24	24	24	-	24
MAYWN	11	13	N/A	11	11	11	11	11	-	11
RFCT	25	3	N/A	25	25	25	25	25	-	25
RFWR	1	23	N/A	1	1	1	1	1	1	-
RRGSCC	12	12	20	32	32	32	32	11	9	23
RRGWB	8	16	11	19	19	19	19	3	3	16
TDJBR	15	13	7	22	22	22	22	22	-	22
TDR27	14	8	7	21	21	21	21	-	-	21
Total	363	174	96	459	459	459	459	359	76	383

Table 3.2. Endophyte status and gene presence by population. Abbreviation index provided in Table 2.1.

Dried plant material as well as fungal cultures from fresh samples were analyzed via PCR for housekeeping gene *tefA*, alkaloid genes *perA*, *perAred*, *dmaW*, and *lolC*, and mating type genes *matA* and *matB*. With 633 total samples screened, 459 were positive for *tefA*, *perA*, *perAred*, and *dmaW* (Table 3.2). Of those 459 endophyte positive samples, 359 were positive for *lolC* (Table 3.2). Absence of *lolC* in positive samples was the only polymorphism found. About a fifth of the 459 endophyte positive samples were of mating type A, the larger majority being mating type B (Table 3.2, 3.3).

Table 3.3 Population Genotypes

Site	E+	E−	Culture	G1: APRDL	G2: BPRDL	G3: APRD−	G4: BPRD−
BCFEP	21	3	2	22	1	-	-
BCFIFT	25	0	10	-	35	-	-
BFJYL	6	18	2	-	4	4	-
BFPF	25	0	4	-	29	-	-
CCWMA	24	0	27	-	14	-	37
CKWMA	8	16	N/A	-	8	-	-
CUGATP	5	0	N/A	5	-	-	-
CUGAWT	8	15	N/A	1	7	-	-
FC	20	0	4	-	-	18	6
LCW	26	0	1	1	26	-	-
LH	17	31	N/A	14	3	-	-
MACAC	24	0	N/A	-	24	-	-
MACAE	20	3	N/A	-	20	-	-
MACAMS	24	0	N/A	-	24	-	-
MACAWPT	24	0	N/A	-	24	-	-
MAYWN	11	13	N/A	-	11	-	-
RFCT	25	3	N/A	-	25	-	-
RFWR	1	23	N/A	1	-	-	-
RRGSCC	12	12	20	9	7	2	14
RRGWB	8	16	11	3	-	-	16
TDJBR	15	13	7	-	22	-	-
TDR27	14	8	7	-	-	-	21
Total	363	174	96	59	286	57	57

Table 3.3. Genotype combinations for each population. (A=Mating type A, B= Mating type B, P = *perA*, R= *perAred*, D = *dmaW*, L = *lolC*). The only variation observed was in mating type and the presence (L) or absence (-) of *lolC* in endophyte-positive samples.

Considering mating type with chemotypic profiles, four genotypes were apparent from our screening. 345 samples were positive for all genes screened (*perA, perAred, dmaW, lolC*), and were either mating type A (*matA;* 59 samples) or mating type B (*matB;* 286 samples) (Table 3.3). The remaining 114 positive samples were absent for *lolC*, still containing the other gene products and evenly split between *matA* and *matB* (Table 3.3). Fourteen populations contained only one genotype and seven sites had two genotypes (Table 3.3, Figure 3.2). Only Red River Gorge Swift Camp Creek (RRGSCC) showed all four *lolC* and mating type combinations. Eight sites contained two genotypes: Berea College Forest East Pinnacle (BCFEP), Bernheim Forest Jackson Yoe Loop (BFJYL), Cane Creek Wildlife Management Area (CCWMA), Cumberland Gap Wilderness Trail (CUGWT), Floracliff (FC), Lilley Cornett Woods (LCW), Laure Hare (LH), and Red River Gorge Whittleton Branch (RRGWB) (Table 3.3, Figure 3.2). CCWMA had 14 samples that were positive for all alkaloid genes tested and *matB*, and 37 samples that were *matA* and missing *lolC* (Table 3.3). RRGWB had three samples that were positive for all alkaloid products and mating type A, along with 16 samples that were mating type B and missing *lolC* (Table 3.3). About 3/4ths of all samples were *matB*, with *matA* samples much less common and no geographical pattern (Figure 3.2). Only two populations (CUGATP and RFWR) were exclusively *matA* (Table 3.3, Figure 3.2).

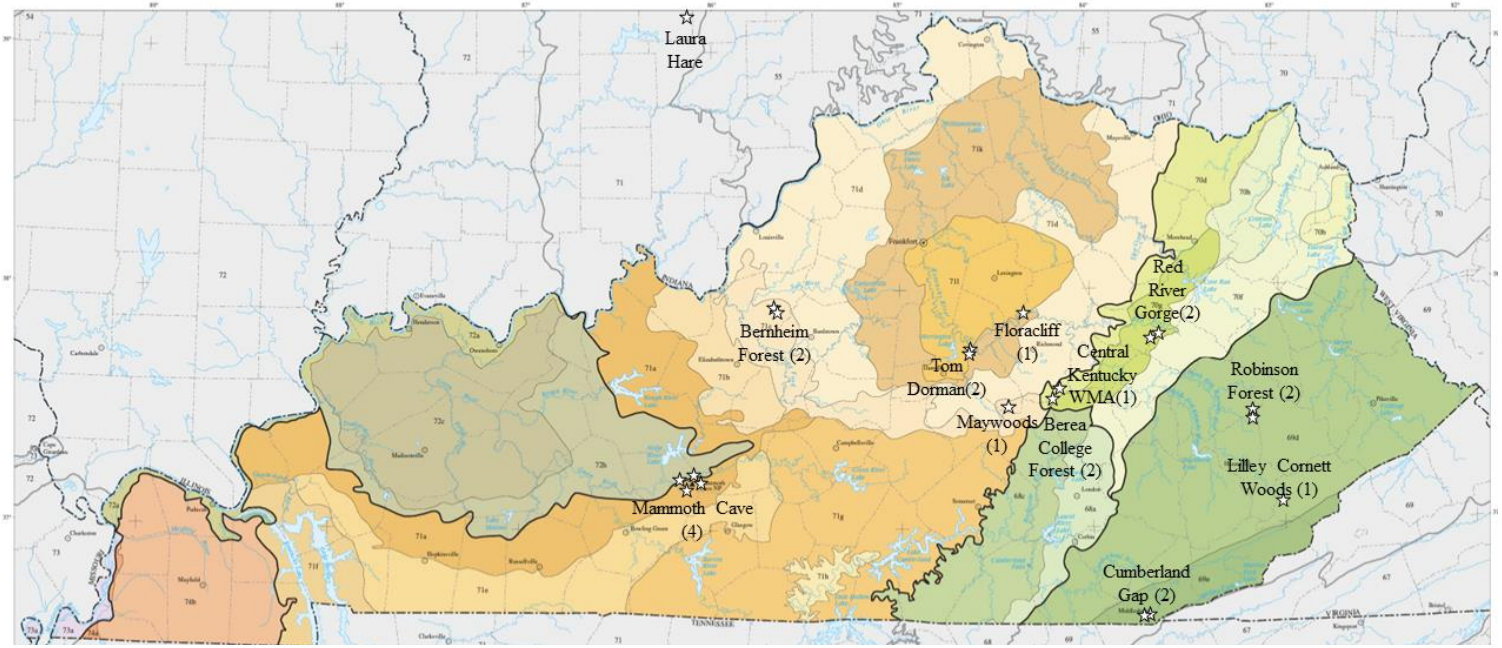

Figure 3.1. Ecoregion map of Kentucky denoted with sampling locations. Numbers in parentheses denote number of populations at that site. Ecoregion map adapted from Woods et al., 2002.

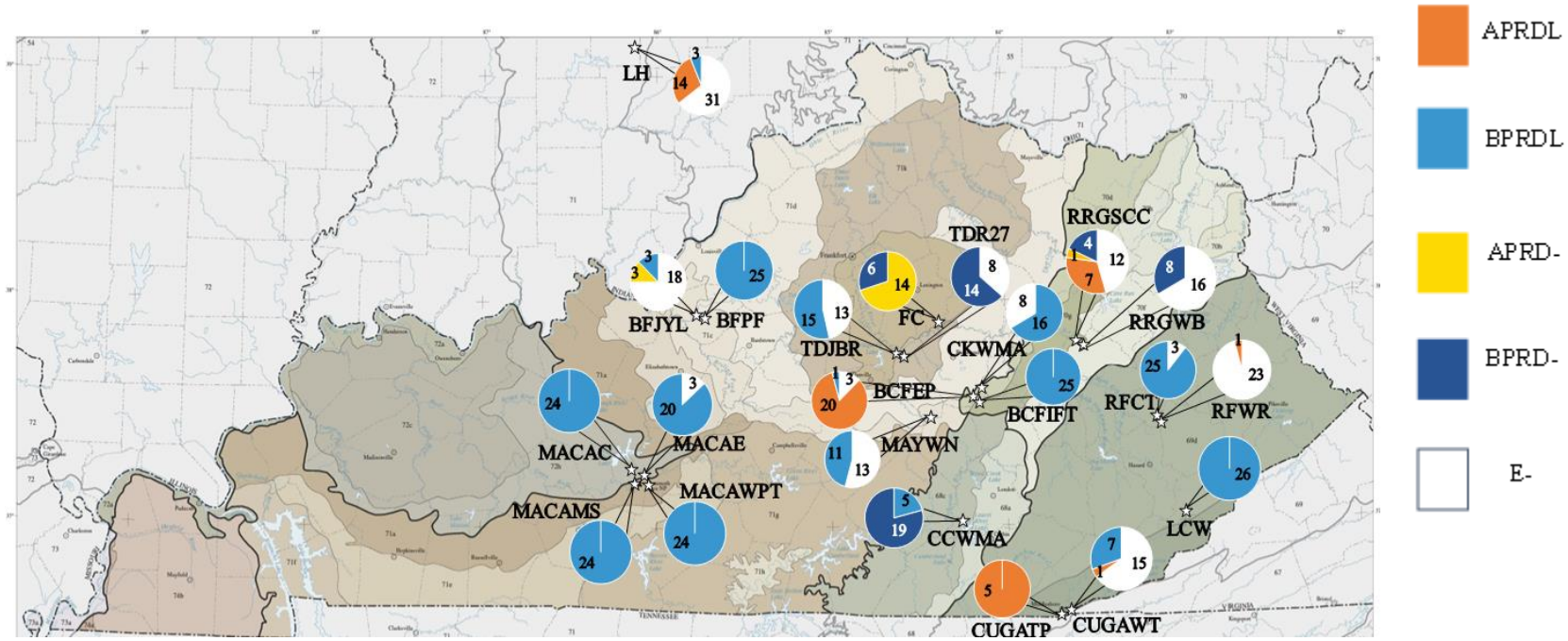

Figure 3.2 Genotype Map. Pie charts of genotypes and endophyte-negative samples present at each population, not including cultures. Ecoregion map adapted from Woods et al., 2002.

Locations were sampled in ten different level four ecoregions (Figure 3.1, Table 3.4). The Shannon Diversity Index (SDI) was calculated (denoted as *H*-value) to measure the endophyte diversity at the different hierarchical levels of population and ecoregion levels III and IV (Table 3.4). Though usually performed to measure the diversity of a population of different species, SDI was used in this case to reflect the number of unique genotypes and how evenly genotypes were distributed among those individuals (similar to work completed in Grof-Tisza et al., 2021). The *H*-value was calculated both with chemotype (all alkaloid genes tested but not mating types) and genotype (including mating types) across individual populations and ecoregions. The higher the *H*-value, the higher the diversity (Kim et al., 2017). CCWMA, RRGSCC, RGGWB, and BFYJL had positive (non-zero) *H*-values for chemotypes (Table 3.4). In addition to those four populations, LCW, CUGATP, BCFEP, LH, and FC had both mating types, reflected in their positive *H*-values for genotypes (Table 3.4). RRGSCC's *H*-value for genotype was the highest of the analyses, being the only site to produce samples of every combination of *lolC* presence/absence and mating types (Table 3.3, 3.4). Looking at groups of different level IV ecoregions, ecoregions 68c, 70g, 71c, and 71l all had positive *H*-values based on chemotype (Table 3.4). Those same groups, along with 69d, 69e, and 70d had positive *H*-values based on genotype (Table 3.4). When grouping across level three ecoregions, levels 68, 70, and 71 had positive *H*-values for chemotype (Table 3.4). All level III ecoregions except ecoregion 72 had positive *H*-values for genotype (Table 3.4). *H*-values of zero occurred when populations in that region did not display chemotypic or genotypic diversity (ex. MACAM and MACAWPT in region 72h) (Table 3.3, 3.4).

Table 3.4 Diversity Measures

Population	Ecoregion	Chemotype *H*	Genotype *H*	Ecoregion	Chemotype *H*	Genotype *H*	Ecoregion	Chemotype *H*	Genotype *H*
CCWMA	68c	**0.588**	**0.588**	68c	**0.588**	**0.588**	68	**0.588**	**0.355**
LCW	69d	0.000	**0.158**	69d	0.000	**0.161**	69	0.000	**0.369**
RFCT	69d	0.000	0.000						
RFWR	69d	0.000	0.000						
CUGATP	69e	0.000	**0.377**	69e	0.000	**0.690**			
CUGAWT	69e	0.000	0.000						
BCFEP	70d	0.000	**0.179**	70d	0.000	**0.673**	70	**0.613**	**0.812**
BCFIF	70d	0.000	0.000						
RRGSCC	70g	**0.643**	**1.224**	70g	**0.625**	**1.126**			
RRGWB	70g	**0.562**	**0.562**						
MACAC	71a	0.000	0.000	71a	0.000	0.000	71	**0.574**	**0.734**
MACAE	71a	0.000	0.000						
BFJYL	71c	**0.693**	**0.693**	71c	**0.231**	**0.590**			
BFPFL	71c	0.000	0.000						
LH	71c	0.000	**0.466**						
MAYWN	71c	0.000	0.000						
CKWMA	71d	0.000	0.000	71d	0.000	0.000			
FC	71l	0.000	**0.562**	71l	**0.625**	**1.089**			
TDJBR	71l	0.000	0.000						
TDR27	71l	0.000	0.000						
MACAM	72h	0.000	0.000	72h	0.000	0.000	72	0.000	0.000
MACAWPT	72h	0.000	0.000						

Table 3.4. *H*-value measure of diversity grouped by ecoregions (levels III and IV) in which the populations were sampled. Chemotype *H* is based on chemotype (alkaloid genes), Genotype *H* is based on genotype (all genes assayed including mating type)

Table 3.5 Soil Types					
Population	**Infection %**	**Genotypes**	**Soil Unit Symbol**	**Soil Description**	**% of Area**
BCFEP	87.5%	20 APRDL 1 BPRDL	uFcrF	Faywood-Cynthia-Rock outcrop complex, 30-65% slopes	83.2%
			WeG	Welkert channery silt loam, 40-80% slopes	16.8%
BCFIFT	100%	25 BPRDL	uFcrF	Faywood-Cynthia-Rock outcrop complex, 30-65% slopes	11.8%
			WeG	Welkert channery silt loam, 40-80% slopes	73.6%
			WpE	Woolper very stony silty clay loam, 12-30% slopes	14.6%
BFJYL	25%	3 BPRDL 3 APRD-	EkC	Elk silt loam, 6-12% slopes	15.8%
			LfE	Lenberg-Carpenter complex, 20-40% slopes	44.7%
			TrD	Trappist silt loam, 12-30% slopes, eroded	39.5%
CCWMA	100%	5 BPRDL 19 BPRD-	LhD	Latham-Lily complex, 6-20 % slopes	17.4%
			SdF	Shelocta-Latham complex, 30-60 % slopes, stony	82.6%
CKWMA	33.33%	8 BPRDL	CsF3	Colyer shaly silty clay loam, 12-50% slopes, severely eroded	63.7%

Table 3.5 Soil Types Cont.					
Population	**Infection %**	**Genotypes**	**Soil Unit Symbol**	**Soil Description**	**% of Area**
CUGA	46.4%	6 APRDL 7 BPRDL	AtF	Alticrest-Totz-Helechawa complex, rocky, 20-55% slopes	38.4%
			HeF	Helechawa-Varilla-Jefferson complex, 35-75% slopes, very rocky	61.6%
FC	100%	14 APRD-6 BPRD-	FaD3	Fairmount very rocky silty clay loam, 6-30% slopes, severely eroded	41.1%
			MpC2	McAfee silty clay loam, 6-12% slopes, eroded	6.8%
			MpD2	McAfee silty clay loam, 12-20% slopes, eroded	12.2%
			Rk	Rock land outcrop-fairmount complex, 20-50% slopes	34.3%
			uBlmB	Bluegrass-Maury silt loams, 2-6% slopes	5.8%
LCW	100%	26 BPRDL	CkF	Cloverlick-Kimper-Highsplint complex, 30-65% slopes, very stony	7.8%
			uShgF	Shelocta-Highsplint-Gilpin complex, 20-70% slopes, very stony	92.2%

Table 3.5 Soil Types Cont.					
Population	**Infection %**	**Genotypes**	**Soil Unit Symbol**	**Soil Description**	**% of Area**
LH	35.42	14 APRDL 3 BPRDL	Be	Beanblossom channery silt loam, occasionally flooded	3.6%
			BgF	Berks-Trevlac-Wellston complex, 20-70% slopes	72.3%
			WaD	Wellston-Berks-Trevlac complex, 6-20% slopes	24.1%
MACAC	100%	24 BPRDL	CoB	Clarkrange silt loam, 2-6% slopes	30.3%
			LyC2	Lily loam, 6-12% slopes, eroded	26.0%
			WbF	Wallen-Bledsoe-Donahue complex, 35-50% slopes, very rocky	31.3%
			WeC2	Wellston silt loam, 6-12% slopes, eroded	12.4%
MACAE	86.96%	20 BPRDL	CkD	Caneyville-Rock outcrop complex, 6-20% slopes	100%

Table 3.5 Soil Types Cont.					
Population	**Infection %**	**Genotypes**	**Soil Unit Symbol**	**Soil Description**	**% of Area**
MACAMS	100%	24 BPRDL	CoB	Clarkrange silt loam, 2-6% slopes	14.9%
			JfD	Jefferson-Lily-Rock outcrop complex, 12-20% slopes	29.2%
			LyC2/D2	Lily loam, 6-20% slopes, eroded	6.0%
			WeB	Wellston silt loam, 2-6% slopes	22.1%
			WeC2	Wellston silt loam, 6 to 12 percent slopes, eroded	17.7%
MACAWPT	100%	24 BPRDL	CoB/C	Clarkrange silt loam, 2-12% slopes	25.3%
			JfE	Jefferson-Lily-Rock outcrop complex, 20-35% slopes	9.9%
			LyC2/D2	Lily loam, 6-20% slopes, eroded	64.8%
MAYWN	45.83%	11 BPRDL	CgE2	Carpenter-Lenberg complex, 12-30% slopes, eroded	62.1%
			CpF2	Colyer-Trappist complex, 25-60% slopes, eroded, very rocky	37.9%

Table 3.5 Soil Types Cont.					
Population	**Infection %**	**Genotypes**	**Soil Unit Symbol**	**Soil Description**	**% of Area**
RFCT	89.29%	25 BPRDL	uCskF	Cloverlick-Shelocta-Kimper complex, 20-80% slopes, very stony	46.6.%
			uMgmF	Matewan-Gilpin-Marrowbone complex, 12-80% slopes, very rocky	53.4%
RFWR	4.17%	1 APRDL	uCskF	Cloverlick-Shelocta-Kimper complex, 20-80% slopes, very stony	18.6%
			uMgmF	Matewan-Gilpin-Marrowbone complex, 12-80% slopes, very rocky	81.4%
RRGSCC	58.33%	7 APRDL 1 APRD- 4 BPRD-	ArF	Alticrest-Ramsey-Rock outcrop complex, 20-65% slopes	10.9%
			GnD	Gilpin silt loam, 12-20% slopes	75.3%
			LaD	Latham silt loam, 8-25% slopes	13.8%
RRGWB	33.33%	8 BPRD-	BsF	Bledsoe-Berks-Rock outcrop complex, 20-70% slopes	66.4%
			HeF	Helechawa-Rock outcrop complex, 35-55% slopes	33.6%

Table 3.5 Soil Types Cont.					
Population	**Infection %**	**Genotypes**	**Soil Unit Symbol**	**Soil Description**	**% of Area**
TDJBR	53.57%	15 BPRDL	FdF2	Fairmount-Faywood-Rock outcrop complex, 25-50% slopes, eroded	100%
TDR27	63.64%	14 BPRD-	FfC2/D2	Faywood-Fairmount complex, phosphatic, 6-25% slopes, eroded, very rocky	100%

Table 3.5 Soil types of each population with infection frequency and genotypes. Information accessed via Staff, 2019.

Figure 3.3. Cane Creek Wildlife Management Area (CCWMA) soil type map. Seven samples were mapped according to their GPS coordinates to estimate the area of interest drawn for soil analysis.

Figure 3.4. Floracliff (FC) soil type map. All samples were mapped according to their GPS coordinates to estimate the area of interest drawn for soil analysis.

Soil types of the sample sites were analyzed to see if there was any relationship that existed between infection frequency, genotype, and soil characteristic(s). While no pattern emerged for infection frequency or number of genotypes, some conclusions were interesting to note. Of the approximately 50 soil types, over 25 were some type of silty/clay loam or loam-complex. Additionally, over 20 of the listed soil types were described as rocky or stony (Table 3.5). Three sites, Mammoth Cave Echo River (MACAE), Tom Dorman Jesse Brims Road (TDJBR), and Tom Dorman Route 27 (TDR27) were made up of only one soil type while all other sites ranged from two to five soil types (Table 3.5). For CCWMA (where more GPS coordinates were recorded), it was noted that all five samples that were *lolC+* (the other 20 samples being *lolC-*) resided in only one of the two soil types present (SdF – Shelocta-Latham complex) rather than being dispersed throughout both (Figure 3.3). Similarly, all of the *matB* samples from FC resided in only one of the five soil types present (Figure 3.4).

3.4 DISCUSSION

Alkaloid gene loci are known to be unstable because of repeat blocks and activity of retroelements, DNA transposons, and miniature inverted-repeat transposable elements (Schardl et al., 2013). It has been suggested that this instability has been selected for as means of chemotypic diversification within epichloid species (Schardl et al., 2013). This diversification can mainly be measured by presence or absence of the alkaloid biosynthesis genes. Diversity within an alkaloid pathway can also be linked with presence of functional domains or component genes (Charlton et al., 2014). In turn, variations in members of biosynthesis pathways could be used to predict the alkaloid-production capabilities of the endophyte (Charlton et al., 2014). Previously, similar characterization studies noted that alkaloid diversity can be high within an individual species of *Epichloë* (Vikuk et al., 2019; Schardl, 2012). Variation in alkaloid profiles

may even be greater in wild grasses because of the heterogeneity present in both endophyte and host genotypes compared to controlled agronomic settings (Malinowski & Belesky, 2019; Saikkonen et al., 2004). For this system, it was entirely unknown if chemotypic diversity would exist, and what would contribute to such diversity if it was present. Additionally, we were interested in possible patterns of distribution for the diversity that could exist, both geographically and by mating types (evidence of sexual vs. clonal populations). Our characterization of the symbiont *Epichloë brachyelytri* within native populations of *Brachyelytrum erectum* revealed a small amount of genotypic diversity that was unevenly distributed across the sampled area. Notably, chemotypic diversity characterized as presence or absence of *lolC* was found mainly in Central Kentucky, whereas all *E. brachyelytri* populations in Eastern Kentucky and most in Western Kentucky tested positive for all screened alkaloid genes in my PCR assay. Additionally, several of the populations were exclusively mating type B (*matB)* including all four in a transect across Mammoth Cave National Park in Western Kentucky.

3.4.1 POSSIBLE EXPLANANTIONS FOR *LOLC* POLYMORPHSIM

The sole contributor to chemotypic diversity in the 459 total positive samples that were screened was absence of *lolC,* a gene required in the preliminary steps of loline alkaloid biosynthesis. The presence/absence polymorphism of *lolC* as well as *dmaW* has been documented before in some isolates of *E. brachyelytri* (Schardl, 2012). The polymorphism for *lolC* was found in samples of both mating types in a total of six populations in Central Kentucky (Table 3.3, Figure 3.2). There may be explanations for the lack of diversity from any other alkaloids screened as well as its geographical distribution, in which two will be described here:

the metabolic load of loline alkaloid production (with cheater endophytes possibly arising) and Muller's ratchet.

Though loline alkaloid levels have not been measured for *E. brachyelytri,* loline alkaloid production and accumulation in endophyte systems is known to occur at high levels, even exceeding endophyte biomass (Schardl et al., 2004). Such high levels help confer resistance to invertebrate feedings and also offer water stress tolerance. Production, however, can be limited by availability of amino acid precursors (Zhang et al., 2009). Alkaloids themselves are metabolically costly to produce and compete with other host processes and nutrients (Faeth & Fagan, 2002). Depending on the pressure of insects that are sensitive to loline alkaloids, as well as drought occurrence, there may be some sites where the tradeoff for loline alkaloid production is not counterbalanced by the advantages they may confer. It may be that at sites where loline alkaloid production was absent, those individuals were under such metabolomic stress that the benefits of loline alkaloids no longer outweighed their production costs, selecting for *lolC*-negative endophytes. In contrast, in environments where loline alkaloids sufficiently counterbalance the metabolic burden of their production, occurrence of *lolC* in most or all of the endophytes is expected. On a similar note, because loline alkaloids may be metabolically expensive, the opportunity arises for cheater endophytes; i.e., those that do not provide the net benefits of their conspecifics but still gain benefit from the endophyte-associated community (Afkhami & Rudgers, 2008). Some individuals may not partake in the costly production of lolines if protection by alkaloid-producing neighbors is sufficient, which could especially be the case in populations that had both *lolC*+ and *lolC*− chemotypes (BFJYL, CCWMA, RRGSCC, RRGWB) (Table 3.4, Figure 3.3).

Previously, an isolate of *E. brachyelytri* from near Mammoth Cave was found to have inactive or nonfunctional *lolO, lolN, lolM,* and *lolP* genes in the 11-gene *LOL* cluster (Schardl et al., 2013). Contrary to the activity of *lolC* in the first reactions of 1-aminopyrrolizidine biosynthesis, *lolN, lolM,* and *lolP* encode loline-decorating (i.e., moiety-addition) genes rather than determinant steps in the loline alkaloid pathway (Schardl et al., 2013; Schardl, 2012). However, the *lolO* gene product is required for the ether bridge that characterizes the lolines in the strict sense (although in this thesis I use the term *lolines* more generally as synonymous with 1-aminopyrrolizidines) (Pan et al., 2018). Genome sequences of that isolate, as well as the *E. brachyelytri ex*-type isolate (ATCC 200753 = E1124) from Sullivan Co., New York (genome sequence in NCBI as accessions CP097975-CP097982), show an inactivating deletion in *lolO*. In strains lacking functional *lolO*, the pathway end-product is 1-acetamidopyrrolizidine (AcAP), which has also been detected in samples from the Palisades Tom Dorman trail (Padmaja Nagabhyru, Tirzah Schanding and C.L. Schardl, unpublished results). Although the lolines have not been characterized for most isolates in this study, this preliminary information suggests that those strains with *lolC* produce AcAP.

Another factor to consider as a possible cause of the distribution of the *lolC* presence/absence polymorphism is Muller's ratchet, a concept that describes the accumulation of deleterious mutations, especially in asexual populations, so that population size and overall fitness gradually decline (Gabriel et al., 1993). Even mildly deleterious mutations can be maintained for long periods of time (Gabriel et al., 1993). If lolines offer the same insecticidal and drought tolerance benefits to *B. erectum* as they do in other grass-*Epichloë* symbioses, the inactivation of the loline biosynthesis pathway would thus be deleterious, but not lethal. Considering that most *E. brachyelytri* populations in this study were one mating type

(predominately *matB*), it is likely that their reproduction in those populations was entirely or almost entirely asexual. Therefore, by the action of Muller's ratchet, the *lolC*− chemotype may have been perpetuated and distributed through and among populations rather than removed by selection, even if it is more deleterious than the AcAP-producing chemotype.

3.4.2 MATING TYPE

In addition to Muller's ratchet, solely asexual reproduction can be disadvantageous because of the restricted ability to respond to changing selection pressures (Saikkonen et al., 2004). Mating type is an important contributor of genetic variation because of the opportunity for genetic recombination, but variance could be further impacted when compounded with chemotypic differences. Although *matA* was rarer than *matB*, it is interesting to note that the *lolC* variation and *matA* genotype did not necessarily correspond geographically (Figure 3.2). The *lolC* variation occurred in 3 ecoregions (68c, 71l, 70g), its distribution lying more east to west. *matA* occurred in 7 ecoregions (68c, 69e, 69d, 70d, 70g, 71c, 71l), its distribution lying more north to south (Table 3.3, Figure 3.1). Selection should be acting on these mostly asexual populations, but presence of the additional mating type suggests that sexual reproduction of *E. brachyelytri* sometimes occurs in some locations (however rarely), thereby introducing genetic variation through recombination. It is thought that in cases of endophytes that have lost sexuality (those that never produce the external stroma required for mating), the host plant is largely in control of the interaction via "sanctions", in which the host can respond on the basis of the behavior of the symbiont (Saikkonen et al., 2004; Afkhami & Rudgers, 2008). In addition to maintaining a limited amount of genetic recombination in the fungus, retaining the ability to sexually reproduce may also protect the endophyte from complete dependence on host

reproduction and host genetic outcrossing, which would otherwise provide the only novel genetic combination in the endophyte-host system.

3.4.3 PERAMINE REDUCTASE

Epichloë spp. commonly display allelic variation along the peramine biosynthesis pathway; often containing one fully functional allele, and/or one with a deletion in the C-terminal reductase (R) domain (Berry et al., 2019). The forms without the R domain catalyze biosynthesis of diketo-pyrrolopyrazines rather than the monoketo-pyrrolopyrazine, peramine (Berry et al., 2019). The variation present in *Epichloë* is an example of transspecies polymorphism, where the allele with the reductase and the allele with the deleted reductase domain constitute two separate clades exhibiting parallel evolution (Berry et al., 2015). Trans-species polymorphism suggests balancing or diversifying selection, and the classic example is the major histocompatibility locus in animals (Cutrera & Lacey, 2007) . Examples are also known in other fungi, including for secondary metabolites (Cutrera & Lacey, 2007). The allelic variation in the peramine reductase domain has not previously been found in *E. brachyelytri*, but only three samples were investigated in that study (Berry et al., 2015). Our screenings revealed that the sampled populations of *E. brachyelytri* still contain the peramine reductase domain and no R-domain deletion was detected. Therefore, it is possible that the only allele for biosynthesis of peramine is present in this species.

3.4.4 OTHER CONSIDERATIONS

To see if there was any relationship that existed between infection frequency, genotype, and soil type(s), soil characteristics for each population was analyzed using the Web Soil Survey application published by the United States Department of Agriculture Natural Resources

Conservation Service. While no pattern emerged for infection frequency or number of genotypes in relation to soil type or diversity, some conclusions were interesting to note. Of the ~50 soil types, over 25 were some type of silty or clay loam or loam-complex. Over 20 other soil types were also described as rocky or stony. Loam soils have been reliably used in greenhouse studies of tall fescue (*Lolium arundinaceum*) and the corresponding endophyte *Epichloë coenophiala* (Arachevaleta et al., 1989; Buyer et al., 2011), so perhaps presence of loam soils of endophyte-infected communities is not surprising. Interestingly, the only soil type to be given a mineral characteristic was the FfC2/D2 (Faywood-Fairmount complex) soil found at TDR27. The soil complex is described as phosphatic for this region. Given that mineral levels were not measured in this study, it would be compelling to analyze pH or mineral levels (like carbon, nitrogen, phosphorus, or calcium) in future work to compare with alkaloid diversity or phenotypic response in infected and uninfected plants.

The chemotypic characterization of *E. brachyelytri* could have exciting implications for future endophytic research. What remains to be known following discoveries of chemotypic diversity is how the diversity affects the competitive ability of the endophyte-host system. When looking at plant volatiles under herbivore pressure on populations of sagebrush, it was found that chemotypic diversity in volatile biosynthesis was negatively correlated to herbivory pressure (Grof-Tisza et al., 2021). In other words, less chemotypic diversity of a community resulted in more damage by herbivores. It would prove valuable to predict how dynamics and consequences of an endophyte-host interaction may change based on the chemotypes in a single species (e.g., pathogen stress, herbivory and feeding pressure, drought tolerance, morphological parameters etc.). This type of research is ongoing with the design of "symbiotically modified organisms" (SMO); the selection of novel endophyte strains that produce none or negligible amounts of

alkaloids harmful to livestock, but still retain host grass tolerance to environmental and biotic stresses (Malinowski & Belesky, 2019; Gundel et al., 2013). As more native endophytes are characterized, natural findings of diversity may help increase options for hybridization of novel endophyte strains. Infected *B. erectum* presents a unique challenge to these types of experiments, as they have never successfully been cultivated in a greenhouse. The information presented is still valuable in the implications to endophyte common garden experiments as well as ecological considerations.

Finally, this evidence supports the postulate that interactions between endophytes and their host plants are not fixed ecologically, geographically, or over evolutionary time. Long-term ecological investigations could examine the stability and trajectory of chemotype variations (e.g., monitoring RRGWB profiles in response to increased herbivore pressure and history of changing water availability). It would be especially pertinent to document these changes alongside environmental changes that alter the stressors on communities (e.g., herbivore pressure, water availability, rising temperatures, or rising atmospheric CO_2 concentrations), therefore altering the competitive ability of the infected grass host. The genetic interactions between endophyte and host, and ecological impacts both on and by the endophyte are multifarious. This project aimed to better capture chemotypic and genotypic diversity that may have been present in native populations of *Brachyelytrum erectum* and *Epichloe brachyelytri,* as well as ponder the implications of such diversity.

CHAPTER 4: CONCLUSION

This project aimed to expand our knowledge on the infection frequencies and geographical distribution of *Epichloe brachyelytri* in cool-season grass host *Brachyelytrum erectum,* how infection may have an impact on functional traits, and chemotypic diversity distributed throughout 22 natural populations of *B. erectum*. Reproductive height, specific leaf area, specific stem density, and tiller count were measured as phenotypes to compare with endophyte status as determined using established PCR methods (Charlton et al., 2012; Charlton et al., 2014). These analyses were performed on individual populations (Figure 2.3), as well as the totals combined from the populations (Figure 2.4). Chemotypes were established based on presence/absence of key genes: *tefA, lolC, dmaW, perA, perA-R, matA,* and *matB.* Although absence of *lolC* and *dmaW* has been previously reported for *E. brachyelytri* (Schardl, 2012), the only contributor to chemotypic diversity in our samples was presence/absence of *lolC.* The persistence of the *lolC*- chemotype may be promoted by cheater endophytes and Muller's ratchet, but also when the cost of loline production is not counterbalanced.

Statistically significant in both the CKWMA population and when all applicable populations were combined, stem specific density was lower in endophyte-positive plants (Figure 2.3C, Figure 2.4C). Considering the tradeoff between low and high stem density (faster growth and increased hydraulic capacity vs. increased structural integrity and biotic stress resistance), it may be that a host can afford to lower stem density and reap the benefits of faster growth while also relying on the endophyte to serve as an alternate form of resistance against pathogens, herbivores, and abiotic stressors. The RRGSCC population had statistically significant differences in the specific leaf area of endophyte positive and negative samples (p=0.00735), with negative samples having higher specific leaf area (Figure 2.2B). From here, we concluded that endophyte presence can, but will not always, impart a significant effect on

specific leaf area when looking at individual populations, and the benefits of hosting an endophyte must outweigh the disruption to a plant's resource allocation strategy that occurs when leaf area is decreased. RRGSCC also contained the highest number of genotype combinations among all 22 populations, showing diversity in both mating type, presence/absence of *lolC,* and endophyte-negative plants (Table 3.3). It may be that this genotypic diversity serves as a way to expand what selection can act on; that is, to find the best combination of genes and/or endophyte presence where the benefits of that endophyte outweigh the disruptions in resource allocation (e.g., changes to leaf specific area and stem specific density, and the metabolic costs of producing alkaloids and/or hosting the endophyte altogether).

In some sites, the genotypic diversity (or lack thereof) was intriguing to consider with physical observations the team made while sampling that may support a metabolic tradeoff of loline production and/or endophyte maintenance. For example, the RRGWB site had plants that were heavily marked with herbivore damage and was in a region commonly has ecological disturbances (e.g. landslides due to flooding in surrounding regions) (Rogers, 2020). At the same time, RRGWB site was also low in infection frequency (33.3%) and displayed the *lolC*− polymorphism in all samples that were positive (Table 2.2, Figure 3.2). Major geological disturbances can change or impact an ecological system in resilience, robustness, or vulnerability (Schoon & Cox, 2012). In this case, the cost and benefit balance of hosting an endophyte and/or producing all alkaloids would be affected. In addition to their insecticidal activity, loline alkaloids are suggested to play a role in reducing the deleterious effects of drought stress by lowering osmotic potential (Malinowski & Belesky, 2019; Bush et al., 1997). If the population is no longer in optimal condition, factors that are usually beneficial in environments (endophyte presence and alkaloid production) with standard drought and herbivore pressures could become

detrimental, thereby selecting for endophyte absence or absence of *lolC.* Though the benefits of endophyte infection in adverse conditions have been discussed, there is also evidence for negative effects on infected hosts when conditions change (Malinowski & Belesky, 2006; Morse et al., 2002). Additionally, to become robust to some types of ecological variability, complex systems (such as endophyte-host relationships) must trade off the ability to cope with other types of variability (Janssen et al., 2007). Perhaps in order for RRGWB plants to adapt to the frequency of ecological disturbances, the benefit of herbivory protection via loline alkaloids must be traded. This could especially be the case if invertebrate pressure (or those that are sensitive to loline alkaloids) was low to begin with. Vertically transmitted endosymbionts, like *E. brachyelytri*, cannot persist in host populations if host fitness is reduced compared to uninfected individuals (Clay & Schardl, 2002). If the environment was changing over time, selection could have transitioned to favor complete loss of the endophyte, in turn reducing the infection frequency of the site. Without resources dedicated for herbivore protection in those plants that maintained the endophyte, feeding activity would increase, which matches the intense defoliation we observed at the site.

These observations would support the concept that evolutionary adaptations of endophyte-infected communities to benefit a host system in a specific environment may completely fail once the community is exposed to contrasting conditions (Malinowski & Belesky, 2006; Hesse et al., 2003). Perhaps there are circumstances where the best option is to reduce an endophyte's effect on resource allocation (but still help deter herbivory), but other environments may also develop where the overall burden imposed by endophyte (even *lolC*−) does not outweigh the cost. Additionally, perhaps endophyte status could be included as a measure in future robustness, resilience, and vulnerability studies due to ecological disturbances.

It is important to remember that one or two years of sampling only provides a snapshot of current population behavior, which is certainly subject to a great quantity of change over evolutionary time.

The Mammoth Cave sites also offered some dramatic findings. A small number of unfertilized stromata were found across the sites. More interesting, however, is that between the four populations that transected the over 50,000-acre national park, there was no variation in chemotype or mating type. All sites were near 100% infected, displayed all alkaloid genes, and were only mating type B (Table 3.3, Figure 3.2). These findings may point to an entirely clonal population. Asexual endophytes that are vertically transmitted exhibit less genetic variation than those that sexually recombine because the same endophyte genotype is transmitted through seeds (Clay & Schardl, 2002). This large population throughout Mammoth Cave appeared to be completely free of the additional mating type, leaving no opportunity for genetic recombination, which could be a factor in the lack of chemotypic diversity. In a different epichloid system, endophyte infected was previously found to directly increase a host plant's propensity for clonal reproduction and self-pollination (Pan & Clay, 2002; Saikkonen et al., 2004). Clonality at Mammoth Cave National Park could also be supported by the high infection frequencies. It is thought that in established populations, a successful host-fungus combination will prevail as incompatible combinations are reduced (Saikkonen et al., 2004). This logic is usually applied to more homogenous agronomic settings but could very well be a component of some natural populations. If the Mammoth Cave sites contain host plants that serve a well-matched endophytic genotype, high infection frequencies would likely be maintained.

Geographic distributions of infection frequency and chemotypes were noted as well (Figure 3.2). Most populations that had endophyte-negative samples were in Northern and

Central Kentucky, though the incidence of 100% infected sites remained higher than initially predicted. Presence of the *lolC*− polymorphism was found mainly in Central Kentucky, whereas all *E. brachyelytri* populations in Eastern Kentucky and most in Western Kentucky consistently had all alkaloid genes tested. In contrast, the rare presence of *matA* over *matB* did not occur in the Eastern regions, reducing the likelihood that diversity from sexual recombination will arise in that region.

This thesis provides valuable work on the knowledge gaps that has existed both on chemotypic diversity and functional trait data on endophyte-host dynamics in natural settings, as well as the infection frequencies and genotypic diversity of *B. erectum* and *E. brachyelytri*. It is my hope that this work can provide data for future use in transcriptional and evolutionary studies using this particular endophyte-host system, along with consideration of endophyte-host relationships in more broad ecological studies.

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VITA

RACHEL SNEED

Education

MS University of Kentucky, Plant Pathology & Plant Molecular Biology — September 2023
Thesis: Assessing Endophyte Frequency Distributions and the Effect of *Epichloë brachyelytri* in the Chemotypic and Genotypic Diversity in *Brachyelytrum erectum*
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BS Southern Illinois University – Edwardsville — December 2020
Genetic, Molecular, and Cellular Biology, Chemistry
Honors Scholar

Professional Experience

Scientist III, HydroGeoLogics, St. Louis, MO — September 2023 - present

Graduate Research Assistant, University of Kentucky, Lexington, KY — August 2021 – August 2023

Laboratory Analyst Technician I, AVESI Inc., St. Louis, MO — May 2021-August 2021

Environmental Science Health Physics Technician II, AVESI, Inc., St. Louis, MO — May 2020-May 2021

Physics I & II Supplemental Instructor, Southern Illinois University – Edwardsville — January 2020- December 2020

Professional Presentations

Oral Presentations

Assessing Endophyte Frequency Distributions and the Effect of *Epichloë brachyelytri* in the Chemotypic Diversity of *Brachyelytrum erectum.* Poisonous Plants Conference, US Department of Agriculture, Logan, UT, 2023.

Assessing Endophyte Frequency Distributions and How Genetic Diversity Influences Functional and Phylogenetic Diversity. Poisonous Plants Conference, Montana State University, Bozeman, MT, 2022.

Poster Presentations

Genomic Analysis of Fungal Population Diversity in Host Communities:

A study of symbiosis between the grass *Bracheylytrum erectum* and endophyte *Epichloë brachyelytri.* Mycological Society of America Annual Meeting, University of Florida, Gainesville, FL, 2022.

Scholastic and Profession Honors

***Eller/Billings Student Research Award,* Appalachian Center of University of Kentucky** April 2023

Amount: $750. Wrote research proposal, estimated budget, and procured two recommendation letters for work related to and benefiting the Appalachian region.

***Professional Development Award for Graduate and Professional Student Organizations,* Graduate Student Congress at the University of Kentucky** November 2022

Amount: $500. Wrote proposal for professional development opportunity to be utilized by the Association of Plant Pathology Scholars at the University of Kentucky.

***Plant Pathology Experiential Department Award,* American Phytopathological Society Foundation Office of Private Sector Relations** April 2021

Amount: $4,000. Co-wrote proposal for travel subsidy for use in professional development to be utilized by the Association of Plant Pathology Scholars at the University of Kentucky.

***Most Outstanding Baritone,* Blue Stars Drum and Bugle Corps** August 2019

Non-monetary. Awarded for leadership, personability, and visual and musical technique.

***Molecular Research and Experimental Design Award,* Southern Illinois University – Edwardsville** March 2019

Non-monetary. Awarded for experimental design midterm project in Cell and Molecular Biology.

***Recognition of Detail Orientation and Note-taking skills,* Southern Illinois University – Edwardsville** August 2018-December 2020

Non-monetary. Recognition by various professors of excellent and detailed notes, organized class materials, and self-made study guides to turn in weekly to Disability Services for use by other students.

***Provost Scholarship,* Southern Illinois University – Edwardsville** May 2017

Amount: Full tuition for 4 years ($88,000). Academic scholarship won on high school achievements, essay, and interview participation.