



Return of the *Phrag-i*: evaluating sexual reproduction mechanisms amenable to dieback recovery and potential invasiveness across *Phragmites australis* haplotypes

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Abstract *Phragmites australis* is one of the most invasive wetland plants on the planet with both native and invasive haplotypes occurring in the United States. Three *Phragmites* haplotypes (Delta-, EU- and Gulf-types) co-occur in marshes of the Mississippi River Delta (MRD), where a recent dieback of *Phragmites* has prompted investigations about the potential for recolonization by seed. In other areas of the US, the invasive EU-type has been shown to spread by seed, yet little is known about reproduction modes of the Delta- and Gulf-types. We conducted a survey at 35 sites along the Mississippi River Delta region in southeast Louisiana to examine the potential

for sexual reproduction across haplotypes as well as the potential for hybridization. Seed and pollen samples were collected from *Phragmites* populations to examine flowering phenology and determine pollen viability of the three lineages. We also conducted a seedbank assay in stands of three haplotypes to test the potential for recruitment by seed. Despite the observed potential for sexual reproduction in Delta- and EU- types, no *Phragmites* seedlings germinated from the seedbank. EU was the only haplotype to exhibit germination from seeds collected from seed heads. Both spatial separation and temporal isolation in flowering times indicate that hybridization between *Phragmites* haplotypes in the lower MRD is unlikely. High pollen production, increased pollen production following dieback, and viable seeds in the EU-type suggest that this invasive haplotype has a greater potential to invade new areas and adapt to stressors through sexual reproduction compared to than Delta- or Gulf haplotypes.

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Introduction

Phragmites australis (Cav.) Trin. Ex Steud is a cosmopolitan large emergent wetland grass that has many genetic haplotypes. Distinguished by a set of DNA variants on a chromosome that are inherited

together, *Phragmites* haplotypes also differ in niche preferences and morphology (Achenbach and Brix 2014; Lynn and Elsey-Quirk 2024). Several haplotypes of *Phragmites* co-occur in the Mississippi River Delta (MRD), also known as the Birdsfoot Delta, region, including the notoriously invasive European haplotype M (EU-type), the Delta-type (haplotype M1) and the Gulf-type (haplotype I2) also known as subspecies *P. australis* subsp. *berlandieri* (E. Fourn.) Saltonst & Hauber. The Delta- and Gulf-types are also non-native to the U.S. and Gulf coast region, despite their names, which reference the local region. In many areas of the U.S., managers are trying to eradicate EU *Phragmites* because it outcompetes other more desirable native plant species (Chambers et al. 1999; Silliman and Bertness 2004) and provides less optimal habitat for waterfowl and other species (Fell et al. 2003; Gratton and Denno 2006; Hazelton et al. 2014). However, in areas along the coast where relative sea-level rise and land loss rates are very high, such as the MRD, *Phragmites* can perform valuable ecosystem services such as reducing erosion, trapping sediment and facilitating accretion, and stabilizing channel edges which helps maintain shipping and navigation routes (Rooth et al. 2003; Kiviat 2013). The Birdsfoot Delta is generally a low-slope environment where *Phragmites* dominate fresher, low elevation areas, where its monotypic stands contribute to bank stability through a dense underground network of rhizomes. Dieback of *Phragmites* was first observed in 2016 in the Birdsfoot Delta where the Mississippi River flows into the Gulf of Mexico (Knight et al. 2018) but has also been documented in European wetlands (van der Putten 1997). The dieback of mature reed stands could compromise the stability of ecosystems in the lower Birdsfoot Delta due to the potential reduction in sediment-trapping and conversion of vegetated land to bare mudflats. Given the diverse roles of *P. australis*, from invasive nuisance plant to ecosystem engineer, it is important that we understand the propensity of sexual reproduction across the different haplotypes so we can assess the potential for invasion into new habitats and recovery following dieback.

Sexual reproduction in plants is a crucial component of invasiveness, as it can facilitate long-distance dispersal and increase genetic diversity to help adapt to novel stressors (Dong et al. 2006; Barrett et al. 2008). Additionally, sexual reproduction seems to

be the optimal mode of reproduction for invasive plants, as the majority of invasives throughout the US and China exhibit this strategy (Gao et al. 2018), and introduced species typically allocate a greater proportion of resources to reproduction compared to native species (Abrahamson 1979). For the invasive EU *Phragmites* haplotype, seed production is crucial for long-distance dispersal to facilitate invasions into new habitats (Albert et al. 2015).

Increased seed production in flowering plants is a common mechanism for progeny to escape stressful conditions, and has been documented in *Phragmites* stands in Europe suffering from dieback syndrome (Reale et al. 2011). Invasive *Phragmites* stands in the US subjected to herbicide treatments also produce a larger crop of viable seeds, as evidenced by a more robust seedbank compared to non-herbicide treated stands (Elsey-Quirk and Leck 2021). However, successful establishment and development of *Phragmites* seeds can be difficult due to increased susceptibility to flooding during the seedling stage (Haslam 1973; Reale et al. 2011). EU-type relies more on sexual reproduction and seed dispersal than the native *Phragmites australis* subsp. *americanus* Saltonst., P.M. Peterson & Soreng in North America (Kettenring and Mock 2012), and recent studies highlight the importance of seed dispersal for colonizing new habitats (Albert et al. 2015). Little is known about the sexual reproductive effort of Delta and Gulf-types, although previous studies have found no viable seeds in *Phragmites* of the northern Gulf of Mexico coast (Pellegrin and Hauber 1999). Here, we explore the reproductive phenology and pollen and seed production of the three dominant *Phragmites* haplotypes in the Birdsfoot Delta to evaluate their propensity to invade new habitats and capacity to recover following dieback.

Sexual reproduction helps plant populations cope with biotic stressors by means of increased genetic variability through recombination (Lei 2010). On the other hand, asexual reproduction is commonly associated with habitats where abiotic stressors outweigh biotic stress (Tilquin and Kokko 2016). Seeds produced through sexual reproduction are typically more robust to stressful environmental conditions than vegetative propagules, and recruitment in the beginning stages of succession and invasion often happens from seedbanks rather than from vegetative propagules such as rhizomes (Alvarez et al. 2005; Nielsen et al.

2006; Pollux et al. 2007). However, it is important to note that asexual reproduction through clonal propagation is considered the dominant mode of reproduction for *Phragmites* and confers its own distinct advantages such as the ability to reproduce without investing in energetically costly sexual reproduction (Daly 1978). Elucidating how sexual reproduction investment differs across *Phragmites* haplotypes will provide clues to which haplotypes are best suited to deal with either biotic or abiotic stressors. This distinction is important because recent *Phragmites* dieback in the MRD is associated with both biotic and abiotic stressors such as scale insects (*Nipponaclerda biwakoensis*) and salt water intrusion (Cronin et al. 2020; Lynn and Elsey-Quirk 2024; Elsey-Quirk et al. 2024).

Genetic observations of North American *Phragmites* suggest no reproductive barriers between introduced and native *Phragmites* lineages, and that introduced *Phragmites* may hybridize with natives (Meyerson et al. 2010a, b). Microsatellite surveys of Gulf Coast *Phragmites* suggest that Delta and Gulf haplotypes form viable hybrids by breeding with EU-type *Phragmites* (Lambertini et al. 2012). The potential for hybridization between *Phragmites* lineages poses a great concern for landscape managers and ecosystem health, as new invasive genotypes can arise from hybridization events (Schierenbeck and Ellstrand 2009). Plant hybrids can also exhibit higher tolerance to stressors from sources such as herbivory, which can contribute to their invasiveness or alternatively facilitate recovery following dieback (Vilà and D'Antonio 1998). We hypothesize that hybridization between *Phragmites* haplotypes could generate more invasive lineages.

To assess sexual reproduction and dieback recovery potential, it is essential that we test seed germination of freshly collected seeds as well as seedbank recruitment from soil samples, where competition from other seedlings is prevalent. Through germinating seed bank samples, we aim to reveal what other plant species are prepared to colonize following *Phragmites* dieback or removal, provided the field conditions are amenable to germination. Seedbank regeneration is typically insufficient to restore entire communities (Bossuyt and Honnay 2008), but is more likely in marshes that experience a complete dry-down, since wetland plants such as *Phragmites* struggle to germinate in completely submerged conditions

(Van Der Valk and David 1978; Weisner and Ekstam 1993; Yu et al. 2012). It is also important to understand if soil properties regardless of hydrology in dieback sites may either contribute to or inhibit germination and recovery of *Phragmites*. To test if soil factors independent of hydrology are contributing to dieback or weakening the *Phragmites*, we compared germination rates of different *Phragmites* lineages planted in soil collected from either healthy or dieback sites.

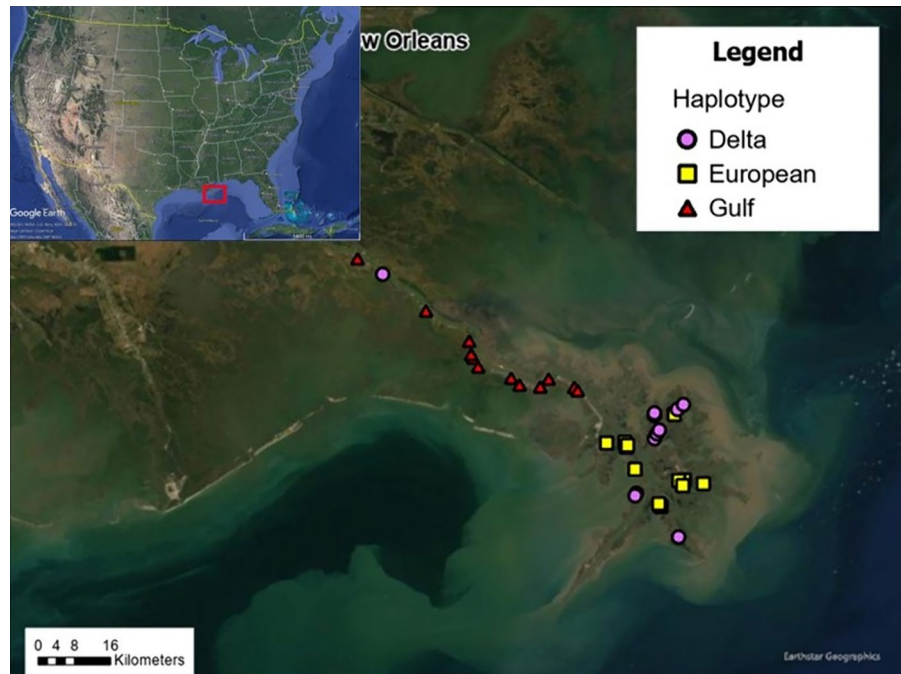
The goal of this study is to examine how *Phragmites* haplotypes across healthy and dieback stands differ in pollen viability, seed set, germination, and seedbank formation in the Birdsfoot Delta. We are also exploring the potential for hybridization based on a phenological survey of all haplotypes. We are testing the hypotheses that (1) non-native EU-type *Phragmites* will exhibit greater sexual reproductive effort than naturalized Gulf and Delta haplotypes, (2) *Phragmites* exposed to dieback conditions will exhibit greater sexual reproductive effort than those in healthy, (3) there is potential for hybridization between the three prominent *Phragmites* haplotypes in the MRD due to spatial and temporal flowering overlap; and (4) *Phragmites* haplotypes form a viable seedbank. Understanding the potential for sexual reproduction, hybridization, and recruitment by seed across haplotypes is important for predicting the spread of the haplotypes and addressing management concerns for eradicating or fostering *Phragmites* growth in areas where it is either unwanted or valuable.

Methods

Pollen and seed collection sites

Phragmites pollen and seed samples for this study were collected at twelve marsh and roadside sites along the Mississippi River from Port Sulfur, Louisiana to the lower Mississippi River in the Birdsfoot Delta (Fig. 1). Sites were selected haphazardly based on proximity to the Birdsfoot Delta and by the status of *Phragmites* plants in each site as either healthy or in a state of dieback. We also preferentially chose sites for each haplotype that were as close as possible to other haplotypes to avoid biasing our estimate of spatial isolation. In the Birdsfoot Delta region, Delta-type *Phragmites* typically occurs in lower marsh

Fig. 1 Map of *Phragmites* field sites by haplotype along the Birdsfoot Delta (Google Earth, 2022)



areas while Gulf- type establishes in more upland roadside ditches. The average distance between EU and Delta sites was 19 km while the average distances between EU and Gulf sites and Delta and Gulf sites were further; both 39 km. EU and Delta also had a minimum distance of 1.3 km between the haplotype sampling sites. Pollen and seed collections occurred from June through December 2021 depending on the flowering stage of each site (Fig. 4). At one EU-type site in the Pass-a-Loutre area of the Birdsfoot Delta, we collected a pollen sample followed by seed sample collection several months later. Additionally, the flowering stage was qualitatively determined at thirty sites from June to December 2021 to document the phenology of each *Phragmites* haplotype and to assess hybridization potential among haplotypes. We also collected several gallons of soil each from one healthy *Phragmites* stand and one dieback stand for the germination experiment.

Pollen viability assessment

Ten visible anthers were collected from one inflorescence per site, and were placed in a microcentrifuge tube filled with Lactophenol Blue dye (Sigma-Aldrich, Burlington, MA) to stain for pollen viability estimation (Melloni et al. 2013). The anthers were

lightly crushed with a microcentrifuge pestle to break up immature anthers and allow the dye to soak into the pollen grains. For each sample, two drops of Lactophenol Blue and pollen mixture and one drop of tap water were pipetted onto a glass slide. A cover glass with a gridded sticker was placed on top of the sample to aid counting, and the sample was examined under a compound light microscope at 400× magnification.

Viable pollen turns a dark blue color when in contact with Lactophenol aniline blue dye and non-viable pollen turns a lighter blue color (Melloni et al. 2013; Fig. 2). Thus, we counted pollen grains as either viable or non-viable to calculate the estimated viability rate by dividing the number of viable grains by total pollen grains per slide. Pollen grains that were broken or not dyed blue were not counted in the pollen viability assessment.

Hybridization potential

We qualitatively assessed the potential of hybridization between any of the three *Phragmites* lineages found in the Birdsfoot Delta. For a total of 94 observations, we noted the haplotype, flowering stage, and date of observation of *Phragmites* stands along the lower MRD. The flowering stages were categorized as no flower, new flower, producing pollen, or

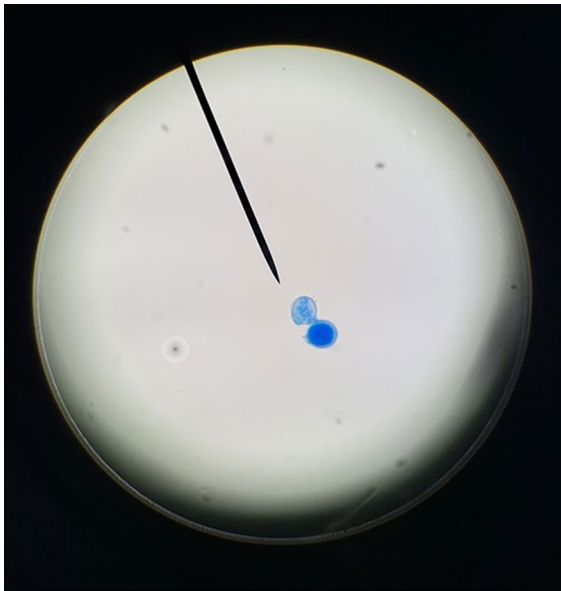


Fig. 2 Viable (dark blue) and non-viable (light blue) *Phragmites* pollen grains stained with Lactophenol aniline blue under a standard light microscope (400× magnification)

producing seed. Many of these stands were also sampled for pollen and seed viability assessment.

Seed collection and floret production assessment

Entire infructescences were cut from *Phragmites* stands in the field and stored in paper bags for transportation back to the lab. To estimate the number of florets and number of seeds per infructescence, we weighed and counted the number of seeds in 50 florets and weighed all of the florets and used the proportional weight of the fifty florets to calculate an estimated total floret count of the infructescence. Pollen viability but not floret production was tested in EU dieback stands.

Seed viability assessment

After weighing, seeds were removed from infructescence and were placed in plastic bags with tap water and autoclaved sand and stratified in cold storage at 4 °C for at least a month since cold-moist treated *Phragmites* seeds have been shown to germinate faster and to higher percentages than seeds that are fresh or warm-dry treated (Kettenring and Whigham 2009). We then randomly selected ten inflorescences

of each *Phragmites* haplotype to plant into either healthy or dieback soil (soil collected in bulk from one healthy site and one dieback site), for a total of 60 inflorescences planted. Each inflorescence was planted into a 6-inch diameter pie tin and placed into a growth chamber on January 25th, 2022, and watered every other day for 30 days.

Germination and growth response

Germination responses of seeds from three *Phragmites* haplotypes (Delta, Gulf, and EU) in the two soil types (dieback and healthy) were tested in a full factorial design. Six haplotype and soil combinations of treatments were tested in growth chambers using diurnal temperature (12 h at 30 °C and 12 h at 10 °C) and light (10 h light and 10 h dark) for 14 days. For *Phragmites* seeds to reach their full germination potential, these optimal temperature fluctuations are required (Ekstam and Forseby 1999). Each sample was randomly assigned a cell within the growth chamber to avoid too many of one haplotype being on the same shelf. We counted the number of new sprouts germinating each day, and after 30 days (February 25, 2022), samples were transplanted into 6" diameter plastic pots and moved into a greenhouse in Baton Rouge, Louisiana. The pots were placed in tubs and given new water twice weekly until several new shoots had formed on each plant. In June, we clipped the stems at the soil surface, stored aboveground biomass in weighed paper bags, and placed them in a drying oven for 4 days. To measure belowground biomass, plant material below the soil surface was rinsed over a 2 mm mesh sieve and separated into live roots and rhizomes (Hester et al. 2001). The rinsed belowground biomass samples were stored in weighed paper bags and placed into a 60 °C drying oven until a constant dry weight was reached (Gu et al. 2020).

Seedbank assay

In March 2020, we collected 55 soil samples (10 cm×10 cm×3 cm depth) from the Birdsfoot Delta. Twelve samples were collected from stands dominated by the Delta-type, 12 samples from the Gulf-type, 13 samples from the EU-type. To examine the potential for viable seeds in the *Phragmites* dieback areas, we collected an additional 12 samples from Delta dieback areas and 6 from EU dieback

sites. Samples were collected across several populations or geographically distinct stands of each haplotype. Samples were kept cold and cold stratified for approximately two weeks before being placed in the greenhouse (Elsey-Quirk and Leck 2021; Leck 2003).

In the greenhouse, each sample was spread on ~2 cm of Perlite in a 20 cm × 20 cm × 4 cm deep aluminum pan with drainage holes. Two pans were randomly assigned to rubber tubs, which were also randomized on a single greenhouse bench. Samples were kept at about field capacity: they were surface watered 2 or 3 times weekly and water level was maintained at about 1 cm depth.

Seedlings were identified to species if possible. Once seedlings were identified, they were removed to prevent overcrowding. Cross-contamination was avoided by removing seedlings before they dispersed seeds. Some seedlings were transplanted to pots with potting soil until identification was possible. Samples were maintained until mid-August after germination had ceased.

Statistical analyses

Pollen viability

To compare pollen viability between EU healthy, EU dieback, and Delta-type *Phragmites*, we conducted a type-III ANOVA test with the statistical software R (R Core Team 2017). We logit transformed the pollen viability percentage to conform to the assumptions for ANOVA. We checked the ANOVA assumption of normality of variance by performing a Shapiro–Wilks test on the residuals of the transformed data and found the data was normally distributed ($p=0.17$). Prior to the ANOVA, we used a Levene’s test to confirm that the data was homoscedastic ($p=0.33$).

Floret production

We conducted an independent t test to compare the amounts of florets produced per inflorescence in Delta haplotype *Phragmites* collected from healthy and dieback sites. Prior to conducting the t test, we verified that the data were normally distributed and samples homoscedastic using Shapiro and F tests, respectively.

Seed germination and growth

Since EU seeds were the only haplotype to germinate, we used an independent t test to compare the germination rate between EU seeds planted into dieback and those planted into healthy soil. We also compared biomass of the seedlings planted into either healthy soil or dieback soil using a t test. We performed separate t tests for the biomass in three different categories: aboveground, belowground roots, and belowground rhizomes. For each test, the data were tested for normality and homoscedasticity using a Shapiro test and F test respectively.

Seedbank assay

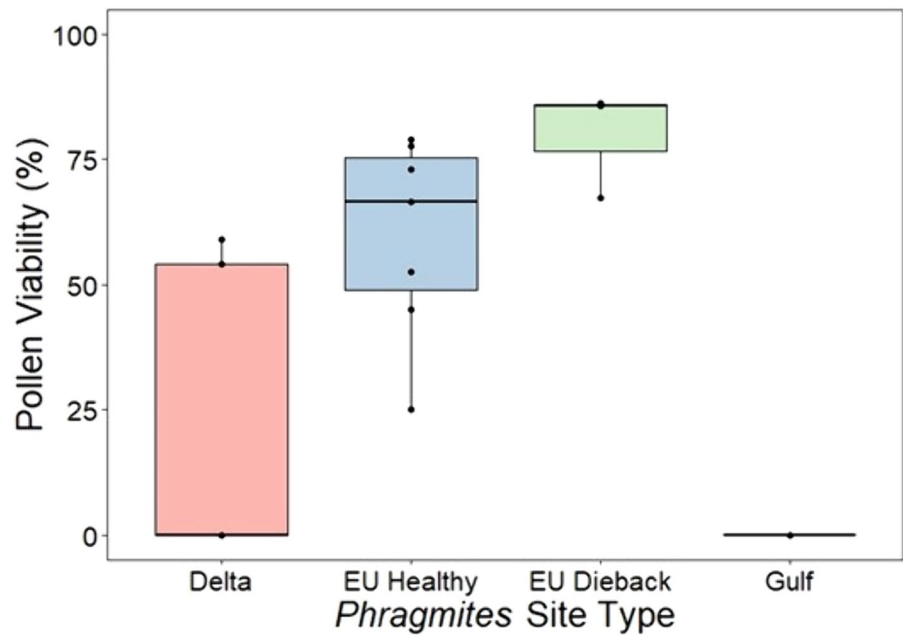
The effect of haplotype was tested on species richness and seedling density of individual species and all species combined using a one-way analysis of variance (ANOVA). Species richness and seedling density data were $\log(x+1)$ transformed to meet the normality assumptions of analysis of variance. The Honestly Significant Difference Tukey test was conducted for all post-hoc comparisons. Separate analyses were conducted for *P. australis* and for all other species combined. To test whether the species composition of seedlings that emerged from the seedbank differed among populations (haplotype, healthy, dieback areas), we used Permutational multivariate analysis of variance in a single-factor design (PERMANOVA, Primer 6; (Clarke and Warwick 2001)). Non-metric multidimensional scaling (MDS) plots were developed to illustrate separation in community structure across transect distances. Pre-treatment of community data included a global logarithmic transformation and a Bray–Curtis similarity resemblance matrix using Primer 6 (Primer-E Ltd, (Clarke and Warwick 2001)).

Results

Pollen viability

The EU haplotype produced the greatest amount of viable pollen ($65.8 \pm 6.20\%$) as compared to Delta-and Gulf-types at healthy sites ($F_{2,12}=7.248$, $p=0.00863$; Fig. 3). The Gulf-type produced no viable pollen. Interestingly, the EU-type collected from dieback sites had the greatest percentage of viable

Fig. 3 Pollen viability of healthy European (EU), dieback EU, Delta, and Gulf haplotypes in the Mississippi River Delta, LA



pollen ($79.8 \pm 6.26\%$), significantly higher than the Delta-type samples ($22.6 \pm 13.9\%$ Fig. 3). We did not include Delta dieback sites in our pollen viability analysis because we were not able to visit a site that was producing anthers. We only collected and analyzed Delta dieback samples in terms of floret production, which occurred months later when no anthers were present.

Floret production

The Delta haplotype floret production was significantly lower in the healthy sites (585 ± 77 florets/infructescence) compared to the dieback sites (1001 ± 205 florets/infructescence; ($F_{1,13} = 4.789$, $p = 0.04749$; Fig. 4).

Hybridization potential

Phragmites haplotypes had either spatially or temporally separated flowering in the Birdsfoot Delta and therefore, little evidence for inter-haplotype hybridization. The Delta and Gulf have overlapping flowering periods yet are spatially isolated from one another (Figs. 1, 5). EU produced viable pollen and seeds several months before the other two haplotypes started flowering (Fig. 5).

Seed germination and growth

Seeds from only the EU haplotype germinated with an average germination rate of $0.99 \pm 0.34\%$ of the florets per infructescence. Germination rates did not differ significantly between seeds planted into dieback and healthy soil ($F_{1,12} = 0.9381$, $p = 0.3519$; Fig. 6). After growing for 5 months, belowground biomass did not differ between EU-type *Phragmites* planted into healthy and dieback soils

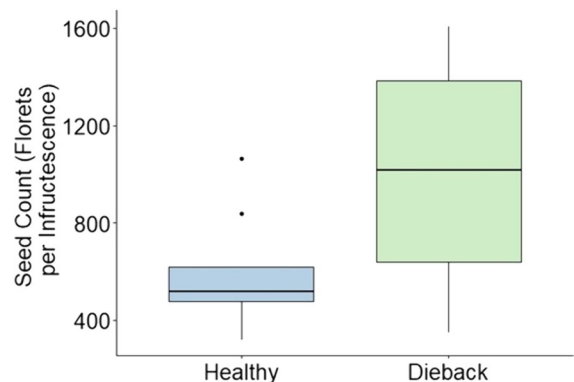


Fig. 4 Floret production of Delta-type *Phragmites* in healthy sites compared to those in dieback sites in the Mississippi River Delta, LA. Delta was the only haplotype with floret samples from both healthy and dieback sites. EU- and Gulf- types were only found at healthy sites

Fig. 5 Observed flowering stages of the three *Phragmites* haplotypes in the Birdsfoot Delta, LA

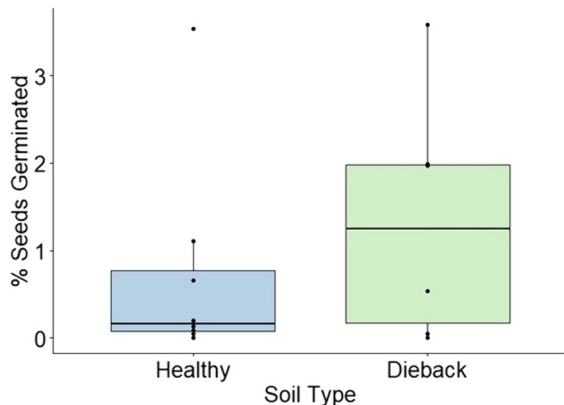
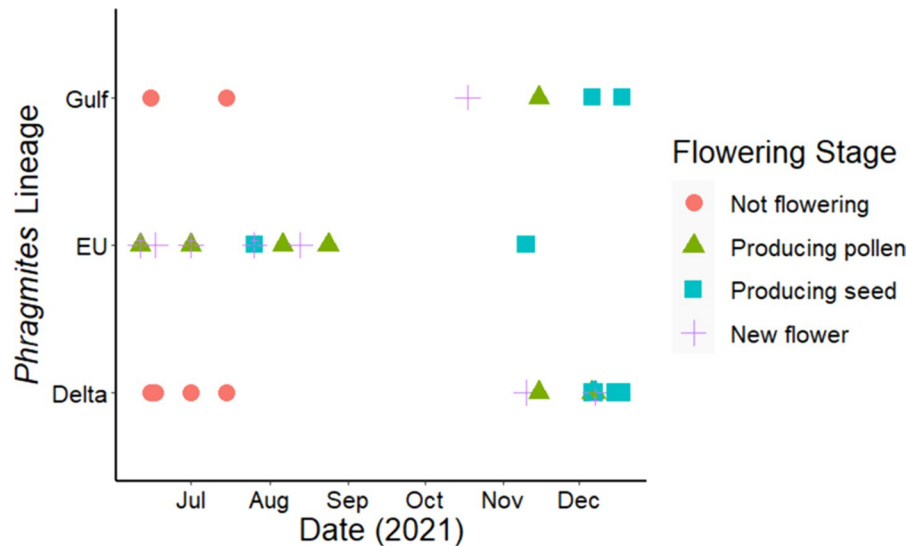


Fig. 6 Seed germination percentages of European-type *Phragmites* planted in healthy and dieback mud collected from *Phragmites* sites in the Birdsfoot Delta, LA (n=15)

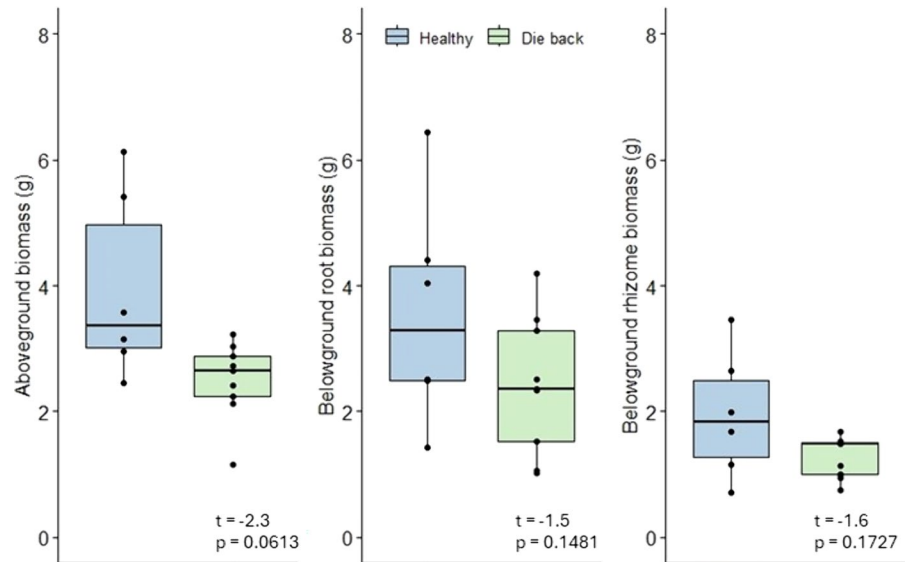
(Fig. 7). Specifically, EU-type *Phragmites* planted into healthy soil had an average aboveground biomass of 3.95 ± 0.60 g, an average belowground root biomass of 3.55 ± 0.73 g, and an average belowground rhizome biomass of 1.94 ± 0.41 g. On the other hand, EU *Phragmites* planted into dieback soil exhibited an average aboveground biomass of 2.49 ± 0.205 , an average belowground root biomass of 2.41 ± 0.364 g, and an average belowground rhizome biomass of 1.28 ± 0.108 g (Fig. 7).

Seedbank assay

Across stands of the different haplotypes including healthy and dieback stands, a total of 27 species emerged from the seedbank. The Gulf haplotype had a larger and more diverse seedbank than the other haplotypes. Species richness per sample averaged two times greater (4.3 ± 0.7 species) than in the healthy and dieback stands of the Delta- and EU-types ($p < 0.001$). Total seedling density was variable across haplotypes and seedling density of Gulf-type was significantly greater than the Delta dieback areas ($F_{4,50} = 4.33$, $p = 0.0047$). Gulf-type had a total seedling density averaging 3242 ± 1235 seedlings m^{-2} , significantly greater than the 122 ± 44 seedlings m^{-2} of the Delta dieback stands.

The density of individual species also varied across haplotypes. Seedlings of *Amaranthus canabinus* averaged 46 ± 18 seedlings m^{-2} in EU stands, which was statistically similar to the EU dieback stands, but greater than the other haplotypes where it was absent from the seedbank ($p = 0.0013$). Seedlings of *Echinochloa crus-galli* emerged in densities of 50 ± 26 seedlings m^{-2} in Gulf stands but were absent from the other stands ($p = 0.0044$). *Sagittaria lancifolia* seedling abundance averaged 164 ± 93 seedlings m^{-2} in Delta stands, while also absent from all other haplotypes with the exception of a single seedling in Gulf stands ($p = 0.0020$). *Samolus valerandi* seedlings were locally abundant in Gulf populations (1400 ± 782 seedlings m^{-2}), found in low densities in

Fig. 7 Aboveground (a), belowground root (b) and rhizome (c) biomass of *Phragmites* grown from seed in healthy and dieback soils (n = 15)



Delta and Delta dieback stands and were absent from EU and EU dieback stands and were absent from EU ($p = 0.0224$). *Schoenoplectus pungens* seedling abundance was greater in Gulf than EU and Delta dieback but similar to Delta and EU dieback stands ($p = 0.0064$) (Fig. 8).

Overall, there was a lot of overlap in the community composition of the seedbank across haplotypes, yet the species compositions significantly differed (Fig. 9). Pairwise comparisons show significant differences between the Delta and EU stands and the EU and Gulf stands ($p < 0.01$, Fig. 9). The species composition of seedlings that emerged from the seedbank in dieback stands of EU and Delta were variable and did not significantly differ from healthy stands.

Discussion

Our research demonstrates stark differences in sexual reproductive effort between the three *Phragmites* lineages, with EU-type more successful in terms of viable pollen production and seed germination. We also determined a low likelihood for inter-haplotype hybridization among the three *Phragmites* haplotypes, due to disjunct flowering periods and lack of pollen production in the Gulf type. Additionally, germination and growth did not differ significantly between EU *Phragmites* seeds planted into dieback and healthy soil. While no *Phragmites* germinated

from seedbank samples, other taxa such as *Sagittaria* spp. or *Colocasia esculenta* in the seedbank may provide some level of recovery following dieback. Overall, we posit that the European *Phragmites* is the most suited to recruitment by seed following a dieback event, and that Gulf and Delta haplotypes have low recovery from seed due to lack of observed sexual reproduction.

European haplotype of *Phragmites australis* was the most prolific seed and pollen producer of all the lineages surveyed. This greater reliance on sexual reproduction compared to other *Phragmites* lineages could provide the European haplotype with the genetic diversity to evolve to withstand herbivory, in addition to other stressors associated with dieback (Lei 2010). This was expected based on previous studies, as European haplotype *Phragmites* is more resistant to the *Nipponaclerda biwakoensis* scale insect implicated in *Phragmites* dieback than the other MRD *Phragmites* haplotypes (Cronin et al. 2020). We posit that the capacity for sexual reproduction in European *Phragmites* will help the plant evolve to withstand biotic stressors from *N. biwakoensis* as well as possible future stressors. Additionally, the increased capacity for sexual reproduction in EU *Phragmites* likely facilitates its ability to invade into new habitats and adapt to deal with novel enemies, features which make it a potent invasive compared to other haplotypes. However, it is worth noting that our relatively low number of inflorescence

Fig. 8 Seedling densities from the seed bank of healthy and dieback stands of three haplotypes of *P. australis* in the Birdsfoot Delta, LA

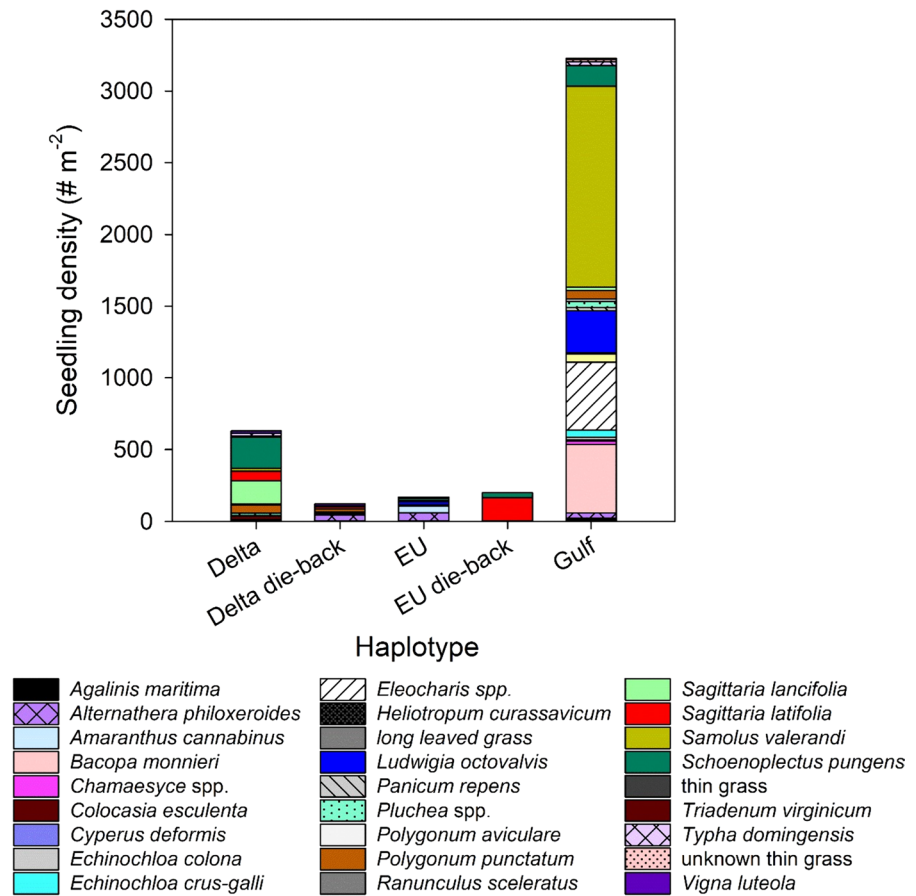
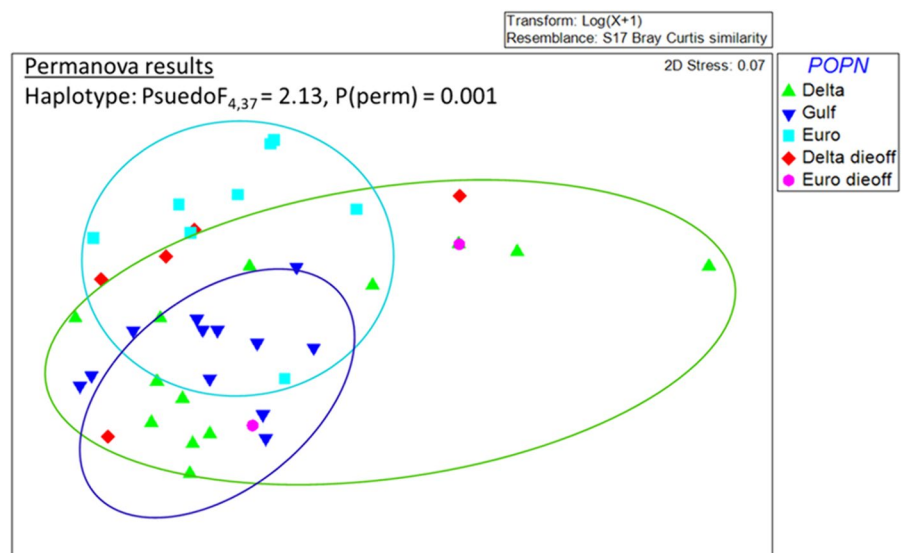


Fig. 9 Results of non-metric multi-dimensional scaling and Permanova analyses examining the species composition of seedlings that emerged from seed bank samples collected from healthy and dieback stands of *Phragmites* haplotypes in the Birdsfoot Delta, Louisiana



samplings per site could be a possible limitation in our assessment of pollen viability.

Delta (M1) is the predominant lineage found in the Birdsfoot Delta, but its geographic span is confined to the Gulf Coast region (Hauber et al. 2011). The lack of sexual reproduction we observed in the Delta-type *Phragmites* likely limits its capability to adapt to deal with biotic stressors (Lei 2010). However, we also saw more floret production, denoting greater sexual reproductive effort, in Delta-type *Phragmites* subjected to dieback conditions. Although we observed no viable seeds produced by the Delta-type *Phragmites*; that could simply be an effect of the particular growing season when we collected the seeds. The presence of some viable pollen in Delta *Phragmites* suggests that this haplotype occasionally produces viable offspring through sexual reproduction. *Phragmites* typically responds to abiotic stressors by producing more seeds as a method to allow the next generation to disperse and escape stressful conditions (Reale et al. 2011), which is reflected in our data showing greater floret production in dieback-afflicted Delta haplotype *Phragmites*. Overall, the low pollen viability and lack of germinating seeds suggest that this haplotype poses a significantly lower threat to native ecosystems compared to EU *Phragmites*.

Gulf haplotype *Phragmites* shows even lower potential for invasive spread through seed production, as we detected no viable pollen from Gulf-type flowers. Furthermore, we did not observe Gulf-type in natural wetlands of the MRD, though it was documented in these habitats by Hauber et al. (2011). In our study, Gulf populations were found in disturbed patches along roadsides, indicating a low potential for invasion into low elevation coastal marshes. Our findings support the hypothesis that EU *Phragmites* is the more invasive lineage of *Phragmites* (Meyerson and Cronin 2013). The EU's successful spread in the MRD is likely bolstered by its ability to sexually reproduce, although it is vastly outnumbered by Delta-type which is better suited to growing in more flooded conditions (Lynn and Elsey-Quirk 2024). To build on our understanding of *P. australis* expansion and recovery following dieback, future studies should explore differences in asexual reproduction strategies between lineages.

Sexual reproductive effort requires more energy investment compared to asexual reproduction (Daly 1978), but can confer several advantages if

germination is successful. First, recombination during sexual reproduction generates greater genetic diversity in a population. Furthermore, sexual reproduction allows for long distance dispersal due to *Phragmites* seeds' adaptation to wind or bird dispersal (Soons 2006). The advantage which sexual reproduction confers cannot be overstated, as European haplotype *Phragmites* predominantly depends on seed dispersal to colonize new habitats (Brisson et al. 2008; Baldwin et al. 2010). The increased capacity to adapt to withstand biotic stressors likely facilitates the invasive nature of the European haplotype (Lei 2010). Therefore, we suggest the lack of sexual reproduction in Gulf and Delta *Phragmites* could contribute to the less invasive nature of those haplotypes. However, there is evidence that the non-invasive *Phragmites australis* subspecies *americanus*, native to more northern wetlands in the USA, does reproduce by seed, albeit with lower germination rates than the often invasive EU haplotype *Phragmites* (Meyerson et al. 2010b).

Flowering phenology observations of *Phragmites* lineages in the Birdsfoot Delta reveal little potential for hybridization. This is due to the combination of temporal and spatial reproductive isolation exhibited by the three haplotypes (Fig. 5). For hybridization to occur, viable pollen and seed production are required from sympatric distributed haplotypes with overlapping pollen production times (Meyerson et al. 2010b). Although pollen transfer in wind-pollinated grasses can happen at large distances up to 21 km, the likelihood of fertilization through pollen transfer drops off dramatically as distance from the pollen source increases (Davis et al. 2004). In our study, we found that EU produced ample viable seed and pollen in the months of June and July, while Gulf and Delta-types set their mostly non-viable pollen and completely non-viable seeds during the months of October through January. Further, Gulf and Delta-types would likely not have success in hybridizing with each other even if they did produce viable pollen and seeds, as Gulf and Delta types are spatially isolated from each other at a distance at which pollen transfer would be unlikely. These findings contrast sharply with studies showing viable hybrids and flowering time (anthesis) overlap between the invasive European haplotype and the native *Phragmites australis* ssp. *americanus* prevalent in northern regions of the USA (Meyerson et al. 2010b).

The lack of hybridization potential detected in our study suggests that novel hybrid *Phragmites* haplotypes will not provide an option for recovery from dieback. On the other hand, low potential for hybridization assuages the concern that EU *Phragmites* hybridizing with a Gulf Coast haplotype could generate new haplotypes with increased invasive capabilities, at least in the MRD region (Meyerson et al. 2010c). Despite our lack of evidence for hybridization, previous microsatellite surveys of Gulf Coast *Phragmites* suggest inter-haplotype hybridization between EU and Gulf type and between EU and Delta type is likely (Lambertini et al. 2012). We suggest that such hybridization, if possible, is rare based on non-overlapping flowering times, and will not lead to a hybrid-swarm scenario in the Birdsfoot Delta as some have posited (Lambertini et al. 2012).

Implications for restoration

Our seed germination trial demonstrates that EU is the only haplotype surveyed that produces viable seeds. If these seeds are able to germinate in Birdsfoot Delta conditions, this viability could help with recovery following dieback. However, *Phragmites* germination from seed is limited by water depth, such that prolonged flooding of at least 3.5 cm can prevent germination and therefore regeneration from seed banks (Van Der Valk and David 1978; Weisner et al. 1993; Baldwin et al. 2010; Yu et al. 2012). Since low elevation presents such a problem for *Phragmites* seed germination, marsh restoration techniques such as thin layer sediment deposition (Ford et al. 1999) followed by planting EU *Phragmites* seeds could prove a viable restoration method. Once established, planted *Phragmites* stands may expand vegetatively into adjacent lower elevation areas (Baldwin et al. 2010).

Hindered germination in flooded conditions could help explain the current distributions of *Phragmites* in the Birdsfoot Delta; for example, EU *Phragmites* is relatively rare in this region and typically only occurs in patches adjacent to Delta *Phragmites*, yet at slightly higher elevations (Jacobs 2022, unpublished). Furthermore, the germination rates we describe were much lower than those observed by Ekstam and Forseby (1999) in *Phragmites* from Sweden, perhaps indicating a greater reliance on reproduction in the lineages extant in southern Louisiana marshes.

Additionally, EU *Phragmites* in the Birdsfoot Delta may spread and rebound from dieback less readily than those invasive *Phragmites* found in the Northeast US due to hydrological differences and rampant subsidence in the Birdsfoot Delta preventing germination and establishment of new stands.

Restoration via seed planting or recruitment from seedbank does not seem to be an option for Gulf- or Delta- type *Phragmites*. However, we only surveyed these for 1 year, and it is possible we missed the optimal window to observe viable seed production in these particular *Phragmites*. In addition to the controlled germination trials, our seedbank study suggests that *Phragmites* recovery by seed is exceedingly rare in nature, especially with other plants present as competitors in the seedbank. However, we posit that plant species other than *Phragmites* may recover following dieback and aid in marsh stabilization, as demonstrated by our seedbank germination experiment. Differences in seedbank community between *Phragmites* haplotypes may be attributable to distinct habitat preferences among the lineages. Alternatively, *Phragmites*' capacity for exerting allelopathic effects or negative plant-soil feedback on surrounding plants (e.g. Rudrappa et al. 2009; Crocker et al. 2017; Gao et al. 2022) may differ between haplotypes, a possible avenue for future research.

Conclusions

Here we detail stark differences in reproductive effort and viability between *Phragmites australis* haplotypes in the Birdsfoot Delta. The haplotype with the most capacity for sexual reproduction is the same haplotype (European) which is wreaking havoc as an invasive plant in other parts of North America, while the Gulf and Delta haplotype display low sexual reproductive capacity, suggesting low potential for invasion. The propensity for sexual reproduction in EU *Phragmites* may facilitate its ability to recover following dieback, which is possible in dieback-affected soils provided the flooding is not too extreme or constant. On the other hand, the production of inter-haplotype *Phragmites australis* hybrids is an unlikely route to dieback restoration or further production of novel invasive haplotypes. This study contributes to a growing body of work displaying the importance of sexual reproduction in the spread and

recovery of *Phragmites australis* in North America and illustrates how different lineages within the *P. australis* species exhibit widely varying propensities for sexual reproduction.

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Data availability The data used for the figures in this study are available upon reasonable request to the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

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