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PHRAGMITES INVASION

Epigenetic variation within *Phragmites australis* among lineages, genotypes, and ramets

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Abstract Epigenetics is likely an important factor in morphological and physiological acclimation, phenotypic plasticity, and potentially ecological dynamics such as invasiveness. We propose that *Phragmites australis* is an ideal model species for studies of epigenetics as a factor in plant invasions and ecology due to natural clonal replication (controlling for genetic variation) and the co-occurrence of subspecies with distinct life history strategies such as differences in invasiveness. In earlier work, genotypes and constituent clonal ramets were identified using microsatellite markers. In this pilot study, we screened the same ramets for epigenetic variation with Methylation-Sensitive AFLPs (MS-AFLPs), a modified type of AFLP dependent on differentially methylation-sensitive restriction enzymes. We found a significant difference in epigenetic signatures between introduced and native subspecies, and found that introduced *P. australis* demonstrated more epigenetic variation than their native counterparts. In both subspecies we observed moderate variation between genotypes relative to the higher degree of epigenetic variation found within genotypes (among ramets), suggesting that epigenotype may be more closely aligned with microhabitat than within-subspecies genotype. Finally, we observed potential epigenetic variation by site. This is the first study to investigate natural variation in DNA methylation patterns of *P. australis* and establishes the baseline in our understanding of the ecological relevance of epigenetics in this species.

Keywords Epigenetics · Plasticity · *Phragmites australis* · Clonal · MS-AFLP

Introduction

The capacity to morphologically or physiologically acclimate to a broad range of conditions can expand ecological niche breadth and has been proposed as one potential mechanism involved in plant invasions (Richards et al. 2006). Evidence is growing that epigenetic regulation may be an important source of such phenotypic plasticity (Massicotte and Angers 2011; Verhoeven and Preite 2014). In response, the need has arisen for research further exploring the ecological role of epigenetics and its contribution to phenotypic plasticity (Bossdorf et al. 2008, Richards 2008; Verhoeven and Preite 2014) and, by extension, plant invasions. *Phragmites australis* is an ideal model species for studies of epigenetics as a factor in plant invasions and ecology (Meyerson et al. 2016, this

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issue). Its character as a facultatively clonal plant with naturally replicated units (ramets) of identical genotypes (Chambers et al. 1999; Douhovnikoff and Hazelton 2014) provides an important control for genetic variation. In addition, the co-occurrence of subspecies with distinct life history strategies and differences in invasiveness allows for informative ecological comparisons. This is the first pilot study investigating natural variation in DNA methylation patterns of *P. australis* and establishes the baseline in our understanding of the ecological relevance of epigenetics in this species.

To date a large portion of research into ecological epigenetics has involved small-scale projects carried out under carefully controlled laboratory conditions (Verhoeven et al. 2010; Herrera and Bazaga 2011; Bossdorf et al. 2010). While informative, such studies are narrow by design and necessarily oversimplify the dynamic ecological factors that exist in wild populations (Bossdorf et al. 2008). Common garden studies, which expose individuals from different environments to a common growth setting, are especially helpful in looking at the heritability of epigenetic markers (Richards 2008). However, large-scale in situ studies offer a broader range of ecological pressures and perspective on the resulting epigenetic acclimation of individual genets or ramets. Some of the few examples of such work include studies in knotweed (Richards et al. 2012) and alligator weed (Gao et al. 2010) both of which are also facultative clonal species. Clonal plants are powerful in situ models for isolating acclimation effects, as they control for genetic variation and leave remaining variability to be explained by epigenetic variation and physiological plasticity (Douhovnikoff and Dodd 2014). There are clear differences in phenotypic plasticity and invasiveness between the two *Phragmites* subspecies (Mozdzer and Megonigal 2012). The proportion of this plasticity that might be attributable to epigenetic variation is unknown.

We examined patterns of global DNA methylation in natural *P. australis* populations at the scale of subspecies, genotype and ramet. As such we identified variation between subspecies, variation of genotypes within subspecies, variation within genotypes (among ramets), and epigenetic differences by site. This is the first in situ epigenetic study comparing two closely related subspecies, one an introduced invasive and the other a native non-invasive. We hypothesized that (1) the introduced *P. australis* subspecies would contain greater epigenetic variation than the native conspecific *P. australis*, suggesting the potential for epigenetic acclimation to a broader range of environments. We also hypothesized that (2) epigenetic variation within a heterogeneous environment is more closely correlated with micro-habitat (ramet) than underlying genotype (genet). In other words, we would expect the bulk of variation to exist within genotypes, not necessarily between them. Finally, we would expect that separate geographic locations with distinct environmental conditions would produce site-specific epigenetic signatures.

Methods

Phragmites australis is recognized as a facultatively clonal species. As such it can reproduce both sexually, which can have important implications for dispersal, and clonally, at what is more often a more localized scale. Clones of *P. australis* may cover areas of 100 m^2 or greater, essentially creating large, naturally propagated stands of genetic replicates that have grown in a heterogeneous habitat of micro-environments (Douhovnikoff and Hazelton 2014). These microenvironments may involve considerable variation in factors such as edaphic conditions, disturbance, and nutrients and can spur epigenetic differentiation within the same genotype.

Subspecies, genotypes, and clonal replicates (ramets) were identified by microsatellite analysis in an earlier study of clonal architecture and diversity in native and introduced stands *of P. australis* (Douhovnikoff and Hazelton 2014). Clonal ramets served as genotype replicates controlling for genetic variation. Clones were replicated only within sites and not across sites. All samples used in this study (collected: $n = 66$ native, $n = 78$ introduced) were collected from the Webhannet and Libby watersheds in midcoast Maine (Webhannet: W 70.585°, N 43.286°; Libby: (W 70.310°, N 43.563°). Both sites are back barrier dune systems, with stands of native and introduced subspecies in close proximity, or overlapping in the case of the Libby marsh (Douhovnikoff and Hazelton 2014). Collection scheme and DNA extraction are as previously described in Douhovnikoff and Hazelton (2014). This study assumed, based on previous studies (Richards et al. 2012) that epigenetic loci may respond

to microhabitat conditions where multiple genotypes exist across a heterogeneous environment.

Methylation-sensitive amplified fragment length polymorphisms (MS-AFLPs)

We screened 96 individuals for epigenetic variation with MS-AFLP, a modified type of AFLP dependent on differentially methylation-sensitive restriction enzymes. (For protocols used refer to Richards et al. 2012). Fragment analysis (performed on ABI 3100 automated sequencer, Life Technologies) returned chromatograph data, which were scored by hand using GeneMapper Software (Version 5.0, Life Technologies) Fingerprints generated through MS-AFLP analysis were further analyzed using the R-based statistical package "msap" (Perez-Figueroa 2013). Through comparisons of the parallel enzymatic digests, msap determines whether or not each recorded fragment is susceptible to methylation (and thus epigenetically informative) or if there is no evidence of methylation. These two types of fragments are referred to as "Methylation sensitive loci" (MSL) and "Non methylated loci" (NML), respectively.

The msap program provides statistical analysis consisting of Shannon Diversity Indices, single-level AMOVAs, and Principle Component Analyses (PCAs). PCAs are visual representations of variation within and between groups: ellipses show the average dispersion of individuals around the centroid for their parent group. PCAs were created using the polymorphic markers from the MSL/NML groups. Additionally, we calculated hierarchical AMOVAs for each site in the study to compare variance between subspecies, genotypes, and ramets using the GenAlEx add-on for Microsoft Excel (Peakall and Smouse 2012). We relied on data from one native and four introduced genotypes at the Webhannet site; of the introduced genotypes, 2 were singlets. At the Libby site we used data from 10 introduced genotypes and three native genotypes; one native genotype and six introduced genotypes were singlets. While singlets were included in PCA analysis at the subspecies and site level, they were removed prior to analysis by hierarchical AMOVA. Our resulting population totals for hierarchical AMOVA included n = 93 for Webhannet (n = 46 invasive, n = 47 native) and n = 30 for the Libby marsh (n = 15 for both invasive and native).

Results and discussion

Earlier work in *P. australis* has observed greater phenotypic plasticity in introduced subspecies as compared to native populations (Mozdzer and Megonigal 2012; Mozdzer and Zieman 2012; Douhovnikoff et al. 2016). As such, we predicted and found a significant epigenetic difference between the introduced and native subspecies at both the Webhannet site ($p = 0.001$, hierarchical AMOVA, Fig. 1) and the Libby site ($p = 0.001$, hierarchical AMOVA, Fig. 1). At both sites, individuals from the introduced subspecies demonstrated more variation in epigenotype than their native counterparts. This variation can be visualized as the spread of samples around the centroid in the PCA graph for each subspecies (Fig. 1). Of our original 408 MS-AFLP markers for the Webhannet site, msap selected 347 as Methylation Sensitive Loci (MSL) (of which 191 were polymorphic) and 61 and Non Methylation Sensitive Loci (NML) (all of which were polymorphic). For the Libby site, 93 of the original 241 markers were identified as MSL (all polymorphic) and 148 were NML (only 13 polymorphic). A hierarchical AMOVA performed on polymorphic MSL markers from the Webhannet and Libby marsh samples revealed that respectively 25 and 37 %

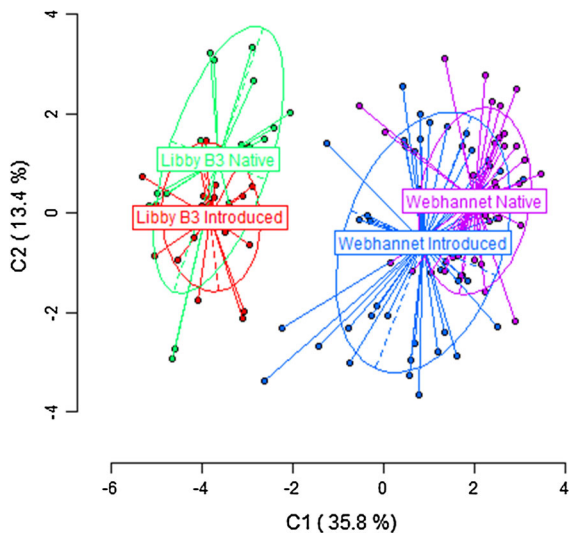

Fig. 1 PCA showing significant differences between individuals from the Webhannet (*blue* and *purple*, n = 95 total) and Libby (*red* and *green*, n = 37 total) sites based on 191 MSL/epigenetic markers from MS-AFLP data (single-level AMOVA, $p < 0.0001$). Samples show spatial segregation according to both site of origin and subspecies

of the total variance could be explained by the segregation of individuals according to subspecies (Table 1). In addition to demonstrating distinct epigenetic signatures, our results support the theory that conspecifics exhibit consistent variation from large-scale morphological characteristics to smaller-scale physiological and epigenetic responses.

Plasticity has been proposed as one characteristic that promotes invasion (Pál 1998; Richards et al. 2006; Davidson et al. 2011; Richards et al. 2012). Being more plastic allows a species to act as a generalist, exploiting a broad niche in its environment. The generalist approach permits the establishment of invasive populations in heterogeneous, unstable or rapidly changing environments (Pál 1998). This appears to be consistent within *P. australis* populations. The native stands are often restricted to growing in low-salinity tidal wetlands, whereas clones of the introduced subspecies can span diverse microhabitats from mesohaline marshes to tidal wetlands to freshwater river systems (Chambers et al. 1999). The broad introduced distribution could be facilitated by high levels of variation in epigenotype. Our results show that the invasive introduced subspecies is more epigenetically plastic than the native, however, at this stage we do not know if this lack of epigenetic plasticity within the native is limiting its expansion.

In both subspecies we observed a higher degree of epigenetic variation within rather than between-genotypes. While environmental variation was not directly measured here, there is considerable evidence in the literature that epigenotype is largely influenced by genotype and environmental factors (Bossdorf et al. 2010; Herrera and Bazaga 2011). However, the limited variation due to genotype we observed suggests that epigenotype may be more closely aligned with environmental factors among ramets. While a hierarchical AMOVA did find that genotype contributed significantly to epigenetic variance ("between genotypes", $p = 0.022$, Table 1), it was not the highest source of variance among samples. Genotype accounted for only 4 % of epigenetic variance at the Webhannet site and 7 % of epigenetic variance at the Libby site (hierarchical AMOVA, Table 1). By comparison, the same hierarchical AMOVA revealed statistically significant variation "among genotypes" ($p = 0.001$ at both sites) that could account for 71 % of variation at Webhannet and 57 % of variation at Libby (Table 1; Fig. 1b). These results show tentative support for the General-Purpose Genotype (GPG) model in *P. australis*. The GPG model suggests that populations with restricted genetic variation might find other mechanisms to extend the plasticity of a single genotype in order to take advantage of a wider ecological niche. The enriched epigenetic diversity within genotypes relative to between them might suggest the use of a GPG strategy by invasive *P. australis* clones.

An earlier study of *P. australis* from multiple watersheds in midcoast Maine showed that clonal growth is important in both native and introduced stands (Douhovnikoff and Hazelton 2014). Because naturally-occurring clones of *P. australis* are very large, they are likely to encounter environmental heterogeneity. As a clone adjusts to optimize its resource extraction and growth, it may prove

Table 1 AMOVA derived from Webhannet and Libby sites, separately, showing percentage variation explained by subspecies, genotypes, and ramets

Source	df	Sum of squares	Mean of squares	Estimated Variance	%	*p* value
Webhannet Marsh (n = 46 invasive, n = 47 native)						
Among subspecies	1	284.932	284.932	5.174	25	0.001
Among genotypes	1	28.908	28.908	0.794	4	0.022
Within genotypes	90	1344.741	14.942	14.942	71	0.001
Total	92	1658.581		20.909	100	
Libby Marsh (n = 15 invasive, n = 15 native)						
Among subspecies	1	86.800	86.800	4.805	37	0.001
Among genotypes	4	41.030	10.258	0.859	7	0.066
Within genotypes	24	178.837	7.452	7.452	57	0.001
Total	29	306.667		13.116	100	

advantageous to differentiate ramets within the genet. Such local specialization would require a mechanism more nimble (fast, reversible, and sensitive to environmental variation) than the presence or absence of a gene, particularly within genetically uniform clones. Variability in epigenetic markers is a means of acclimation, potentially more rapid and responsive than adaptation, and thus practical over short to moderate amounts of time and space (Douhovnikoff and Dodd 2014).

Natural selection acts to increase or decrease plasticity depending on environmental conditions, the rate at which conditions change, and the character of the species (Alpert and Simms 2002; Davidson et al. 2011; Herman et al. 2013). The limited distribution of native *P. australis*, large clone sizes, and relatively lower plasticity may indicate a life history strategy more dependent on stability, where it could be more advantageous to specialize in a narrow niche (Alpert and Simms 2002, Douhovnikoff and Dodd 2014). However, this does not minimize the importance of epigenetics in the native. The rate of response and reversibility in traits (malleability) will have variable optima based on the life history of the organism and the stability of its environment (Donohue 2014). It is important to note that despite exhibiting less variation than the invasive subspecies, native *P. australis* still displays considerable phenotypic and epigenetic diversity.

Finally, we observed strong site-specific epigenetic patterns between sampled marshes. The Libby and Webhannet groups, when compared based on both subspecies and location, showed significantly different epigenetic fingerprints (Fig. 1). A single-level AMOVA performed in msap that compared sampling sites showed a significant difference between the sites ($p < 0.0001$, Table 1). This suggests that there are macroscale shifts in epigenotypes, possibly in response to overall environmental conditions. However, these methods do not control for genotype across sites, making it unclear what role genetic variation, potential epigenetic drift, and local adaption may play in this observation. If adaptation is not a major factor in this variation then these site to site differences could be an indicator of the broader epigenetic potential variation of *P. australis*. Reciprocal transplant research into both the extent and character of these site-specific differences would go a long way to revealing *P. australis*' full capacity for epigenetic acclimation. Additionally, future in situ studies undertaken across a span of several years could provide real-time competition data and a greater understanding of which variables appear most important for real-world inter-species interactions.

Conclusion

This study shows that *P. australis* is an excellent candidate for further studies into the ecological dynamics of epigenetic differentiation. Clonality provides a means to control for genetic variation, and the distinct life history strategies of co-occurring subspecies permits the design of powerful comparative ecological studies. Our analysis showed clear tendencies of individual plants toward site- or subspecies-correlated epigenetic fingerprints. The small scope of this study, being focused as it is on two relatively small sites within a bounded region of Maine, acts as a regional proof of concept and further work is necessary before extrapolating these observations to populations from other regions. We encourage other researchers interested in ecological epigenetics to incorporate *P. australis* as a model species. There is evidence in other systems that epigenetics is a potential mechanism for controlling ecologically advantageous phenotypic responses to environmental cues (Bossdorf et al. 2010; Herrera and Bazaga 2011). A more in-depth understanding of *P. australis* epigenetics is necessary if we are to more fully appreciate the role clonal growth and plasticity play in its ecology. More specifically, how variation in epigenotype confers a competitive advantage on the introduced subspecies might help conservation groups and land managers across North America as they fight its invasion and displacement of native plant species. *P. australis* will serve as a useful model for further investigation of the ecological relevance of epigenotypes and epigenetic diversity.

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