11-9-2015

Population Genetics and Epigenetics of Two Salt Marsh Plant Species along an Environmental Gradient

Christy M. Foust
University of South Florida, foust.christy@yahoo.com

Population Genetics and Epigenetics of Two Salt Marsh Plant Species along an Environmental Gradient

by

Christy M. Foust

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
with a concentration in Ecology and Evolutionary Biology
Department of Integrative Biology
College of Arts and Sciences
University of South Florida

Co-Major Professor: V. Jody Harwood, Ph.D.
Co-Major Professor: Christina L. Richards, Ph.D.
Gordon Fox, Ph.D.
Marc Lajeunesse, Ph.D.
Aaron W. Schrey, Ph.D.

Date of Approval:
October 15, 2015

Keywords: epigenetics, DNA methylation, *Spartina alterniflora*, *Borrichia frutescens*

DEDICATION

I dedicate this dissertation to those who believed and me and supported me during some of the darkest times of my life, which also coincided with my graduate education: my mother, my friends, my brothers and sisters in Graduate Assistants United, Dr. Daniel C. Moon, and Dr. Aaron W. Schrey.

I also dedicate this dissertation to the memory of those who passed while I pursued my degree: my grandfather, C. Tom Bartlett; my great-grandmother, Thelma (Tillie) Iler; my grandmother, Ruth Foust; and my father, Gordon (Heavy) Foust.

ACKNOWLEDGMENTS

I am extremely thankful to my doctoral advisor, Dr. Christina L. Richards, who did not give up on me and who has been extremely supportive in helping me reach my goals. I am grateful for Dr. Jody Harwood stepping up to be a Co-advisor due to last minute logistical issues. I am also thankful to my committee members, Drs. Gordon Fox, Marc Lajeuneusse, and Aaron W. Schrey, for sharing their valuable time and wisdom to help me complete my project. I am also appreciative of my fellow lab members' insight and camaraderie through the years we've worked together towards our goals.

I am also appreciative of the financial support I received from: the Aylesworth Foundation, the National Science Foundation through the Global Invasions Network Research Exchange (Grant No. 0541673), the Georgia Coastal Ecosystems Long-Term Ecological Research program (Grant No. OCE-0620959), the Department of Integrative Biology travelgrant program, the Student Government conference presentation grant program, the Society for Integrative and Comparative Biology, and the Fern Garden Club of Odessa.

TABLE OF CONTENTS

LIST OF TABLES iii

LIST OF FIGURES iv

ABSTRACT v

CHAPTER 1: Population Epigenetics 1

CHAPTER 2: Epigenetic variation is correlated with habitats in natural populations of the foundation salt marsh plant *Spartina alterniflora* 2
- AUTHORS 2
- ABSTRACT 2
- INTRODUCTION 3
- MATERIALS AND METHODS 5
  - Study species and sampling sites 5
  - AFLP and MS-AFLP protocol 6
  - Genetic and epigenetic analysis 8
  - Comparison between genetic and epigenetic variation 10
- RESULTS 11
  - Genetic diversity and structure 11
  - Epigenetic diversity and structure 12
  - Comparison between genetic and epigenetic variation 12
- DISCUSSION 13
- ACKNOWLEDGMENTS 18
- LITERATURE CITED 19
- TABLES AND FIGURES 23

CHAPTER 3: Disentangling genetic and epigenetic variation across salt marsh environmental gradients in replicate populations of *Borrichia frutescens* 28
- AUTHORS 28
- ABSTRACT 28
- INTRODUCTION 29
- MATERIALS AND METHODS 32
  - Study species and sampling sites 32
  - AFLP and MS-AFLP protocol 33
  - Genetic and epigenetic analysis 35
  - Comparison between genetic and epigenetic variation 37
- RESULTS 38
  - Genetic diversity and structure 38

Epigenetic diversity and structure 39
Comparison between genetic and epigenetic variation 39
DISCUSSION 40
Structure of population genetic and epigenetic variation 41
Disentangling genetic and epigenetic variation 41
ACKNOWLEDGMENTS 46
LITERATURE CITED 47
TABLES AND FIGURES 52

APPENDIX A: Population Epigenetics 59

APPENDIX B: Copyright permission from Springer Science for use of this manuscript in dissertation 74

LIST OF TABLES

Table 2.1: Mean AFLP haplotype and MS-AFLP epi-genotype diversity (*h*) and percent polymorphic loci by site (% P), based on 96 polymorphic AFLP loci and 80 polymorphic MS-AFLP loci in *Spartina alterniflora* sampled from three habitats (i.e. low, intermediate, and high salt) within five sites on Sapelo Island, GA. (SE = standard error). 23

Table 2.2: Pairwise Φ_{ST} values for comparisons among 15 sub-populations at AFLP (above the diagonal) and MS-AFLP (below the diagonal). ^{NS} indicates a non-significant Φ_{ST} value, all others are significant based on FDR = 0.05. 24

Table 3.1: Mean AFLP haplotype (n=288) and MS-AFLP epi-genotype (n=288) diversity (*h*) and percent polymorphic loci by site (%P), based on 64 AFLP loci and 47 MS-AFLP loci in *Borrichia frutescens* sampled from three habitats (i.e. low, intermediate, and high salt) within five sites on Sapelo Island, GA. (SE = standard error). SE=standard error. 52

Table 3.2: Pairwise Φ_{ST} values for comparisons among 15 sub-populations at AFLP (below the diagonal) and MS-AFLP (above the diagonal). ^{NS} indicates a non-significant Φ_{ST} value, all others are significant based on FDR = 0.05. S, M, and T represent short, medium, and tall plants (i.e. high, intermediate, and low salt) respectively. 53

Table 3.3: Genetic and epigenetic correlations to variation in habitat (i.e. low, medium, high salt) calculated separately for each of the five populations on Sapelo Island, GA and combined across the five populations using partial Mantel tests. The correlation between genetic and epigenetic variation was calculated in a separate Mantel test. *Q≤0.05, based on FDR. 54

Table 3.4: Three-level hierarchical analysis of molecular variance (AMOVA). Hierarchical Φ-statistics and percentage of genetic and epigenetic variance explained by each hierarchical level are given. Φ-statistics were calculated using 9,999 permutations. d.f.=degrees of freedom; **p≤0.001, ***p≤0.0001. 55

LIST OF FIGURES

Figure 2.1: Map of the five sampling sites on Sapelo Island, GA with the results of Bayesian clustering from the program Structure. Population assignment to two groups is indicated by the shaded portion of the circle (Group 1=light grey, Group 2=dark grey). 25

Figure 2.2: Genetic and epigenetic correlations (r value when significant and p-value) to variation in habitat (i.e. low, medium, high salt) using partial Mantel tests. The correlation between genetic and epigenetic variation was calculated in a separate Mantel test. 26

Figure 2.3: Frequencies of (A) AFLP and (B) MS-AFLP loci that were significantly correlated with habitat using a generalized linear model. 27

Figure 3.1: Map of the five sampling sites on Sapelo Island, GA with the results of Bayesian clustering from the program Structure. Population assignment to two groups is indicated by shaded portion of the circle. Group 1=light grey, Group 2=dark grey. 56

Figure 3.2: Genetic and epigenetic correlations to variation in habitat (i.e. low, medium, and high salt) across five populations using partial Mantel tests. The correlation between genetic and epigenetic variation was calculated in a separate Mantel test. NS=not significant, r=correlation coefficient when significant, and p-value. 57

Figure 3.3: Frequencies of (A) AFLP and (B) MS-AFLP loci that were significantly correlated with habitat across five populations using a generalized linear model. 58

ABSTRACT

Phenotypic plasticity is the ability of a given genotype to exhibit different phenotypes in response to environmental variables, which can impact population level processes. Plasticity of ecologically-relevant traits is important to an organism's environmental response; however, the underlying mechanisms of plasticity are largely unknown. Ecological epigenetics may offer mechanisms (e.g. DNA methylation) underlying phenotypic plasticity. Epigenetics can be defined as the underlying molecular mechanisms that allow one genotype to exhibit different phenotypes. Differential DNA methylation is one epigenetic mechanism that has been correlated with a number of ecologically-relevant traits; including, differential herbivory in *Viola cazorlensis*, spinescence in *Ilex aquifolium*, flower morphology in *Linaria vulgaris*, and fitness in *Arabidopsis thaliana*. The epigenetic correlations with traits found in these studies are interesting, but they are also partially confounded by a potential correlation between genetic and epigenetic variation.

Teasing apart the correlation between genetic and epigenetic variation is one of the challenges within ecological epigenetics. This correlation has resulted in epigenetic variation being partitioned into three types by researchers: obligate, facilitated, and pure. Changes in obligate epigenetic variation are directly correlated with genetic variation. Changes in pure epigenetic variation are completely independent from genetic variation. Changes in facilitated epigenetic variation are partially dependent on genetic variation, but the outcome of the phenotype is context-dependent based on environmental conditions. Since our predictions about

the outcome of phenotypic variation are driven largely by population genetics theories, which make no room for variation that operates in non-Mendelian ways, epigenetics research needs to utilize unique ways to tease apart the interaction between genetic and epigenetic variation where facilitated or pure epigenetic variation exists outside of the realm of population genetics theory.

To address these issues, I performed a literature review and two research-based studies. In Chapter 1 I performed a literature review on the topic of population epigenetics addressing the correlation with genetic variation and recommending an extension to the Modern Synthesis to accommodate the non-Mendelian nature of DNA methylation. While population genetics has approximately 85-years of data to support it, epigenetics is beginning to show some of the limitations associated with predictions made using populations genetics models. One of these limitations is that population genetics as defined by the Modern Synthesis does not allow for violations of Mendelian genetics (i.e. random assortment and segregation of alleles). This limitation does not allow for phenotypic variation that is directly due to environmental conditions; however, recent ecological epigenetics data shows that this can, indeed, occur. Within this review I propose epigenetic questions that we should focus on at the population level, and I make recommendations for how to approach these questions in future studies.

In the second and third research-based chapters, I investigated whether an independent component of epigenetic variation was correlated with habitat, while controlling for a correlation with genetic variation, for *Spartina alterniflora* and *Borrichia frutescens*, respectively. Previous work has shown that there is no consistent genetic response to environment in these species. I, therefore, hypothesized that there would be a significant epigenetic correlation with habitat instead. To test this hypothesis, I collected leaf samples from five different sites for each species on Sapelo Island, GA. Within each site I established three 10m transects (n=20 for each

microhabitat) in low, middle, and high marsh microhabitats, respectively. Plants of both species exhibit different phenotypes for height (tall, intermediate, short, respectively) based on their location within the marsh. I screened AFLP and methylation-sensitive AFLP (MS-AFLP) markers for genetic and epigenetic variation, respectively. I used a variety of statistical tests to attempt to tease apart a potential correlation between genetic and epigenetic variation and found that when genetic population structure is controlled for, significant epigenetic population structure persists across all populations for *S. alterniflora* and within 3 of 5 populations for *B. frutescens*. These results suggest that regulation of certain genomic elements via DNA methylation may play an important role in dealing with environmental variables. To fully determine the significance of these findings, future studies should examine the interaction between environmentally-mediated epigenetic variation and gene expression to determine its importance to phenotypic plasticity and habitat differentiation.

The body of work I produced supports that epigenetics may play a role in environmental response in populations within relatively small spatial scales. I used a combination of statistical tests to control for potential correlations with genetic variation which allowed me to see patterns that may normally be hidden. These findings expand upon traditional views of evolution by suggesting that environment can play a role in phenotypic variation, and other research supports that the variation due to epigenetic mechanisms can be inherited in future generations. Much of the current epigenetic research is based upon studies involving model species in highly controlled studies. While this research is been incredibly informative about some of the mechanisms underlying epigenetics, to fully understand the role of epigenetics to environmental response and evolution we must pair these data with field studies of non-model organisms. Only then will we begin to see the full role of epigenetics in organisms.

CHAPTER 1:

Population Epigenetics

Note to Reader:

This chapter has been previously published: Foust, C.M., Schrey, A.W., Richards, C.L. 2015. Population Epigenetics. In: *Nuclear Function in Plant Transcription, Signaling, and Development*. O. Pontes, H. Jin, eds. Springer. 165-179. See Appendix A for the PDF of the published document.

CHAPTER 2:

Epigenetic variation is correlated with habitats in natural populations of the foundation salt marsh plant *Spartina alterniflora*

AUTHORS

Christy M. Foust, Veronica Preite, Aaron W. Schrey, Koen J.F. Verhoeven, Christina L. Richards

ABSTRACT

Understanding the relationship between the environment and natural phenotypic variation has long been of interest to ecologists. Some ecological studies have demonstrated that natural selection can result in genetic associations with habitat type within populations; however, this pattern is not always apparent along natural environmental gradients. Using AFLP and MS-AFLP markers, we tested the hypothesis that epigenetic variation in natural populations of *Spartina alterniflora* is structured by the extreme environmental gradients of the salt marsh, rather than genetic variation. With separate AMOVAs, we detected genetic and epigenetic differentiation among habitats across Sapelo Island. However, when analyzed simultaneously, we found that the correlation between epigenetic variation and habitat was significant and there was no correlation between genetic variation and habitat. Further, there were more epigenetic

loci correlated to habitat compared to genetic loci. These results suggest that epigenetic variation plays a role in response to habitat variation by *S. alterniflora*.

INTRODUCTION

Ecologists have long been interested in the relationship between the environment and natural phenotypic variation (Turreson 1922; Clausen et al. 1948; Bradshaw 1965; Antonovics & Bradshaw 1970). Studies have demonstrated that natural selection in different microhabitats within potentially interbreeding populations can result in associations of genotypes, or alleles of candidate genes, with habitat types (e.g., Hamrick & Allard 1972; Schmidt & Rand 1999; Schmidt et al. 2008). These studies support the evolutionary theory that heritable phenotypic variation for traits that increase tolerance to local conditions can result in adaptation to local conditions. Strong selection should act on these traits leading to genetic differentiation, and therefore, to locally adapted populations even in the face of gene flow (Levene 1953; Hedrick 1976; Caisse & Antonovics 1978; Feder et al. 1997). However, studies across a diversity of taxa have found either no association of genetic differences with habitat (e.g. Richards et al. 2004; examples in Schmidt et al. 2008) or that low levels of molecular level diversity were not associated with decreased phenotypic variation or habitat variation (Dlugosch & Parker 2008; Richards et al. 2012).

The predicted relationship between genetic variation and environmental conditions rests on the assumption that the heritable variation in traits that responds to selection along gradients is based only on DNA sequence differences (Schmidt et al. 2008; Fischer et al. 2013; Roda et al. 2013). However, the actual mechanisms that underlie heritable phenotypic variation are not completely understood, particularly those attributable to factors other than genotype (Richards et

al. 2010a; Keller 2014). One important source of phenotypic variation is phenotypic plasticity, which allows for variation in phenotype from the same genotype in response to different environmental conditions. The ability to express plasticity is modulated at a molecular level, and there is considerable evidence that both genetic and epigenetic effects contribute to phenotypic plasticity (Bossdorf et al. 2010; Herrera & Bazaga 2013; Zhang et al. 2013; Nicotra et al. 2015). In ecological studies, the most studied epigenetic effect is DNA methylation (Schrey et al. 2013). DNA methylation has been shown to increase in variance in response to exposure to stress (Verhoeven et al. 2010; Dowen et al. 2012) and affect ecologically important phenotypes (Cortijo et al. 2014; Kilvitis et al. 2014). Because epigenetic states can be altered in organisms facing stressful conditions, epigenetic effects could allow for rapid phenotypic response to dynamic environments without any change in genetic variation, thereby affecting the evolutionary potential of natural populations (Bossdorf et al. 2008; Richards et al. 2010a).

Coastal salt marshes have been ideal systems for examining how organisms respond to environmental variation because they contain severe environmental gradients that have been correlated to variation within species, as well as zonation patterns among species (Pennings & Bertness 2001; Richards et al. 2005, 2010b). In particular, the dominant salt marsh plant, *Spartina alterniflora*, has a large native range that extends along the Atlantic to Gulf coasts of North America and is tolerant to a wide array of environmental conditions (Pennings & Bertness 2001; Richards et al. 2005). Previous studies have shown that *S. alterniflora* displays variation in many traits correlated with environmental drivers; such as inundation, nutrients, and salinity gradients (e.g. Richards et al. 2005). Replicate populations along the east coast of North America provide a unique opportunity to explore how natural selection can shape the genetic make-up of populations (Schmidt et al. 2008). Our previous work in these salt marshes found no association

between habitat and multi-locus genotypes (i.e. alleles at allozyme loci), even though plants across the gradient harbor high levels of genetic and genotypic variation and are actively reproducing (Richards et al. 2004).

In this study, our objective was to determine if epigenetic variation is correlated to habitat along a salinity gradient, which includes the wide environmental tolerance of *S. alterniflora.* We screened *S. alterniflora* from three habitat types (i.e. low, medium, and high salt) within and among five native populations on Sapelo Island, GA at both genetic (amplified fragment length polymorphism, AFLP) and epigenetic (methylation sensitive (MS)-AFLP) markers. We tested the hypothesis that epigenetic differentiation occurs among habitats along the gradient, while genetic differentiation does not.

MATERIALS AND METHODS

Study species and sampling sites

We conducted this study at five sites separated by approximately 5-20 km on Sapelo Island, GA, USA (31° 28’ N, 81° 14’ W) in May 2011 (Fig. 2.1). The salt marshes within each site were typical of those in the south-eastern United States and *Spartina alterniflora* was the dominant plant species (Richards et al. 2004, 2005). We defined each site as a distinct population that consists of potentially interbreeding individuals. Within each population, *S. alterniflora* occurs across a broad range of environmental conditions (e.g. soil salinities range from 20 to >100 ppt), and exhibits a broad range phenotypic variation (heights range from approximately 20 to 200 cm; Richards et al. 2005) that is correlated to environmental factors.

Our sampling scheme allowed for the examination of genetic and epigenetic variation at population and habitat-level within populations (i.e. sub-population) spatial scales. Within each

population, we established a 10 m transect within each of the low, intermediate, and high salt habitats (sensu Richards et al. 2004). This sampling scheme allowed us to use the relative height of plants as a proxy for salinity (e.g. Richards et al. 2005), and it assumes that the relative salinity between habitats within a population is approximately the same across all populations. We collected plant tissue at 1 m increments on both sides of each transect, resulting in 20 samples each from a) high salt, which were the shortest plants adjacent to salt pans, b) intermediate salt, which were intermediate height plants at least 3 m from high and low salt transects, and c) low salt, which were the tallest plants adjacent to a tidal creek. We collected the second or third fully expanded leaf on each plant that did not exhibit signs of herbivory. All samples were frozen in liquid nitrogen in the field within 10 minutes of being harvested and stored at -20°C until further analysis.

AFLP and MS-AFLP protocol

We screened a total of 287 individuals for genetic variation using AFLP. We performed duplicate DNA extractions from each of the plant samples with the Qiagen DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). We screened each of the duplicates through the entire AFLP protocol following Richards et al. (2012), using *Eco*RI+AGC (6-FAM) and +ACG (HEX) labeled primers with *Mse*I+CAA unlabeled primers, to ensure reliable scoring of AFLP fragments. We sent selective PCR products to Iowa State University DNA facility for fragment analysis on an ABI3130XL.

We used the R package RawGeno 2.0 (Arrigo et al. 2009) to score AFLP fragments. We manually set bin widths using the graphic interface with the minimum relative fluorescence units for band identification set at 50. These parameters resulted in 96 reliably scored AFLP bands,

with bands present coded as “1”, absent coded as “0”. Where duplicates did not match at a certain locus, the band was recorded as present to minimize the chance of missing a present band because of inefficient PCR amplification. Throughout, we use “locus” to indicate a specific fragment size in the AFLP and MS-AFLP results. We use “haplotype” to indicate the collection of binary variable positions (dominant genotypes) for each individual at AFLP loci, and “epi-genotype” to indicate the collection of binary variable positions at MS-AFLP loci.

For MS-AFLP, we screened 298 individuals using the same duplicate DNA extractions used for AFLP. In two reactions for each duplicate DNA extraction, we replaced *Mse*I with either *Msp*I+TCAT or *Hpa*II+TCAT. *Msp*I and *Hpa*II are isochizomers (restriction site CCGG) with different sensitivities to cytosine methylation. Cleaving by *Hpa*II is blocked when the inner or outer C is methylated at both strands, while cleaving by *Msp*I is blocked when the outer cytosines are fully or hemi-methylated; cleaving in both enzymes is blocked when both cytosines are methylated. We scored four methylation states with MS-AFLP: Type 1 is when both enzymes cut (no methylation); Type 2 if *Hpa*II does not cut and *Msp*I does cut (restriction site has a methylated internal C), Type 3 if *Hpa*II does cut and *Msp*I does not (restriction site has a hemi-methylated outer C); and Type 4 is when no band is produced, indicating either hypermethylation or a sequence mutation at the restriction site. We pooled data into two categories, methylated (Type 2 and Type 3) or not methylated (Type 1) restriction sites. We treated Type 4 as missing data, because the methylation state cannot be specified (Salmon et al. 2008). While some advocate for discriminating between Type 2 and Type 3 methylation (Schulz et al. 2014; Medrano et al. 2014), recent work indicates that Type 2 and 3 variation cannot be simply interpreted as CG versus CHG methylation, because what looks like CHG methylation is

often caused by differential CG methylation at internal restriction sites nested within fragments (Fulnecek & Kovarik 2014).

Genetic and epigenetic analysis:

Although we initially assumed that each of the five sites represented unique potentially interbreeding populations, we tested this assumption by calculating standard population genetics statistics within and among populations and habitats. We used GenALex 6.5 (Peakall & Smouse 2012) to estimate haplotype diversity (*h*-AFLP), epigenetic diversity (*h*-MS-AFLP), and percent polymorphisms by site (Table 2.1). We also used GenALex to estimate genetic differentiation and epigenetic differentiation using hierarchical analysis of molecular variance (AMOVA). We conducted hierarchical AMOVA treating each of the habitats within sites as if they were separate sub-populations (total = 15 sub-populations). This analysis assessed if physical geography structured genetic or epigenetic differences by comparing variances among populations (Φ_{RT}), among sub-populations (i.e. habitats) within populations (Φ_{PR}), and among sub-populations (Φ_{PT}). We used 9999 permutations to estimate statistical significance and the initial alpha of 0.05. We also estimated Φ_{ST} pairwise among sub-populations with GenALex. Following the pairwise analysis, we determined how many pairwise comparisons were significantly different from each other by the false discovery rate method (FDR = 0.05: Benjamini & Hochberg 1995).

While the hierarchical AMOVA can identify whether plants in different habitats are differentiated within populations, AMOVA cannot determine repeated patterns in response to a gradient among populations. Instead, the different habitats are treated as three random sub-populations within a site. Relatedness within populations may also mask the repeated fine scale differences that might occur in replicate populations with similar environmental gradients. In

order to specifically test for repeated patterns across similar conditions, we ran a permutational multivariate analysis of variance (MANOVA) using the Adonis function within the Vegan package of R. This analysis allowed us to specifically examine the repeated genetic and epigenetic response to “high”, “medium” and “low” salt habitats. As a component of this and following analyses, we used the Euclidean genetic distance matrix (with interpolation for missing values) generated by GenALex as our input data so that comparisons among analyses would be more consistent.

For the genetic data only, we performed Bayesian clustering using Structure v.2.3.4 to estimate population structure among individuals (Pritchard et al. 2000; Falush et al. 2003). Structure estimates the number of groups (k) present among individuals, and assigns individuals to each k using Bayesian modeling. We tested eight populations (k = 1-8), three more than the maximum number of anticipated populations (Evanno et al. 2005), with five independent runs at each k using both the log probability of observing the data (ln Pr(x|k)) method of Structure and Delta k (Evanno et al. 2005) to determine the number of populations that best fit the data. We incorporated sampling location data in our model to assist in detecting weak amounts of differentiation. We performed clustering with the admixture model, 30,000 burn-in steps, 100,000 post burn-in steps, and allowed correlated allele frequencies. We assigned individuals to groups based on the highest q-value. We also performed a Mantel test using ZT software (Bonnet & Van der Peer 2002) to determine if genetic differentiation was driven by isolation-by-distance. We used the Euclidean genetic distance matrix generated by GenALex, and we created a pairwise geographic distance matrix among the sub-populations using distances obtained via Google Earth.

Comparison between genetic and epigenetic variation

We simultaneously analyzed the correlation between genetic variation, epigenetic variation and habitat by performing a partial Mantel test using ZT software (Bonnet & Van der Peer 2002). The partial Mantel test determines correlations between two distance matrices while controlling for correlations with a third matrix. In this case, to create the habitat distance matrix we created a step-wise distance matrix to represent the relative salinity difference between habitats within a population. We use zero (0) for distance within the same habitat, one (1) for the distance between low and medium or between medium and high salt habitats, and two (2) for the distance between low and high salt habitats. We tested for a relationship between genetic variation and habitat while controlling for correlations between genetic variation and epigenetic variation and epigenetic variation and habitat. Likewise, we tested for a relationship between epigenetic variation and habitat while controlling for correlations between genetic variation and epigenetic variation and genetic variation and habitat. We also performed a Mantel test using the ZT software (Bonnet & Van der Peer 2002) to test for a relationship between genetic and epigenetic variation. In all cases, we used the Euclidean genetic and epigenetic distance matrices generated by GenALex.

To determine if specific AFLP and MS-AFLP loci were correlated with habitat, we ran a generalized linear model using the "glm" function in R. "Population" was set as a random effect, and the distribution family was set as "binomial." This allowed us to isolate specific loci that might otherwise be swamped out by genome wide analyses.

RESULTS

Genetic diversity and structure

Across all populations, genetic diversity was high (Table 2.1). Nearly every individual (n = 287) exhibited a unique haplotype across 96 polymorphic loci and haplotype diversity (*h*-AFLP) ranged from 0.317-0.412 among the five sampling sites (Table 2.1). Population structure was detected at every hierarchical level: among populations (Φ_{RT} = 0.088, p < 0.001), among sub-populations (habitats) within populations (Φ_{PR} = 0.071, p = 0.0001), and among sub-populations (Φ_{PT} = 0.152, p = 0.0001). In pairwise comparisons of the 15 sub-populations, 88.6% of the tests were significant (FDR = 0.05; Table 2.2). Thus, samples from the different habitats within each population (e.g. Apex population: low, medium, and high salt habitats) were typically different from each other and also from the samples from each habitat in other populations. Despite the significant sub-population structure within populations, the permutational MANOVA showed no effect of habitat type, which suggests a lack of consistent genetic differentiation to habitat type.

Structure identified two genetic groups. The maximum ln Pr(X|K) was K2 (-13498.5) and the maximum Delta K was K2 (6,823.4). Bayesian clustering showed a pattern of weak genetic isolation by distance among the different sites, which suggests geographic-based isolation by distance between the north-east (i.e. Apex and Cabretta) and south-west (i.e. Hunt Camp, Lighthouse, and Marsh Landing) portions of the island or a north-south gradient in genetic make-up on the island (Fig. 2.1). A Mantel test comparing genetic distance and geographic distance showed a highly significant correlation between geographic distance and genetic variation (r = 0.20, p < 0.0001).

Epigenetic diversity and structure

We found a high level of epigenetic variation across sites. Nearly every individual (n = 298) exhibited a unique epi-genotype across 80 polymorphic loci, and epi-genotype diversity (*h*-MSAFLP) ranged from 0.336-0.402 among the five sampling sites (Table 2.1). Hierarchical AMOVA revealed epigenetic structure among populations (Φ_{RT} = 0.045, p = 0.0001), among sub-populations (habitats) within populations (Φ_{PR} = 0.056, p = 0.0001), and among sub-populations (Φ_{PT} = 0.098, p = 0.0001). In post-hoc pairwise comparisons, 95.2% of the tests were significant (FDR = 0.05, Table 2.2). Thus, nearly all sub-populations were significantly differentiated at epigenetic loci. Despite the significant sub-population structure within populations, the permutational MANOVA showed no effect of habitat, which suggests no consistent epigenetic differentiation to habitat type.

Comparison between genetic and epigenetic diversity

We detected an independent portion of epigenetic variation that was weakly, but significantly correlated with habitat when we controlled for the correlation with genetic variation (partial Mantel test, r = 0.026, p = 0.0047: Fig. 2.2). Partial Mantel tests on each site showed stronger correlations to support this trend ($0.057 < r < 0.213$; $p \leq 0.034$), with the exception of Marsh Landing. We found a stronger correlation between genetic and epigenetic variation (r = 0.424, p < 0.001) than between epigenetic variation and habitat. However, there was no relationship between genetic variation and habitat when we controlled for the correlation with epigenetic variation. Further, the generalized linear model identified only 8.3% of the AFLP loci were significantly correlated with habitat (Fig. 2.3A), while16.3% of the MS-AFLP loci were significantly correlated with habitat (Fig. 2.3B). Thus, while epigenetic and genetic variation

were strongly correlated, habitat differences showed stronger correlation with epigenetic than with genetic variation (Fig. 2.2).

DISCUSSION

In recent years, several lines of evidence have suggested that epigenetic mechanisms could contribute to response to dynamic or stressful environments independent of genetic variation, but these ideas have rarely been tested in natural populations. While we found significant genetic and epigenetic population and sub-population structure, we identified a weak but significant independent correlation between epigenetic variation and habitat, and no independent correlation between genetic variation and habitat when we statistically control for the correlation between genetic and epigenetic variation. Considering the complex relationship between genetic and epigenetic variation (Richards 2006; Richards et al. 2010b), the application of partial Mantel tests was critical to isolate the independent relationships between genetic and epigenetic variation with habitat type.

We found high levels of genetic and epigenetic variation in *Spartina alterniflora*, and contrary to our prediction we found significant genetic and epigenetic structure among populations and among habitats within populations. However, AMOVA tells us only that the sub-populations are different from each other and cannot detect ordered response across replicate populations. Therefore, detecting significant structure within populations does not address repeated response to similar location along the environmental gradient. In order to test for repeated response to similar conditions along the environmental gradients within populations, we used permutational MANOVA. This analysis revealed that there was no repeated change in genetic or epigenetic structure in response to habitat when each was analyzed separately; though,

a generalized linear model on each locus revealed individual AFLP and MS-AFLP loci that are correlated with habitat. The presence of nearly twice as many epigenetic loci correlated with habitat suggests that DNA methylation may be an important source of variation in response to environmental conditions. Isolating how much this source of variation is due to a correlation with genetic variation versus independent of genetic variation requires further work.

Previous studies that have found relationships between epigenetic variation and environmental factors have often benefitted from naturally low levels of genotypic diversity (e.g. Verhoeven et al. 2010; Richards et al. 2012; Herrera & Bazaga 2013) or artificially increased variation in methylation within replicate isogenic lines (Zhang et al. 2013; Cortijo et al. 2014). As we expand our studies of epigenetics into a variety of non-model organisms, including those with high levels of genetic variation, researchers will be faced with the confounding problem of the potential correlation between genetic and epigenetic variation: a problem that has already been highlighted as one of the important issues in the field of ecological epigenetics (Bossdorf et al. 2008, 2010; Richards et al. 2010a).

To disentangle these two sources of variation, Richards (2006) categorized the possible relationships between genetics and epigenetics as obligate, facilitated, or pure. Obligate epigenetic variation is directly correlated with genetic, pure is independent from genetic, and facilitated is partially correlated with genetic variation but the outcome of the epigenetic state is context dependent. Careful design of experiments and analyses are therefore required to determine the complex interactions between genetic and epigenetic variation in natural populations (e.g. Bossdorf et al. 2010; Herrera & Bazaga 2013; Nicotra et al. 2015).

In our *S. alterniflora* populations, high levels of both genetic and epigenetic diversity made it challenging to parse out which mechanisms may be independently contributing to

environmental response. AFLP and MS-AFLP determine genome-wide patterns of variation, but epigenetically mediated functional responses may be restricted to a few specific loci that we cannot standardize across diverse genotypes. For example, each individual possessed a unique genotype, but similarly-sized fragments across the individuals could be related to similar function. We may not be able to make that same assumption about MS-AFLP loci. Additionally, it is difficult to evaluate genetic and epigenetic variation simultaneously and account for the correlation between the two. To address this, we used a partial Mantel test across populations to accomplish this simultaneous analysis and showed that independent epigenetic variation is significantly, if weakly, correlated with habitat, when we control for a correlation with genetic variation. Separate partial Mantel tests on each population showed even stronger support for significant, independent epigenetic correlations with habitat in four out of five populations when the correlation with genetic variation was controlled. These findings suggest that assessing AFLP and MS-AFLP data sets separately may misrepresent the roles of genetic and epigenetic variation. Analyses that accommodate a comparison between both data sets and allow for controlling for the correlation between genetic and epigenetic data can reveal significant relationships that are otherwise obscured.

Our results support the potential importance of DNA methylation in *S. alterniflora* habitat response, even in the presence of genetic population structure, but also generate additional questions. For example, are these loci associated with specific phenotypes that are ecologically-relevant? Are the epigenetic loci associated with identifiable, functional genes or gene networks that are important in response to environmental conditions? Is this epigenetic variation constitutively passed on to the next generation or necessarily induced by the environment?

Answering these questions will be challenging in naturally, wind pollinated, and outcrossing plants like *S. alterniflora* that are long lived and have few genomics resources. Although we currently face limitations in many non-model species, data from other systems suggest that DNA methylation is important for many questions in ecology and evolution (Kilvitis et al 2014; Robertson & Richards 2015). For example, there is mounting evidence that DNA methylation is associated with complex ecologically important traits. One of the clearest examples is the epimutation in the *Lcyc* gene in *Linaria vulgaris* which transforms the flower from the wild type bilateral symmetry to radial symmetry (Cubas et al. 1999). In *Helleborus foetidus*, global methylation levels have been significantly associated with size and fecundity-related traits (Alonso et al. 2014). Chemically reduced methylation levels on epigenetic recombinant inbred lines (epiRILs) developed in a single genotype of *Arabidopsis thaliana* have resulted in decreased phenotypic plasticity, size, biomass, and fecundity-related traits; while increasing flowering time and mortality (Bossdorf et al. 2010). Further experiments on these epiRILs have identified epigenetic quantitative trait loci (QTLepi), which account for 90% of the heritability of flowering time and 60% of the heritability of primary root length (Cortijo et al. 2014).

While recent studies support an important role of DNA methylation in ecologically and evolutionarily relevant traits, most are limited to the understanding of the complex relationship between genetic variation and epigenetic variation because they rely on systems naturally limited in genetic variation. For species that do exhibit genetic diversity (e.g. *A. thaliana*) the genome and epigenome are atypical compared to most plants that have been surveyed (Alonso et al. 2015). While several authors have suggested that DNA methylation is strictly under genetic control (e.g. Dubin et al. 2015; Hagmann et al. 2015), we have very little data in any system that

can address to what extent there is a component of epigenetic variation independent of genetic variation that contributes to organismal function. Our study demonstrates that we can expose an independent epigenetic component using MS-AFLPs – the next step will be to use higher-resolution methods to understand the functional consequences of that independent component. An important step in this process is identifying the functional relationship between DNA methylation and gene expression, and ultimately phenotype (Alvarez et al. 2015). While further work is required to determine if there is a causal link between DNA methylation and the phenotypic differences exhibited across the environmental gradient in *S. alterniflora*, this work will inform our understanding of how complex genomes translate into functional variation in natural environments.

As more field studies and studies on non-model organisms are done, researchers must be mindful of the potential correlation between genetic and epigenetic variation. This correlation is one of the important issues in the field of ecological epigenetics, and it may prove difficult to define epigenetic variation as obligate, facilitated, or pure (Richards 2006, Richards et al. 2010a). Our study shows that statistical approaches can reveal correlations between epigenetic variation and habitat when the correlation with genetic variation is controlled for. Though more studies are needed to determine if this correlation between DNA methylation and habitat is also correlated with differential gene expression and ecologically-relevant traits, the current body of epigenetic data supports that epigenetics could play a role at several levels of biological hierarchy. We are beginning to address these questions using sequencing techniques, to identify relationships between DNA methylation, functional genomic elements and ecologically relevant traits.

ACKNOWLEDGEMENTS

We thank Mariano Alvarez and Ashley Shayter for assistance with plant sampling on Sapelo Island, GA and the Georgia Coastal LTER for access to field sites. We also thank Mariano Alvarez, Marta Robertson, Holly Kilvitis, and Nancy Sheridan for offering helpful feedback on this manuscript. This work was partially supported by the Global Invasions Network Research Exchange funded by the National Science Foundation (Grant No. 0541673).

LITERATURE CITED

1. Alonso, C., Pérez, R., Bazaga, P., Medrano, M. & Herrera, C.M. (2014). Individual variation in size and fecundity is correlated with differences in DNA cytosine methylation in the perennial herb *Helleborus foetidus* (Ranunculaceae). *Am. J. Bot.*, 101, 1309-1313.
2. Alonso, C., Pérez, R. Bazaga, P. & Herrera, C.M. (2015). Global DNA cytosine methylation as an evolving trait: phylogenetic signal and correlated evolution with genome size in angiosperms. *Front. in Genetics*, 6, 4.
3. Alvarez, M., Schrey, A.W. & Richards, C.L. (2015). Ten years of transcriptomics in wild populations: What have we learned about their ecology and evolution? *Mol. Ecol.*, 24, 710-725.
4. Antonovics, J. & Bradshaw, A.D. (1970). Evolution in closely adjacent plant populations. *Heredity*, 25, 349-362.
5. Arrigo, N., Tuszynski, J.W., Ehrich, D., Gerdes, T. & Alvarez, N. (2009). Evaluating the impact of scoring parameters on the structure of intra-specific genetic variation using RawGeno, an R package for automating AFLP scoring. *BMC Bioinformatics*, 10, 10-33.
6. Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. Roy. Statist. Soc. B*, 57, 289-300.
7. Bonnet, E. & Van de Peer, Y. (2002). zt: a software tool for simple and partial Mantel tests. *J. Statist. Software*, 7, 1-12.
8. Bossdorf, O., Richards, C.L. & Pigliucci, M. (2008). Epigenetics for ecologists. *Ecol. Lett.*, 11, 106-115.
9. Bossdorf, O., Arcuri, D., Richards, C.L. & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evol. Ecol.*, 24, 541-553.
10. Bradshaw, A.D. (1965). Evolutionary significance of phenotypic plasticity in plants. *Adv. Genet.*, 13, 115-155.
11. Caisse, A. & Antonovics, J. (1978). Evolution in closely adjacent plant populations. *Heredity*, 40, 371-384.
12. Clausen, J.D., Keck, D. & Heisey, W.M. (1948). Experimental studies on the nature of species. III. Environmental responses of climatic races of *Achillea*. *Carnegie Institution of Washington Publication 520*.
13. Cortijo, S., Wardenaar, R., Colomé-Tatché, M., Gilly, A., Etcheverry, M., Labadie, K., Caillieux, E., Hospital, F., Aury, J. –M., Wincker, P., Roudier, F., Jansen, R.C., Colot, V. & Johannes, F. (2014). Mapping the epigenetic basis of complex traits. *Science*, 343, 1145-1148.
14. Cubas, P., Vincent, C. & Coen, E. (1999). An epigenetic mutation responsible for natural variation in floral symmetry. *Nature*, 401, 157-161.
15. Dlugosch, K.M. & Parker, I.M. (2008). Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol. Ecol.*, 17, 431-449.
16. Dowen, R.H., Pelizzola, M., Schmitz, R.J., Lister, R., Dowen, J.M., Nery, J.R., Dixon, J.E. & Ecker, J.R. (2012). Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl. Acad. Sci. USA* 109, E2183-2191.

17. Dubin, M.J., Zhang, P., Meng, D., Remigereau, M.-S., Osborne, E.J., Casale, F.P., Drewe, P., Kahles, A., Vilhjálmsson, B., Jagoda, J., Irez, S., Voronin, V., Song, Q., Long, Q., Rätsch, G., Stegle, O., Clark, R.M. & Nordborg, M. (2015). DNA methylation variation in *Arabidopsis* has a genetic basis and shows evidence of local adaptation. *Elife 4*.
18. Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.*, 14, 2611-2620.
19. Falush, D., Stephens, M. & Pritchard, J.K. (2007). Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Mol. Ecol. Notes*, 7, 574-578.
20. Feder, J.L., Roethele, J.B., Wlazlo, B. & Berlocher, S.H. (1997). Selective maintenance of allozyme differences among sympatric host races of the apple maggot fly. *Proc. Natl. Acad. Sci. USA*, 94, 11417-11421.
21. Fisher, M.C., Rellstab, C., Tedder, A., Zoller, A., Gugerli. F., Shimizu, K.K., Holderegger, R. & Widmer, A. (2013). Population genomic footprints of selection and associations with climate in natural populations of *Arabidopsis halleri* from the Alps. *Mol. Ecol.*, 22, 5594–5607.
22. Fulneček, J. & Kovařík, A. (2014). How to interpret Methylation Sensitive Amplified Polymorphism (MSAP) profiles? *BMC Genetics*, 15, 2-9.
23. Hagmann, J., Becker, C., Müller, J., Stegle, O., Meyer, R.C., Wang, G., Schneeberger, K. Fitz, J., Altmann, T., Bergelson, J., Borgwardt, K. & Weigel, D. (2015). Century-scale methylome stability in a recently diverged *Arabidopsis thaliana* lineage. *PLoS Genetics*, 11(1), e1004920.
24. Hamrick, J.L. & Allard, R.W. (1972). Microgeographical variation in allozyme frequencies in *Avena barbata*. *Proc. Natl. Acad. Sci. USA*, 69, 2100-2104.
25. Hedrick, P.W. (1976). Genetic variation in a heterogeneous environment. 2. Temporal heterogeneity and directional selection. *Genetics*, 84, 145-157.
26. Herrera, C.M. & Bazaga, P. (2013). Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (Aquifoliaceae) trees. *Bot. J. Linean Soc*, 171, 441-452.
27. Keller, E.F. (2014). From gene action to reactive genomes. *J. Physiol*, 592.11, 2423–2429.
28. Kilvitis, H.J., Alvarez, M., Foust, C.M., Schrey, A.W., Robertson, M.H. & Richards, C.L. **(**2014). Ecological epigenetics. In: *Ecological Genomics*. C.R. Landry & N. Aubin-Horth, eds. Springer. Series: *Adv. in Exper. Med. and Biol.*, 781: 191-210.
29. Levene, H. (1953). Genetic equilibrium when more than one ecological niche is available. *Am. Nat.*, 87, 331-333.
30. Nicotra, A.B., Segal, D., Hoyle, G.L., Schrey, A.W., Verhoeven, K.J.F. & Richards, C.L. (2015). Adaptive plasticity in response to warming in an Australian alpine herb. *Ecol. Evol*., 5, 634–647.
31. Medrano, M., Herrera, C.M. & Bazaga, P. (2014). Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. *Mol. Ecol.*, 23, 4926–4938.
32. Peakall, R. & Smouse, P.E. (2012). GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics*, 28, 2537-2539.

33. Pennings, S.C. & Bertness, M.D. (2001). Salt marsh communities. In *Marine Community Ecology.* Bertness, M.D. & Gaines, S.D. eds., Sinauer, Sunderland, Massachusetts, USA.
34. Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155, 945-959.
35. Richards, C.L., Hamrick, J.L., Donovan, L.A. & Mauricio, R. (2004). Unexpectedly high clonal diversity of two saltmarsh perennials across a severe environmental gradient. *Ecol. Lett.*, 7, 1155–1162.
36. Richards, C.L, Pennings, S.C. & Donovan, L.A. (2005). Habitat range and phenotypic variation in salt marsh plants. *Plant Ecol.*, 176, 263-273.
37. Richards, C.L., Bossdorf, O. & Verhoeven, K.J.F. (2010a). Understanding natural epigenetic variation. *New Phytol.*, 187, 562-564.
38. Richards, C.L., White, S.N., McGuire, M.A., Franks, S.J., Donovan, L.A. & Mauricio, R. (2010b). Plasticity, not adaptation to salt level, explains variation along a salinity gradient in a salt marsh perennial. *Est. Coasts*, 33, 840-852.
39. Richards, C.L., Schrey, A.W. & Pigliucci, M. (2012). Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecol. Lett*., 15**,** 1016-1025.
40. Richards, E.J. (2006). Inherited epigenetic variation – revisiting soft inheritance. *Nature Rev. Genetics*, 7, 395-401.
41. Robertson, M.H. & Richards, C.L. (2015). Non-genetic inheritance in evolutionary theory the importance of plant studies. *Non-Genet. Inherit.*, 2, 3–11.
42. Roda, F., Ambrose, L., Walter, G.M., Liu, H.L., Schaul, A., Lowe, A., Pelser, P.B., Prentis, P., Rieseberg, L.H. & Ortiz-Barrientos, D. (2013). Genomic evidence for the parallel evolution of coastal forms in the *Senecio lautus* complex. *Mol. Ecol.*, 22, 2941–2952.
43. Salmon, A., Clotault, J., Jenczewski, E., Chable, V. & Manzanares-Dauleux, M.J. (2008). *Brassica oleracea* displays a high level of DNA methylation polymorphism. *Plant Sci.*, 174, 61-70.
44. Schmidt, P.S. & Rand, D.M. (1999). Intertidal microhabitat and selection at MPI: Interlocus contrasts in the northern acorn barnacle, *Semibalanus balanoides*. *Evolution*, 53, 135-136.
45. Schmidt, P.S., Serrão, E.A., Pearson, G.A., Riginos, C., Rawson, P.D., PD, Hilbish, T.J., Brawley, S.H., Trussel, G.C., Carrington, E., Wethey, D.S., Graham, J.W., Bonhomme, F. & Rand, D.M. (2008). Ecological genetics in the north Atlantic: Environmental genetics and adaptation at specific loci. *Ecology, 89*, S91–S107.
46. Schrey A.W., Alvarez M., Foust C.M., Kilvitis H.J., Lee J.D., Liebl A.L., Martin, L.B., Richards, C.L. & Robertson M.H. (2013). Ecological epigenetics: Beyond MS-AFLP. *Integ. Comp. Biol.*, 53, 340-350.
47. Schulz, B., Eckstein, R.L. & Durka, W. (2014). Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Mol. Ecol.*, 14, 3523–3537.
48. Turesson, G. (1922). The genotypical response of the plant species to the habitat. *Hereditas*, 3, 211–350.
49. Verhoeven K.J.F., Jansen, J.J., van Dijk, P.J. & Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytol*, 185, 1108-1118.

50. Zhang, Y. Y., c, M., Colot, V. & Bossdorf, O. (2013). Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *New Phytol*, 197(1), 314-322.

TABLES AND FIGURES

Table 2.1: Mean AFLP haplotype and MS-AFLP epi-genotype diversity (*h*) and percent polymorphic loci by site (% P), based on 96 polymorphic AFLP loci and 80 polymorphic MS-AFLP loci in *Spartina alterniflora* sampled from three habitats (i.e. low, intermediate, and high salt) within five sites on Sapelo Island, GA. (SE = standard error)

	***h*-AFLP (SE)**	**AFLP % P**	***h*-MS-AFLP (SE)**	**MS-AFLP % P**
Apex	0.410 (0.011)	100.00%	0.402 (0.014)	98.75%
Cabretta	0.412 (0.011)	100.00%	0.374 (0.012)	100.00%
Hunt Camp	0.400 (0.011)	98.96%	0.358 (0.015)	95.00%
Lighthouse	0.338 (0.017)	93.75%	0.389 (0.016)	95.00%
Marsh Landing	0.317 (0.018)	91.67%	0.336 (0.017)	95.00%
Average	0.375 (0.006)	96.88%	0.372 (0.007)	96.75%

Table 2.2: Pairwise Φ_{ST} values for comparisons among 15 sub-populations at AFLP (above the diagonal) and MS-AFLP (below the diagonal). ^{NS} indicates a non-significant Φ_{ST} value, all others are significant based on FDR = 0.05. The first letter of the sub-population labels is the site (i.e. Apex, Cabretta, Hunt Camp, Lighthouse, Marsh Landing), and the second letter is the habitat (i.e. high, medium, and low salinity).

AH	AM	AL	CH	CM	CL	HH	HM	HL	LH	LM	LL	MH	MM	ML
	0.04^{NS}	0 ^{NS}	0.09	0.03^{NS}	0.07	0.12	0.09	0.17	0.18	0.14	0.30	0.25	0.22	0.29
0.04		0.04^{NS}	0.09	0.07	0.10	0.11	0.11	0.16	0.15	0.13	0.24	0.18	0.19	0.26
0 ^{NS}	0.07		0.07	0.02^{NS}	0.07	0.10	0.08	0.16	0.17	0.13	0.28	0.23	0.21	0.29
0.11	0.09	0.12		0.04	0.01^{NS}	0.08	0.04	0.14	0.14	0.10	0.24	0.20	0.16	0.22
0 ^{NS}	0.02	0 ^{NS}	0.03		0.05 ^{NS}	0.11	0.05	0.17	0.16	0.11	0.27	0.23	0.18	0.26
0.11	0.08	0.10	0.01^{NS}	0.04		0.10	0.02^{NS}	0.17	0.16	0.14	0.25	0.21	0.19	0.26
0.10	0.10	0.11	0.07	0.07	0.08		0.11	0.03^{NS}	0.06	0.06	0.14	0.10	0.10	0.16
0.09	0.06	0.07	0.05	0.01^{NS}	0.08	0.08		0.18	0.19	0.14	0.28	0.23	0.19	0.28
0.14	0.11	0.16	0.10	0.11	0.06	0.03	0.13		0.02^{NS}	0.02^{NS}	0.15	0.09	0.08	0.17
0.05	0.05	0.07	0.09	0.03	0.10	0.04	0.09	0.03		0.02^{NS}	0.11	0.08	0.06	0.15
0.07	0.10	0.09	0.15	0.08	0.17	0.07	0.12	0.13	0.06		0.18	0.13	0.08	0.19
0.11	0.12	0.11	0.16	0.08	0.17	0.14	0.12	0.19	0.13	0.10		0.07	0.07	0.12
0.08	0.09	0.09	0.12	0.05	0.15	0.07	0.07	0.12	0.06	0.04	0.07		0.08	0.09
0.09	0.11	0.09	0.16	0.06	0.18	0.14	0.09	0.20	0.11	0.07	0.06	0.03		0.08
0.12	0.14	0.12	0.15	0.10	0.16	0.13	0.09	0.18	0.14	0.11	0.07	0.03	0.05	

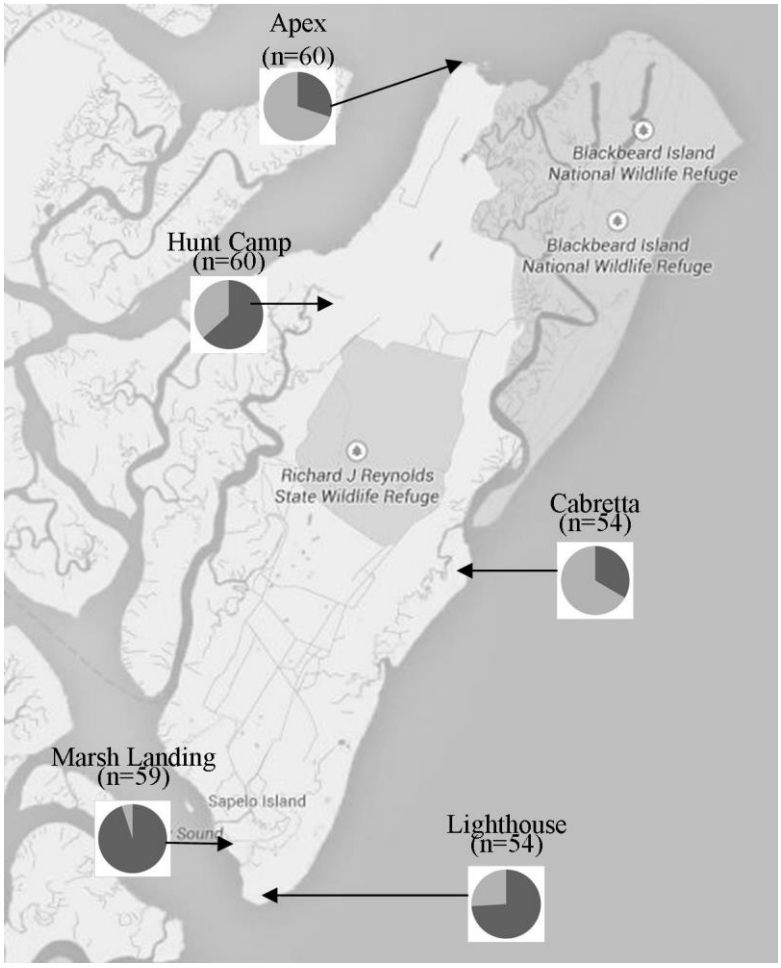

Figure 2.1: Map of the five sampling sites on Sapelo Island, GA with the results of Bayesian clustering from the program Structure. Population assignment to two groups is indicated by shaded portion of circle (Group 1=light grey, Group 2=dark grey).

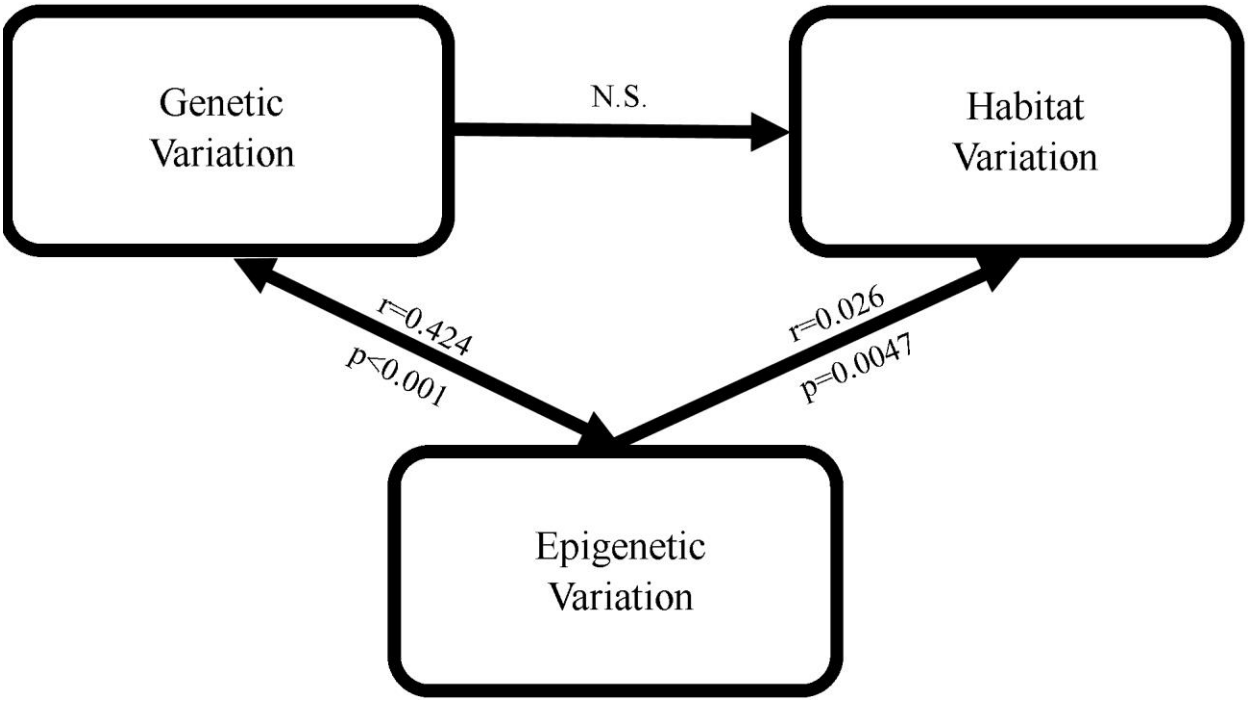

Figure 2.2: Genetic and epigenetic correlations (r value when significant and p-value) to variation in habitat (i.e. low, medium, high salt) using partial Mantel tests. The correlation between genetic and epigenetic variation was calculated in a separate Mantel test.

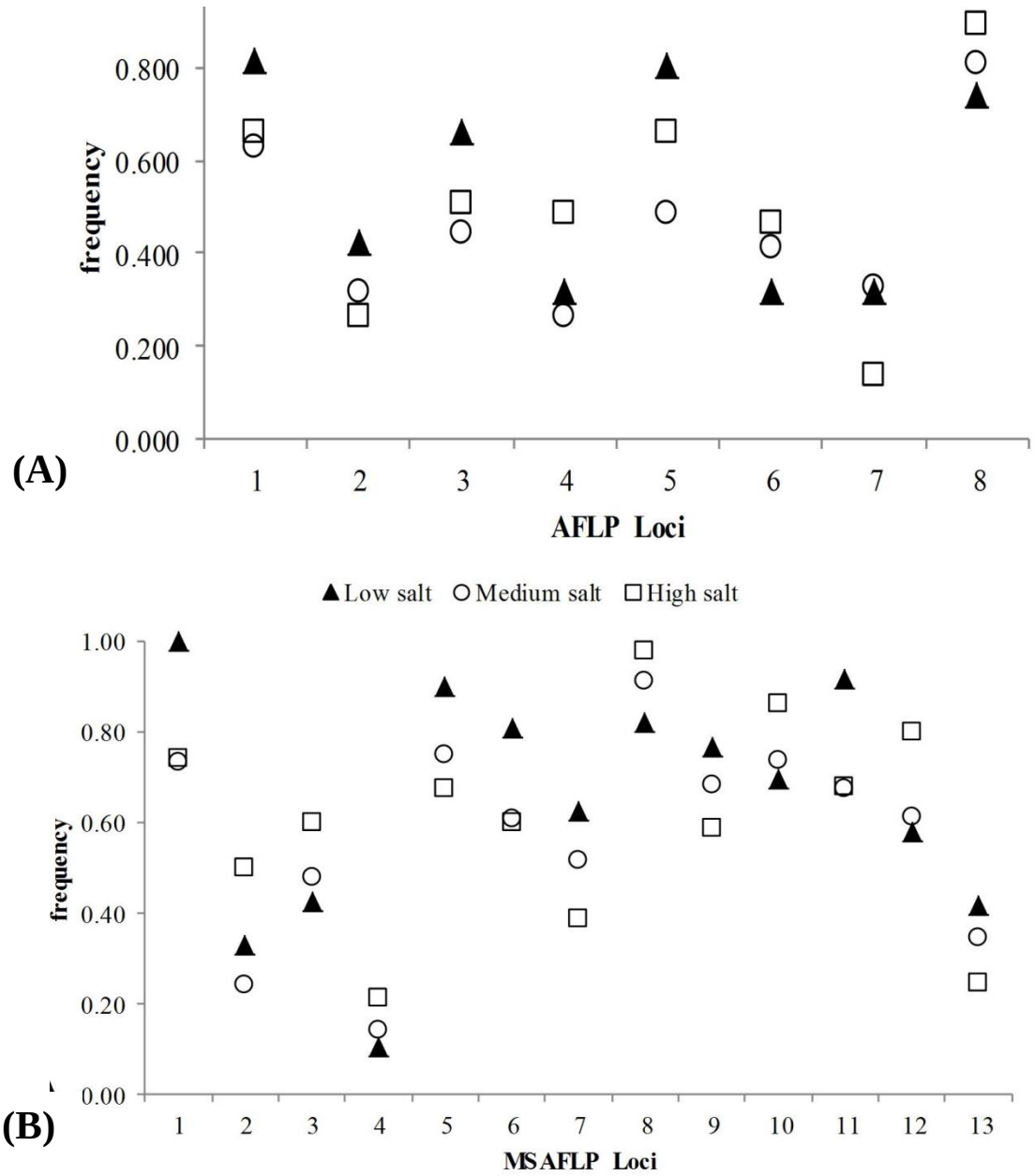

Figure 2.3: Frequencies of (A) AFLP and (B) MS-AFLP loci that were significantly correlated with habitat using a generalized linear model.

CHAPTER 3:

Disentangling genetic and epigenetic variation across salt marsh environmental gradients in replicate populations of *Borrichia frutescens*

AUTHORS

Christy M. Foust, Veronica Preite, Aaron W. Schrey, Koen J.F. Verhoeven, Christina L. Richards

ABSTRACT

Variation in ecologically-relevant traits allows organisms to cope with environmental variation; however, the underlying molecular mechanisms of this response are largely unknown. While we know that both traits and plasticity in traits are genetically based, investigating epigenetic mechanisms, like DNA methylation, may provide more nuanced understanding of the mechanisms underlying response to environment. Variation in DNA methylation can result in phenotypic variation, allow for response to environmental variation, and be stably transmitted across generations. Using AFLP and methylation-sensitive AFLP (MS-AFLP) to assess genetic and epigenetic variation, we tested the hypothesis that *Borrichia frutescens* exhibits epigenetic differentiation, but not genetic differentiation, to habitats along natural salt marsh environmental gradients. With separate AMOVAs, we detected genetic and epigenetic differentiation among habitats across Sapelo Island, GA. However, when genetic and epigenetic variation were analyzed simultaneously across the five populations combined, we found no significant

correlations between genetic or epigenetic variation and habitat. Yet, we found significant correlations between epigenetic variation and habitat and/or genetic variation and habitat in four out of five populations. These analyses suggest that site-specific conditions and historical contingency may cloud our ability to detect response in replicate populations with similar environmental gradients. Additionally, genetic (4.7%) and epigenetic (17%) loci were significantly correlated to habitat, which may better reflect response to conditions along gradients of the salt marsh than genome-wide population structure. Future studies controlling for the correlation between genetic variation and DNA methylation will be critical to disentangling the contributions of genetic and epigenetic response to environmental gradients.

INTRODUCTION

Ecological theory posits that a large proportion of phenotypic variation in natural populations may represent adaptive matching of phenotypes to environmental conditions (Turesson 1922; Clausen et al. 1948; Bradshaw 1965; Antonovics & Bradshaw 1970). To understand adaptation, ecological studies have typically focused on either the genetically-based specialization of putatively adaptive traits or the plasticity of putatively adaptive traits. Yet the two strategies are not mutually exclusive and even individuals with highly specialized traits can adjust to local conditions through phenotypic plasticity (Jump & Peñuelas 2005; van Kleunen & Fischer 2005; Richards et al. 2010b). Further, phenotypic plasticity is genetically based and can be acted on by natural selection if it allows individuals to maintain fitness in multiple habitats (i.e. “adaptive plasticity”; Sultan 2003; van Kleunen & Fischer 2005; Richards et al. 2006; 2010b; Herman et al. 2014).

Given that both trait specialization and plasticity are genetically based, the relative contributions of these strategies lead to different predictions about population genetic structure. Evolutionary models predict, and empirical studies support, that natural selection should lead to locally adapted populations. This is reflected in genetic differentiation among populations in different habitats (Levene 1953; Hedrick 1976; Feder et al. 1997; e.g. Fisher et al 2013; Roda et al. 2014). On the other hand, with sufficient genetic variation, modest gene flow, and the frequency of habitat variation, theory predicts plasticity should be favored in heterogeneous environments leading to highly plastic generalists or general-purpose genotypes that can inhabit a wide variety of environmental conditions (van Tienderen 1991; Sultan and Spencer 2002; Herman et al. 2014). In such systems, studies have found a lack of population genetic structure associated specifically with habitat (e.g. Richards et al. 2004, 2012; Schmidt et al. 2008).

Despite the theoretical and empirical progress from the application of population genetics, the actual mechanisms underlying response to complex environmental conditions are not well understood (Keller 2011, 2014). Response to environment is modulated at the molecular level, and evidence is mounting that both genetic and epigenetic mechanisms play a role in controlling phenotype (Bossdorf et al. 2010; Nicotra et al. 2010, 2015; Herrera & Bazaga 2012, 2013). DNA methylation has been the most-studied epigenetic mechanism in ecology (Schrey et al. 2013), and it can effect ecologically-relevant traits and trait plasticity (Herrera & Bazaga 2012, 2013; Zhang et al. 2013; Cortijo et al. 2014; Xie et al. 2015). Variance in DNA methylation has also been shown to increase in response to ecologically relevant stressors (Verhoeven et al. 2010; Dowen et al. 2012; Herrera et al. 2012; Xie et al. 2015). As such, DNA methylation can provide a mechanism of rapid response for organisms living in stressful

conditions (Rapp & Wendel 2005; Bossdorf et al. 2008), potentially influencing the evolutionary rate and trajectory of natural populations (Klironomos et al. 2013; Platt et al. 2015).

The Atlantic coastal salt marshes provide natural replication of gradients in environmental conditions that allows for robust analyses of how population genetic and epigenetic markers may be structured by environmental stress and heterogeneity at different spatial scales (Pennings & Bertness 2001; Richards et al. 2004, 2005; Schmidt et al. 2008). In a survey of eight replicate populations of 12 salt marsh species, we found variation in many traits that reflect the ability to maintain nutrient uptake, conserve water, and make osmotic adjustments allowing intertidal plants to persist under the toxic and osmotic effects of inundation and high salinity (Flowers et al. 1977; Cavalieri & Huang 1979; Richards et al. 2005). Trait variation across all 12 species was significantly correlated with environmental drivers such as inundation, nutrients, and salinity gradients. One of the 12 species, *Borrichia frutescens* (Asteraceae: sea-oxeye daisy) demonstrated among the largest amount of phenotypic variation that was correlated with wide environmental breadth (Richards et al. 2005). In another study, we showed that *B. frutescens* has average levels of expected heterozygosity and high clonal diversity in natural populations (Richards et al. 2004). Although we expected that high levels of diversity across environmental gradients might lead to adaptive differentiation, in a glass house study we found that putative salt tolerance traits were extremely plastic in response to controlled salinity treatments. There was also no heritable variation in trait means across half-sibling families collected from high and low salt habitats (Richards et al. 2010b). Therefore, we found no evidence of specialization of salt tolerance traits or adaptation to salt level, but instead that highly plastic reaction norms for these traits are important for *B. frutescens* to live across a broad range of salinity.

Our previous studies provided no support for genetically based differentiation among habitats in *B. frutescens* at both functional genetic markers and putatively adaptive traits. Here, we intended to determine if epigenetic variation contributes to the wide environmental tolerance of *B. frutescens* instead. We sampled individuals from three different habitats (i.e. low, medium, and high salt) within five different sites and used amplified fragment length polymorphism (AFLP) and methylation-sensitive (MS)-AFLP to quantify both genetic and epigenetic variation. We compared patterns of genetic and epigenetic differentiation separately and combined to test the hypothesis that repeated patterns of epigenetic differentiation to habitat would exist in the absence of genetic differentiation to habitat.

MATERIALS AND METHODS

Study species and sampling sites

We performed this study among five sites on Sapelo Island, GA, USA (31° 28' N, 81° 14' W) separated by approximately 5-15 km in May 2011 (Figure 3.1). The salt marshes within each site were typical of those in the south-eastern United States, and we defined each site as a distinct population that consists of potentially interbreeding individuals. Within each population, *B. frutescens* typically exists in freestanding mounds that span a broad of range environmental conditions (e.g. salinity ranges from 4 ppt to hypersaline conditions of 127 ppt; Antlfinger 1981; Richards et al. 2005). In our previous study within and among mounds, environmental conditions were correlated with variation in height, number of leaves, and length, width and thickness of three fully emerged leaves (e.g. heights of mature individuals ranged on a gradual cline from 12.5 to 100 cm), and the salinity of the soil occupied by these individuals was the strongest predictor of variation in all traits measured (Richards et al. 2005). *Borrichia frutescens*

reproduces both clonally by rhizomes (Pennings & Callaway 2000) and sexually through bee and butterfly pollinated, completely outcrossed seeds (Antlfinger 1982). The relative contribution of each type of reproduction is unknown, but populations have average levels of genetic diversity and heterozygosity for species with similar life history characteristics (Richards et al. 2004).

Our sampling allowed for the examination of genetic and epigenetic variation at the spatial scales of population and habitat-level within populations (i.e. putative "sub-populations"). Within each population, we established transects within each of the low, intermediate, and high salt habitats (sensu Richards et al. 2004). At the Cabretta, Hunt Camp, Marsh Landing and Shell Hammock sites, we sampled at 1-m intervals along a 20m transect. At the Lighthouse site, *B. frutescens* grew in larger mounds, so we established 10m transects and sampled at 1-m increments on both sides. This sampling scheme resulted in 20 samples each from a) high salt, which were the shortest plants on the edge of mounds adjacent to the salt pan, b) intermediate salt, which were intermediate height plants equidistant and separated at least 1-m from low and high salt transects, and c) low salt, which were tall plants in the center of mounds. We collected fully expanded leaves that did not exhibit signs of herbivory, froze the samples in liquid nitrogen in the field within 10 minutes of being harvested and stored the samples at -20°C until later analysis.

AFLP and MS-AFLP protocol

We screened a total of 288 individuals for genetic variation using AFLP. We isolated duplicate DNA extractions from each plant sample with the Qiagen DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). We screened each of the duplicates through the entire AFLP protocol following Richards et al. (2012), using *Eco*RI+AGC (6-FAM) and +ACG (HEX) labeled primers with *Mse*I+CAA unlabeled primers to ensure reliable scoring of AFLP

fragments. We sent selective PCR products to Iowa State University DNA facility for fragment analysis on an ABI3130XL.

To score AFLP fragments, we used the RawGeno 2.0 (Arrigo et al. 2009) R package. We manually set bin widths using the graphic interface with the minimum relative fluorescence units for band identification set at 50. These parameters resulted in 64 AFLP bands that were consistent across duplicates, with bands coded as '1' or '0' for present and absent, respectively. Where duplicates did not match at a certain locus, the band was recorded as present, which minimized the chance of missing a present band because of inefficient PCR amplification. Throughout we use "locus" to indicate a specific fragment size in the AFLP and MS-AFLP results. We use "haplotype" to indicate the binary variable positions (dominant genotypes) for each individual's collection of AFLP loci, and "epi-genotype" to indicate the collection of binary variable positions of MS-AFLP loci.

For MS-AFLP we screened the 288 individuals using the same duplicate DNA extractions used for the AFLP protocol, which resulted in 47 AFLP bands that were repeatable across duplicates. In two separate reactions, we replaced the *Mse*I with either *Msp*I or *Hpa*II. *Msp*I and *Hpa*II are isochizomers (restriction CCGG) with different sensitivities to cytosine methylation. Cleaving by *Hpa*II is blocked when the inner or outer cytosine is methylated on both strands, while cleaving by *Msp*I is blocked when the outer cytosines are fully or hemi-methylated. Cleaving by both enzymes is blocked when both cytosines are methylated. Following standard methods (Salmon et al. 2008; Richards et al. 2012), we scored four methylation states: Type 1 is when both enzymes cut (no methylation); Type 2 when *Hpa*II does not cut but *Msp*I does cut (methylated internal cytosine); Type 3 when *Hpa*II does cut but *Msp*I does not (hemi-methylated outer cytosine); and Type 4 when no band is produced (either hemi-

methylation or sequence mutation). We pooled these methylation states into two categories: methylated (Type 2 and Type 3) and unmethylated (Type 1) restriction sites. We treated Type 4 as missing data, because the methylation state cannot be specified (Salmon et al. 2008). Some advocate for discriminating against Type 2 and Type 3 methylation because based on enzyme specificities they are expected to capture methylation in CG versus CHG contexts which are associated with different functionality in plants (Schulz et al. 2014; Medrano et al. 2014). However, recent work indicates that Type 2 and 3 variation cannot simply be interpreted as CG versus CHG methylation. This is because what looks like CHG methylation is often caused by the nesting of internal restriction sites that exhibit differential methylation within MS-AFLP fragments (Fulnecek & Kovarik 2014).

Genetic and epigenetic analysis

We initially assumed that each of the five sampling sites represented unique, potentially interbreeding populations. We tested this assumption by calculating standard population genetics statistics within and among populations and habitats using GenALex 6.5 (Peakall & Smouse 2012). We estimated haplotype diversity (*h*-AFLP), epi-genotype diversity (*h*-MS-AFLP) and percent polymorphisms by site (Table 1). We also used GenALex to estimate genetic and epigenetic differentiation using hierarchical analysis of molecular variance (AMOVA). We treated each of the habitats within a site (low, intermediate, and high salt) as if they were sub-populations within the sites (total=15 sub-populations). This analysis assessed if spatial location structured genetic or epigenetic differences by comparing variances among populations (Φ_{RT}), among sub-populations (i.e. habitats) within populations (Φ_{PR}), and among all sub-populations (Φ_{PT}). We used 9,999 permutations to estimate statistical significance and an initial alpha of

0.05. We also estimate pairwise Φ_{ST} among sub-populations, and determined how many pairwise comparisons were significantly different from each other by the false discovery rate method (FDR=0.05; Benjamini & Hochberg 1995).

The hierarchical AMOVA can only identify whether plants in different sub-populations are differentiated within populations, but cannot determine repeated patterns in response to a gradient among populations since the different sub-populations are treated as random sub-populations within a population. To specifically test for repeated patterns across similar conditions, we ran a permutational multivariate analysis of variance (MANOVA) using the Adonis function within the Vegan package of R. This analysis allowed us to examine the repeated genetic and epigenetic response to "high," "medium," and low" salt habitats across the five populations. We used the Euclidean genetic and epigenetic distance matrices (with interpolation of missing values) generate by GenALex as the basis for this analysis so that comparisons among analyses would be consistent.

For the genetic data only, we performed Bayesian clustering using Structure v.2.3.4 to estimate population structure among individuals (Pritchard et al. 2000; Falush et al. 2003). Structure estimates the number of groups (k) present among individuals, and assigns individuals to each k using Bayesian modeling. We tested eight populations (k=1-8), three more than the expected number (Evanno et al. 2005), with five independent runs at each k using both the log probability of observing the data (ln Pr(x|k)) method of Structure and Delta k (Evanno et al. 2005), which allowed us to determine the number of populations that best fit the data. We incorporated sampling location in our model to assist in detecting weak differentiation. We performed clustering with the admixture model, 30,000 burn-in steps, 100,000 post-burn-in steps, and allowed correlated allele frequencies. We assigned individuals to groups based on the

highest q-value. We also performed a Mantel test to determine if genetic differentiation was driven by isolation-by-distance. Again, we used the Euclidean genetic distance matrix generated by GenALex as the basis of this analysis, and we created a pairwise geographic distance matrix among the sampling sites using distances obtained via Google Earth.

Comparison between genetic and epigenetic variation

We analyzed the correlation between genetic variation, epigenetic variation, and habitat by performing Mantel and partial Mantel tests using ZT software (Bonnet & Van der Peer 2002). To test for a relationship between genetic and epigenetic variation, we performed a simple Mantel test using ZT software. The partial Mantel test determines correlations between two distance matrices while controlling for correlations with a third matrix. In this case, to create the habitat distance matrix we used zero (0) for distance within the same habitat, one (1) for the distance between low and medium or between medium and high salt habitats, and two (2) for the distance between low and high salt habitats. This design allowed us to determine if there were correlations between genetic and/or epigenetic variation with habitat across all sites, but makes the assumption that the relative salinity between habitats within a site is similar among all sites. We tested for a relationship between genetic variation and habitat while controlling for epigenetic variation. Likewise, we tested for a relationship between epigenetic variation and habitat while controlling for correlations with genetic variation. In all cases we used the Euclidean genetic and epigenetic distance matrices generated by GenALex.

To determine if specific AFLP and MS-AFLP loci were correlated with habitat, we ran a generalized linear model using the "glm" function in R. "Population" was set as a random effect,

and the distribution family was set as "binomial." This allowed us to isolate specific loci that might otherwise be masked by a genome-wide analysis.

RESULTS

Genetic diversity and structure

Genetic diversity across all sites was high (Table 3.1). Nearly every individual (n=288) exhibited a unique haplotype across 64 polymorphic loci, and haplotype diversity (h-AFLP) ranged from 0.249-0.316 among the five sampling sites (Table 3.1). We detected population structure at every level of hierarchy (explaining 8% and 7% of the variation at the population and sub-population within population levels; Table 3.2). In pairwise comparison of the 15 sub-populations we found that 83.8% of the tests were significant (FDR=0.05: Table 3.3). This indicates that samples from different habitats within each population (e.g. Cabretta low, medium, and high salt) were typically different from each other and also from samples from each habitat in other populations. Though we found significant sub-population structure within populations, the permutational MANOVA showed no effect of habitat type. This finding suggests a lack of consistent genetic differentiation to habitat type.

Structure identified two genetic groups. The maximum ln Pr(X|K) was K2 (-7,864.7) and the maximum Delta K was K2 (2,697.9). Bayesian clustering showed a pattern of weak genetic isolation by distance among the different sites, which suggests geographic-based isolation by distance between the north (i.e. Cabretta and Hunt Camp) and south (i.e. Lighthouse, Marsh Landing, and Shell Hammock) portions of the island, or a north-south gradient in genetic make-up on the island (Figure 1). A Mantel test comparing genetic distance and geographic distance

failed to detect a correlation between genetic variation and geographic distance ($r = 0.004$, $p < 0.38$).

Epigenetic diversity and structure

We found a high level of epigenetic variation across sites (Table 3.1). Nearly every individual (n=288) exhibited a unique epi-genotype across 47 polymorphic loci, and epi-genotype diversity (h-MSAFLP) ranged from 0.280-0.339 across all sampling sites. We detected epigenetic structure at every level of hierarchy (explaining 6% and 7% of the variation at the population and sub-population within population levels; Table 3.2). In pairwise comparisons of the sub-populations we found that 94.3% the tests were significantly different (FDR=0.05). This indicates that nearly all sub-populations were different from other sub-populations within the same site, as well as different from sub-populations in other sites. Though we found significant sub-population structure within populations, the permutational MANOVA showed no effect of habitat, suggesting a lack of consistent epigenetic differentiation to habitat type across the island.

Comparison between genetic and epigenetic variation

In a combined analysis across all five populations, we detected a significant correlation between genetic and epigenetic variation (Mantel test, $r = 0.107$, $p = 0.001$), but neither was independently correlated with habitat (Figure 3.2). However, in separate analyses for each population, we did find independent correlations between epigenetic variation and habitat in three of the five populations (Hunt Camp, Lighthouse and Marsh Landing; Table 3.4). In two of these three populations (Hunt Camp and Marsh Landing) and in a third (Cabretta), we also found significant correlations between genetic variation and habitat independent of epigenetic

variation. In these four populations where there was an independent correlation with either genetic or epigenetic variation or both, there was no correlation between genetic and epigenetic variation. Only in Shell Hammock did we find a correlation between epigenetic and genetic variation, and in that site there was no correlation between genetic variation and habitat or epigenetic variation and habitat.

Analyses with GLM showed that only 3 of the 64 (4.7%) polymorphic AFLP loci were significantly correlated with habitat (Figure 3A), while for MS-AFLP loci a significantly higher proportion (8 of the 47 (17%)) showed a significantly correlation with habitat (Fisher's exact test p= 0.051; Figure 3.3B).

DISCUSSION

Though evidence is accumulating that epigenetic mechanisms play a role in responding to different environmental conditions (Verhoeven et al. 2010; Richards et al. 2008, 2012; Herrera et al. 2012; Platt et al. 2015; Xie et al. 2015), there is still little evidence for the independent role of epigenetics in natural populations (Foust et al. 2015). The salt marsh perennial *B. frutescens* provides a unique opportunity to examine the role of epigenetics because it spans a broad range of environmental conditions in replicate populations, without association of genotypes or alleles at allozyme loci or specialization of putatively adaptive traits with habitats (Richards et al. 2004, 2005, 2010b). We predicted that epigenetic mechanisms independent of genetic variation might underlie the broad environmental tolerance of this species response to environmental conditions, and that epigenetic variation would be structured by environmental gradients. While we found support for this prediction in three of five populations, the independent importance of genetic variation, epigenetic variation, and the correlation between the two varies widely by population.

Structure of population genetic and epigenetic variation

We found high levels of both genetic and epigenetic diversity in *B. frutescens*, and contrary to our prediction we found significant genetic and epigenetic structure among populations and among habitats within populations. However, AMOVA is limited in that it can only determine that sub-populations are different from each other and cannot identify repeated response to similar location along environmental gradients in replicate populations. To confirm that we had not only differentiation among habitats within sub-populations but repeated patterns of genetic and epigenetic variation that correlated to environmental conditions, we used a permutational MANOVA. This analysis showed that there was not a consistent response to environment across the island. However, a generalized linear model on each locus revealed that several genetic and epigenetic loci were significantly correlated to habitat. The fact that nearly three times as many MS-AFLP as AFLP loci were correlated to environment across the five populations indicates that DNA methylation at specific loci may be important component of response to different habitats in this species. Further work is required to determine if the patterns at these loci are merely a downstream readout of genetic variation versus independent of genetic variation (see also Foust et al. on review).

Disentangling genetic and epigenetic variation

Several studies have found relationships between epigenetic variation and environment by taking advantage of clonal organisms or organisms with low levels of genetic variation (Verhoeven et al. 2010; Richards et al. 2012; Herrera & Bazaga 2012, 2013; Verhoeven & Preite 2014) or artificially increasing the variation in DNA methylation within isogenic lines (Latzel et al. 2013; Zhang et al. 2013). As ecological epigenetics expands into a larger variety of non-

model organisms, researchers must further investigate the correlation between genetic and epigenetic variation (Bossdorf et al. 2008; Richards et al. 2010a; Foust et al. 2015). To address this problem, Richards (2006) defined three classes of epigenetic variation at a given locus: obligatory, facilitated, or pure. In the case of obligatory epigenetic variation, epigenotype is strictly determined by genotype. In facilitated epigenetic variation, epigenotype depends on the genotype, but the epigenotype is context dependent. Pure epigenetic variation is created and operates independently from genetic variation. Considering that facilitated and pure epigenetic variation can operate via non-Mendelian segregation, Richards' (2006) framework helps to clarify when epigenetic variation might contribute a previously unappreciated source of heritable phenotypic variation: either facilitated by or independent of genetic variation (Robertson & Richards 2015b).

In this study, we found high levels of both genetic and epigenetic diversity challenging our ability to parse out which mechanisms may be independently contributing to environmental response. For example, since each individual was a unique genotype, fragments of slightly different size across individuals could be analogous in function. In addition, it is difficult to evaluate genetic and epigenetic variation simultaneously and account for the correlation between the two. We used partial Mantel tests to combine analysis of genetic and epigenetic variation and tease out the independent correlations between genetic variation and habitat and epigenetic variation and habitat while controlling for the correlation between genetic and epigenetic variation (determined by a Mantel test). Combined across the five sites, we found only a direct correlation between genetic and epigenetic variation. In separate Mantel and partial Mantel tests on each population, this result was only repeated in one site (Shell Hammock). In three of the other populations, we found significant correlations between epigenetic variation and habitat.

However, the correlations were in opposite directions and weak in two populations (Lighthouse: $r = 0.083$, $p = 0.009$; Marsh Landing: $r = -0.070$, $p = 0.02$), and more strongly positive in the third (Hunt Camp: $r = 0.353$, $p < 0.0001$). Further, we found weak but significant correlations between genetic variation and habitat in two of these populations (Hunt Camp and Marsh Landing) and a third (Cabretta; $r = 0.11$- 0.16). These findings indicate that assessing AFLP and MS-AFLP data sets separately may misrepresent the roles of genetic and epigenetic variation. Analyses that accommodate a comparison between both data sets and allow for controlling for the correlation between genetic and epigenetic data can reveal significant relationships that are otherwise obscured.

Our findings across populations in *B. frutescens* are partially congruent to results from a parallel study on the widespread salt marsh grass *Spartina alterniflora* (see Chapter 2). In *S. alterniflora*, we found similar levels of genetic and epigenetic structure across populations and sub-populations, but epigenetic variation was correlated with habitat when we controlled for genetic variation across the five populations. Separate partial Mantel tests on each population showed even stronger support for significant, independent epigenetic correlations with habitat when the correlation with genetic variation was controlled for in four out of five populations ($r = 0.057$ - 0.213, $p \leq 0.034$). The more consistent patterns of response might be facilitated in *S. alterniflora* which is an outcrossing wind-pollinated grass with high levels of genetic diversity that are about average for plants with these life history characteristics (expected heterozygosity (He) = 0.205; Richards et al. 2004; Foust et al. in review). In contrast, although *B. frutescens* is completely outbreeding, pollinator flight distributions suggested restricted pollen flow and the effective neighborhood size was 20–30 individuals with a neighborhood area of less than one square meter (Antlfinger 1982). In addition, *B. frutescens* lives higher in the intertidal where its

gravity-dispersed seeds will be less likely to travel between populations. Therefore, both pollen and seed mediated gene flow in *B. frutescens* may allow for modest mixing within populations across the environmental gradient, but are unlikely to result in homogeneity among sites. Considering the much more restricted gene flow for *B. frutescens* and lower levels of species level genetic diversity (H_e = 0.089; Richards et al. 2004), site-specific conditions and historical contingency may interfere with detecting response in replicate populations with similar environmental gradients (Losos et al. 1998; Hendry & Kinnison 2001; Gutschick & BassiriRad 2003; Jackson et al 2009). Further research may reveal why epigenetic variation independent of genetic is correlated to habitat in some populations and not at all in others.

In order to truly dissect how epigenetic variation might relate to response to environment, the next step in ecological epigenetics is to develop tools that can associate differential DNA methylation with functional genes or gene networks that allow for translation into ecologically important phenotypic variation (Schrey et al 2013; Alvarez et al. 2015). *Linaria vulgaris* is one of the clearest examples where an epimutation in one gene transforms the flower from the wild type bilateral symmetry to radial symmetry (Cubas et al. 1999). However, this study does not explore if environmental conditions play a role in inducing this epigenetic variation. Studies with epigenetic recombinant inbred lines (epiRILs) developed in a single genotype of *Arabidopsis thaliana* have shown that epigenetic quantitative trait loci (QTLepi) account for 90% of the heritability of flowering time and 60% of the heritability of primary root length (Cortijo et al. 2014), and that variation in DNA methylation alone contributes to response to drought and nutrients (Zhang et al. 2013). However, no study has yet definitively explored how functional epi-alleles independent of genetic variation contributes to organismal function in response to real complex environments. The resources available in *A. thaliana* have provided insight into

components of this question, but the genome and epigenome of *A. thaliana* are atypical for most plants that have been surveyed (Alonso et al. 2015), so the generality of these findings may be limited. Given that MS-AFLP offer a limited number of loci, and that it is difficult to draw functional interpretation form the anonymous loci, reduced representation bisulfite sequencing approaches will be an important next step in exploring the functional significance of epigenetic variation in natural populations (e.g. Platt et al. 2015).

Field studies, and studies on non-model organisms, are necessary to reveal the true importance of genetic and epigenetic mechanisms for organismal response. As more of these studies are done, researchers must be mindful of the potential correlation between genetic and epigenetic variation, but it may prove difficult to define epigenetic variation as obligate, facilitated, or pure (Richards et al. 2010a; Robertson & Richards 2015b). Our study demonstrates that we can expose an independent epigenetic component using MS-AFLPs – the next step will be to use higher-resolution sequencing methods to understand the functional consequences of pure epigenetic mechanisms.

ACKNOWLEDGEMENTS

We thank Steve Pennings, Jacob Shalack, Caroline Reddy, Justin Manley, Mariano Alvarez and Ashley Shayter for access to field sites and assistance with plant sampling on Sapelo Island, GA. This work was partially supported by the Global Invasions Network Research Exchange (Grant No. 0541673), and the Georgia Coastal Ecosystems Long-Term Ecological Research program (Grant No. OCE-0620959), both funded by the National Science Foundation. This is contribution number xxx from the University of Georgia Marine Institute.

LITERATURE CITED

1. Alonso, C, Pérez, R Bazaga, P , Herrera, CM (2015) Global DNA cytosine methylation as an evolving trait: phylogenetic signal and correlated evolution with genome size in angiosperms. *Front in Genetics*, 6, 4.
2. Alvarez M, Schrey AW, Richards CL (2015) Ten years of transcriptomics in wild populations: What have we learned about their ecology and evolution? *Mol Ecol,* 24, 710-725.
3. Antlfinger AE (1981) The genetic basis of microdifferentiation in natural and experimental populations of *Borrichia frutescens* in relation to salinity. *Evolution,* 35, 1056-1068.
4. Antlfinger AE (1982) Genetic neighborhood structure of the salt marsh composite, *Borrichia frutescens*. *J Hered,* 73, 128-132.
5. Antonovics J, Bradshaw AD (1970) Evolution in closely adjacent plant populations. *Heredity*, 25, 349-362.
6. Arrigo N, Tuszynski JW, Ehrich D, Gerdes T, Alvarez N (2009) Evaluating the impact of scoring parameters on the structure of intra-specific genetic variation using RawGeno, an R package for automating AFLP scoring. *BMC Bioinformatics*, 10, 10-33.
7. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J Roy Statist Soc B,* 57, 289-300.
8. Bonnet E, Van de Peer Y (2002) zt: a software tool for simple and partial Mantel tests. *J Statist Software*, 7, 1-12.
9. Bossdorf O, Richards CL, Pigliucci M (2008) Epigenetics for ecologists. *Ecol Lett,* 11, 106-115.
10. Bossdorf O, Arcuri D, Richards CL, Pigliucci M (2010) Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evol Ecol*, 24, 541-553.
11. Bradshaw AD (1965) Evolutionary significance of phenotypic plasticity in plants. *Adv Genet,* 13, 115-155.
12. Caisse A, Antonovics J (1978) Evolution in closely adjacent plant populations. *Heredity*, 40, 371-384.
13. Cavalieri AJ, Huang, AHC (1979) Accumulation of proline and glycinebetaine in *Spartina alterniflora* Loisel. in response to NaCl and nitrogen in the salt marsh. *Oecol,* 49, 224-228.
14. Clausen JD, Keck D, Heisey WM (1948) Experimental studies on the nature of species. III. Environmental responses of climatic races of *Achillea*. *Carnegie Institution of Washington Publication 520*.
15. Cortijo S, Wardenaar R, Colomé-Tatché M, Gilly A, Etcheverry M, Labadie K, Caillieux E, Hospital F, Aury J –M, Wincker P, Roudier F, Jansen RC, Colot V, Johannes F (2014) Mapping the epigenetic basis of complex traits. *Science*, 343, 1145-1148.
16. Cubas P, Vincent C, Coen E (1999) An epigenetic mutation responsible for natural variation in floral symmetry. *Nature*, 401, 157-161.
17. Dlugosch KM, Parker IM (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol Ecol,* 17, 431-449.
18. Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR (2012) Widespread dynamic DNA methylation in response to biotic stress. *Proc Natl Acad Sci USA* 109, E2183-2191.

19. Dubin MJ, Zhang P, Meng D, Remigereau M-S, Osborne EJ, Casale FP, Drewe P, Kahles A, Vilhjálmsson B, Jagoda J, Irez, S, Voronin V, Song Q, Long Q, Rätsch G, Stegle O, Clark RM, Nordborg M (2015) DNA methylation variation in *Arabidopsis* has a genetic basis and shows evidence of local adaptation. *Elife 4.*
20. Evanno, G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol Ecol,* 14, 2611-2620.
21. Falush, D, Stephens M, Pritchard, JK (2007) Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Mol Ecol Notes,* 7, 574-578.
22. Feder JL, Roethele JB, Wlazlo, B, Berlocher SH (1997) Selective maintenance of allozyme differences among sympatric host races of the apple maggot fly. *Proc Natl Acad Sci USA,* 94, 11417-11421.
23. Fisher MC, Rellstab C, Tedder A, Zoller A, Gugerli F, Shimizu KK, Holderegger R, Widmer A (2013) Population genomic footprints of selection and associations with climate in natural populations of *Arabidopsis halleri* from the Alps. *Mol Ecol,* 22, 5594–5607.
24. Flowers TJ, Troke, PF, Yeo, AR. (1977) The mechanism of salt tolerance in halophytes. *Annu Rev Plant Physiol*, 28, 89-121.
25. Foust CM, Priete V, Verhoeven KJF, Schrey AW, Richards CL *In review* Epigenetic, not genetic, variation is correlated with habitats in natural populations of the foundation salt marsh plant *Spartina alterniflora*.
26. Foust CM, Schrey AW, Richards CL (2015) Population epigenetics. In: *Nuclear Function in Plant Transcription, Signaling, and Development*. O Pontes, H Jin, eds. Springer.165-179.
27. Fulneček, J, Kovařík, A (2014) How to interpret Methylation Sensitive Amplified Polymorphism (MSAP) profiles? *BMC Genetics*, 15, 2-9.
28. Hagmann J, Becker C, Müller J, Stegle O, Meyer RC, Wang, G, Schneeberger K Fitz, J, Altmann T, Bergelson J, Borgwardt K, Weigel, D (2015) Century-scale methylome stability in a recently diverged *Arabidopsis thaliana* lineage. *PLoS Genetics*, 11(1), e1004920.
29. Hamrick, JL, Allard, RW (1972) Microgeographical variation in allozyme frequencies in *Avena barbata*. *Proc Natl Acad Sci USA,* 69, 2100-2104.
30. Hedrick, PW (1976) Genetic variation in a heterogeneous environment. 2. Temporal heterogeneity and directional selection. *Genetics*, 84, 145-157.
31. Herman JJ, Spencer HG, Donohue K, Sultan SE (2014) How stable 'should' epigenetic modifications be? Insights from adaptive plasticity and bet hedging. *Evolution,* 68, 632-643.
32. Herrera, CM, Bazaga, P (2013) Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (Aquifoliaceae) trees. *Bot J Linean Soc*, 171, 441-452.
33. Herrera CM, Pozo MI, Bazaga P (2012) Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. *Mol Ecol*, 21, 2602-2616.
34. Jump, AS, Pañuelas J (2005) Running to stand still: adaptation and the response of plants to rapid climate change. *Ecol Lett,* 8, 1010-1020.
35. Keller EF (2011) Genes, genomes, and genomics. *Biol Theory*, 6, 132-140.

36. Keller EF (2014) From gene action to reactive genomes. *J Physiol*, 592.11, 2423–2429.
37. Kilvitis HJ, Alvarez, M, Foust CM, Schrey AW, Robertson MH, Richards CL **(**2014) Ecological epigenetics. In: *Ecological Genomics*. CR Landry N Aubin-Horth, eds. Springer. Series: *Adv in Exper Med and Biol*, 781: 191-210.
38. van Kleunen M, Fischer M (2005) Constraints on the evolution of adaptive phenotypic plasticity in plants. *New Phytol*, 166, 49–60
39. Klironomos FD, Berg, J, Collins S (2013) How epigenetic mutations can affect genetic evolution: Model and mechanism. *BioEssays*, 35, 571-578.
40. Latzel V, Allan E, Silveira AB, Colot V, Fischer M, Bossdorf O (2013) Epigenetic diversity increases the productivity and stability of plant populations. *Nature Comm*, 4, 2875
41. Levene H (1953) Genetic equilibrium when more than one ecological niche is available. *Am Nat*, 87, 331-333.
42. Losos JB, Jackman TR, Larson A, de Queiroz K, Rodriguez-Schettino L (1998) Contingency and determinism in replicated adaptive radiations of island lizards. *Science*, 279, 2115–2118.
43. Medrano, M, Herrera, CM, Bazaga, P (2014) Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. *Mol Ecol*, 23, 4926–4938.
44. Moon DC, Stiling, P (2002) The effects of salinity and nutrients on a tritrophic salt marsh system. *Ecology*, 83, 2465-2476.
45. Nicotra, AB, Atkin OK, Bonser SP, Davidson AM, Finnegan EJ, Mathesius U, et al (2010) Plant phenotypic plasticity in a changing climate. *Trends Plant Sci*, 15, 684-692.
46. Nicotra, AB, Segal, D, Hoyle GL, Schrey AW, Verhoeven KJF, Richards CL (2015) Adaptive plasticity in response to warming in an Australian alpine herb. *Ecol Evol*, 5, 634–647.
47. Peakall, R, Smouse PE (2012) GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics*, 28, 2537-2539.
48. Pennings SC, Bertness MD (2001) Salt marsh communities. In *Marine Community Ecology.* Bertness MD, Gaines SD eds, Sinauer, Sunderland, Massachusetts, USA.
49. Pennings SC, Callaway RM (2000) Salt marsh plant zonation: The relative importance of competition and physical factors. *Ecology*, 73, 681-690.
50. Platt A, Gugger P, Sork V (2015) Genome-wide signature of local adaptation linked to variable CpG methylation in oak populations. *Mol Ecol*, 24, XXX-XXX.
51. Pritchard, JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, 155, 945-959.
52. Rapp, RA, Wendel, JF (2005) Epigenetics and plant evolution. *New Phytol*, 168, 81-91.
53. Richards CL, Hamrick, JL, Donovan LA, Mauricio, R (2004) Unexpectedly high clonal diversity of two saltmarsh perennials across a severe environmental gradient. *Ecol Lett*, 7, 1155–1162.
54. Richards CL, Pennings SC, Donovan LA (2005) Habitat range and phenotypic variation in salt marsh plants *Plant Ecol*, 176, 263-273.
55. Richards CL, Bossdorf O, Muth NZ, Gurevitch J, Pigliucci M (2006) Jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. *Ecol Let*, 9, 981-993.
56. Richards CL, Walls RL, Bailey JP, Parameswaran R, George T, Pigliucci M (2008) Plasticity in salt tolerance traits allows for invasion of novel habitat by Japanese

knotweed S.L. (*Fallopia japonica* and *F. xbohemica*, Polyconaceae). *Am J Bot*, 95, 931-941.
57. Richards CL, Bossdorf O, Verhoeven KJF (2010a) Understanding natural epigenetic variation. *New Phytol*, 187, 562-564.
58. Richards CL, White SN, McGuire MA, Franks SJ, Donovan LA, Mauricio, R (2010b) Plasticity, not adaptation to salt level, explains variation along a salinity gradient in a salt marsh perennial. *Est Coasts*, 33, 840-852.
59. Richards CL, Schrey AW, Pigliucci M (2012) Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecol Lett*, 15**,** 1016-1025.
60. Richards EJ (2008) Population epigenetics. *Curr Opin Genetics Dev*, 18, 221-226.
61. Robertson MH, Richards CL (2015a) Non-genetic inheritance in evolutionary theory the importance of plant studies. *Non-Genet Inherit*, 2, 3–11.
62. Robertson MH, Richards CL (2015b) Opportunities and challenges of Next Generation Sequencing applications in ecological epigenetics. *Mol Ecol*, 24, XXX-XXX.
63. Roda, F, Ambrose L, Walter GM, Liu, HL, Schaul, A, Lowe A, Pelser PB, Prentis P, Rieseberg, LH, Ortiz-Barrientos D (2013) Genomic evidence for the parallel evolution of coastal forms in the *Senecio lautus* complex. *Mol Ecol*, 22, 2941–2952.
64. Salmon A, Clotault J, Jenczewski E, Chable V, Manzanares-Dauleux, MJ (2008) *Brassica oleracea* displays a high level of DNA methylation polymorphism. *Plant Sci*, 174, 61-70.
65. Salzman AG (1985) Habitat selection in a clonal plant. *Science*, 228, 603-604.
66. Scheiner SM (1993) Genetics and evolution of phenotypic plasticity. *Annu Rev Ecol Syst*, 24, 35-68.
67. Schmidt PS, Rand DM (1999) Intertidal microhabitat and selection at MPI: Interlocus contrasts in the northern acorn barnacle, *Semibalanus balanoides. Evolution*, 53, 135-136.
68. Schmidt PS, Serrão EA, Pearson GA, Riginos C, Rawson PD, Hilbish TJ, Brawley SH, Trussel GC, Carrington E, Wethey DS, Graham JW, Bonhomme F, Rand DM (2008) Ecological genetics in the north Atlantic: Environmental genetics and adaptation at specific loci. *Ecology*, *89*, S91–S107.
69. Schrey AW, Alvarez M, Foust CM, Kilvitis HJ, Lee JD, Liebl AL, Martin LB, Richards CL, Robertson MH (2013) Ecological epigenetics: Beyond MS-AFLP. *Integ Comp Biol*, 53, 340-350.
70. Schulz, B, Eckstein RL, Durka, W (2014) Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Mol Ecol*, 14, 3523–3537.
71. Sultan SE (2003) Phenotypic plasticity in plants: A case study in ecological development. *Evol Devel*, 5, 25–33.
72. Sultan SE, Spencer HG (2002) Metapopulation structure favors plasticity over local adaptation. American Naturalist 160: 271–283.
73. Turesson G (1922) The genotypical response of the plant species to the habitat. *Hereditas*, 3, 211–350.
74. van Tienderen PH (1991) Evolution of generalists and specialists in spatially heterogeneous environments. *Evolution*, 45, 1317–1331.
75. Verhoeven KJF, Jansen JJ, van Dijk PJ, Biere A (2010) Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytol*, 185, 1108-1118.

76. Verhoeven KJF, Preite V (2014) Epigenetic variation in asexually reproducing organisms. *Evol*, 68, 644–655.
77. Xie HJ, Li AH, Liu AD, Dai WM, He JY, Lin S, Duan H, Liu LL, Chen SG, Song XL, Valverde BE, Qiang S (2015) ICE1 demethylation drives the range expansion of a plant invader through cold tolerance divergence. *Mol Ecol*, 24, 835–850.
78. Zhang YY, Fischer M, Colot V, Bossdorf O (2013) Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *New Phytol*, 197(1), 314-322.

TABLES AND FIGURES

Table 3.1. Mean AFLP haplotype (n = 288) and MS-AFLP epi-genotype (n = 288) diversity (*h*) and percent polymorphic loci by site (%P), based on 64 AFLP loci and 47 MS-AFLP loci in *Borrichia frutescens* sampled from three habitats (i.e. low, intermediate, and high salt) within five sites on Sapelo Island, GA. SE = standard error.

Site	***h*-AFLP (SE)**	**AFLP %P**	***h*-MSAFLP (SE)**	**MS-AFLP %P**
Cabretta	0.249 (0.023)	81.25%	0.339 (0.020)	97.87%
Hunt Camp	0.299 (0.020)	98.44%	0.282 (0.025)	89.36%
Lighthouse	0.308 (0.019)	100.00%	0.318 (0.023)	97.87%
Marsh Landing	0.316 (0.018)	100.00%	0.289 (0.022)	95.74%
Shell Hammock	0.263 (0.023)	81.25%	0.280 (0.025)	93.62%
Average	0.287 (0.009)	92.19%	0.301 (0.010)	94.89%

Table 3.2. Pairwise Φ_{ST} values for comparisons among 15 sub-populations at AFLP (below the diagonal) and MS-AFLP (above the diagonal). ^{NS} indicates a non-significant Φ_{ST} value, all others are significant based on FDR = 0.05. S, M, and T represent short, medium, and tall plants (i.e. high, intermediate, and low salt) respectively.

CS	CM	CT	HS	HM	HT	LS	LM	LT	MS	MM	MT	SS	SM	ST
	0.07	0.11	0.03	0.12	0.25	0.17	0.12	0.13	0.06	0.07	0.08	0.10	0.14	0.19
0.12		0.05	0.10	0.12	0.19	0.14	0.09	0.07	0.12	0.02^{NS}	0.10	0.13	0.11	0.15
0.03	0.19		0.11	0.05	0.24	0.20	0.23	0.13	0.12	0.08	0.17	0.14	0.09	0.11
0.07	0.20	0.03		0.04	0.21	0.19	0.15	0.12	0.05	0.06	0.16	0.09	0.10	0.13
0.10	0.20	0.08	0.10		0.22	0.20	0.23	0.14	0.12	0.11	0.22	0.09	0.09	0.09
0.17	0.27	0.11	0.07	0.16		0.07	0.13	0.04	0.15	0.11	0.23	0.21	0.20	0.16
0.26	0.32	0.20	0.14	0.27	0.05		0.03	0.04	0.13	0.09	0.14	0.11	0.11	0.12
0.20	0.30	0.15	0.08	0.18	0.02^{NS}	0.07		0.06	0.10	0.05	0.09	0.13	0.16	0.20
0.28	0.35	0.22	0.17	0.28	0.06	0.04	0.10		0.10	0.01^{NS}	0.11	0.12	0.13	0.12
0.17	0.27	0.11	0.07	0.16	0.00^{NS}	0.05	0.02^{NS}	0.06		0.06	0.06	0.10	0.10	0.15
0.23	0.31	0.19	0.14	0.27	0.06	0.03	0.10	0.00^{NS}	0.06		0.06	0.12	0.10	0.14
0.22	0.30	0.17	0.09	0.20	0.03	0.04^{NS}	0.00^{NS}	0.10	0.03	0.10		0.16	0.17	0.24
0.26	0.34	0.21	0.16	0.27	0.07	0.06	0.11	0.00^{NS}	0.07	0.00^{NS}	0.12		0.02^{NS}	0.02^{NS}
0.26	0.34	0.20	0.16	0.28	0.05	0.00^{NS}	0.09	0.01^{NS}	0.05	0.00^{NS}	0.08	0.04		0.05
0.27	0.34	0.22	0.18	0.30	0.08	0.01^{NS}	0.12	0.01^{NS}	0.08	0.00^{NS}	0.11	0.02^{NS}	0.00^{NS}	

Table 3.3. Genetic and epigenetic correlations to variation in habitat (i.e. low, medium, high salt) calculated separately for each of the five populations on Sapelo Island, GA and combined across the five populations using partial Mantel tests. The correlation between genetic and epigenetic variation was calculated in a separate Mantel test. *Q ≤ 0.05, based on FDR.

Site	**Genetic vs. epigenetic variation**	**Genetic variation vs. habitat**	**Epigenetic variation vs. habitat**
	r	**r**	**r**
Cabretta	0.045	0.11*	0.028
Hunt Camp	0.067	0.16*	0.353*
Lighthouse	0.0158	0.043	0.083*
Marsh Landing	0.130	0.13*	-0.070*
Shell Hammock	0.148*	-0.013	0.052
Combined	0.107*	0.0054	-0.017

Table 3.4. Three-level hierarchical analysis of molecular variance (AMOVA). Hierarchical Φ-statistics and percentage of genetic and epigenetic variance explained by each hierarchical level are given. Φ-statistics were calculated using 9,999 permutations. d.f.= degrees of freedom; ** $P \leq 0.001$, *** $P \leq 0.0001$.

	Φ statistics	**% variation**	**d.f.**
Genetic variation			
Among populations	0.080***	8%	4
Among subpopulations within populations	0.076***	7%	10
Within subpopulations		85%	273
Epigenetic variation			
Among populations	0.056**	6%	4
Among subpopulations within populations	0.075**	7%	10
Within subpopulations		87%	273

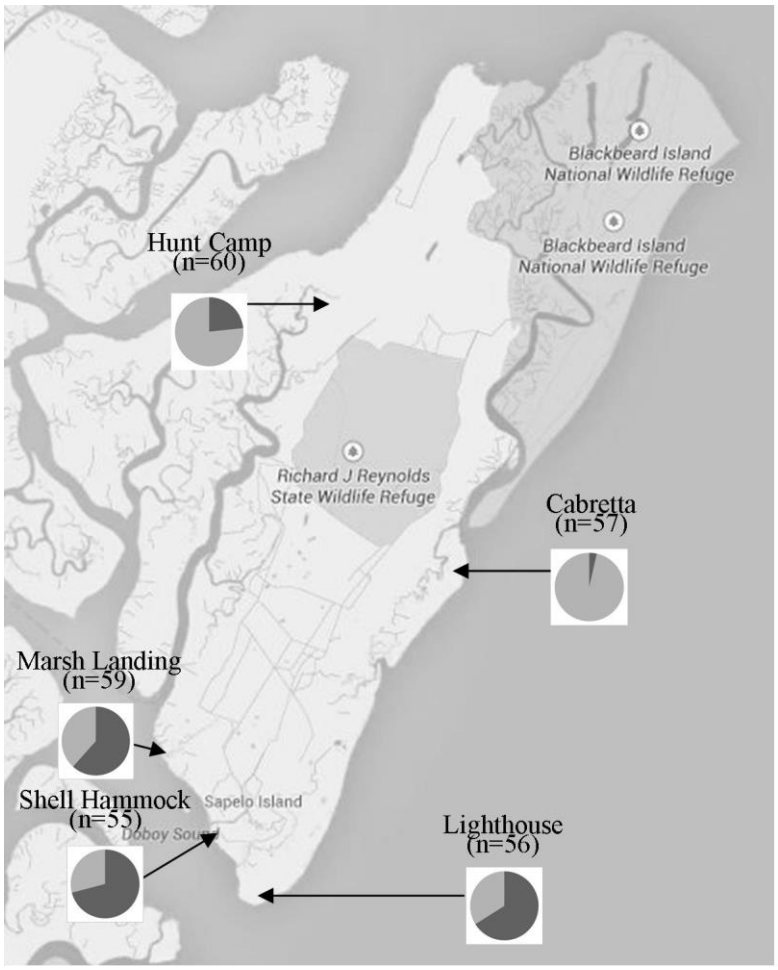

Figure 3.1. Map of the five sampling sites on Sapelo Island, GA with the results of Bayesian clustering from the program Structure. Population assignment to two groups is indicated by shaded portion of circle. Group 1 = light grey, Group 2 = dark grey.

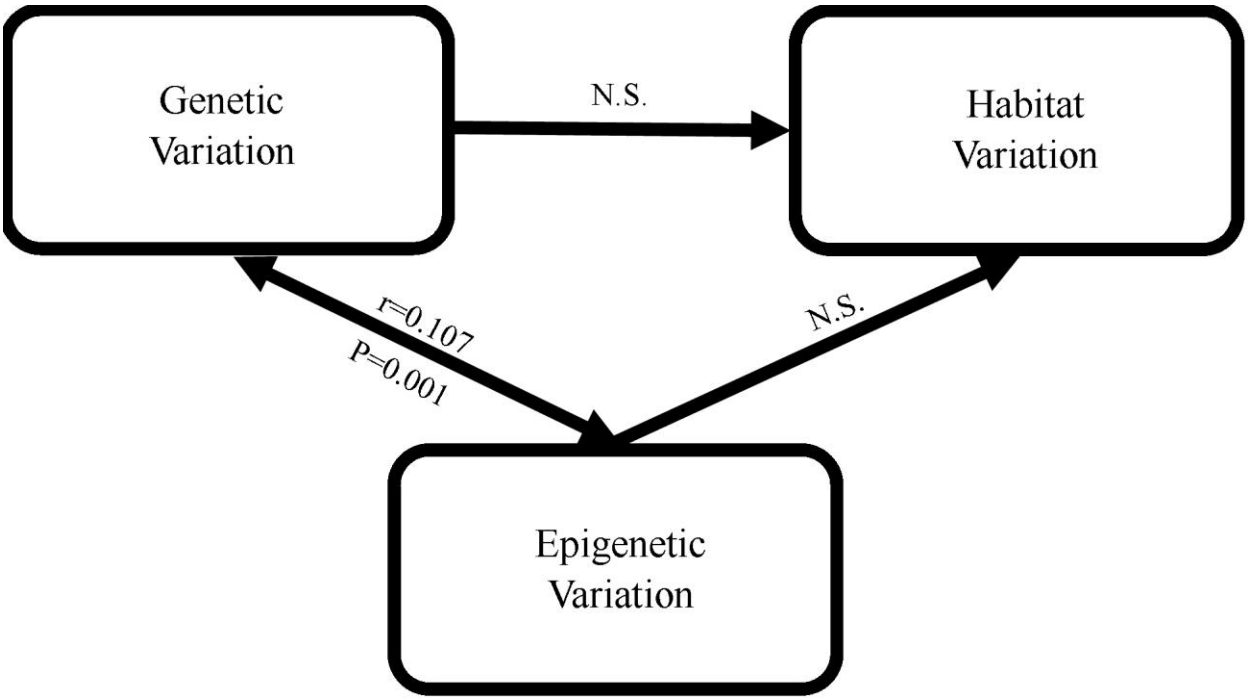

Figure 3.2. Genetic and epigenetic correlations to variation in habitat (i.e. low, medium, high salt) across five populations using partial Mantel tests. The correlation between genetic and epigenetic variation was calculated in a separate Mantel test. NS = not significant, r = correlation coefficient when significant and p-value.

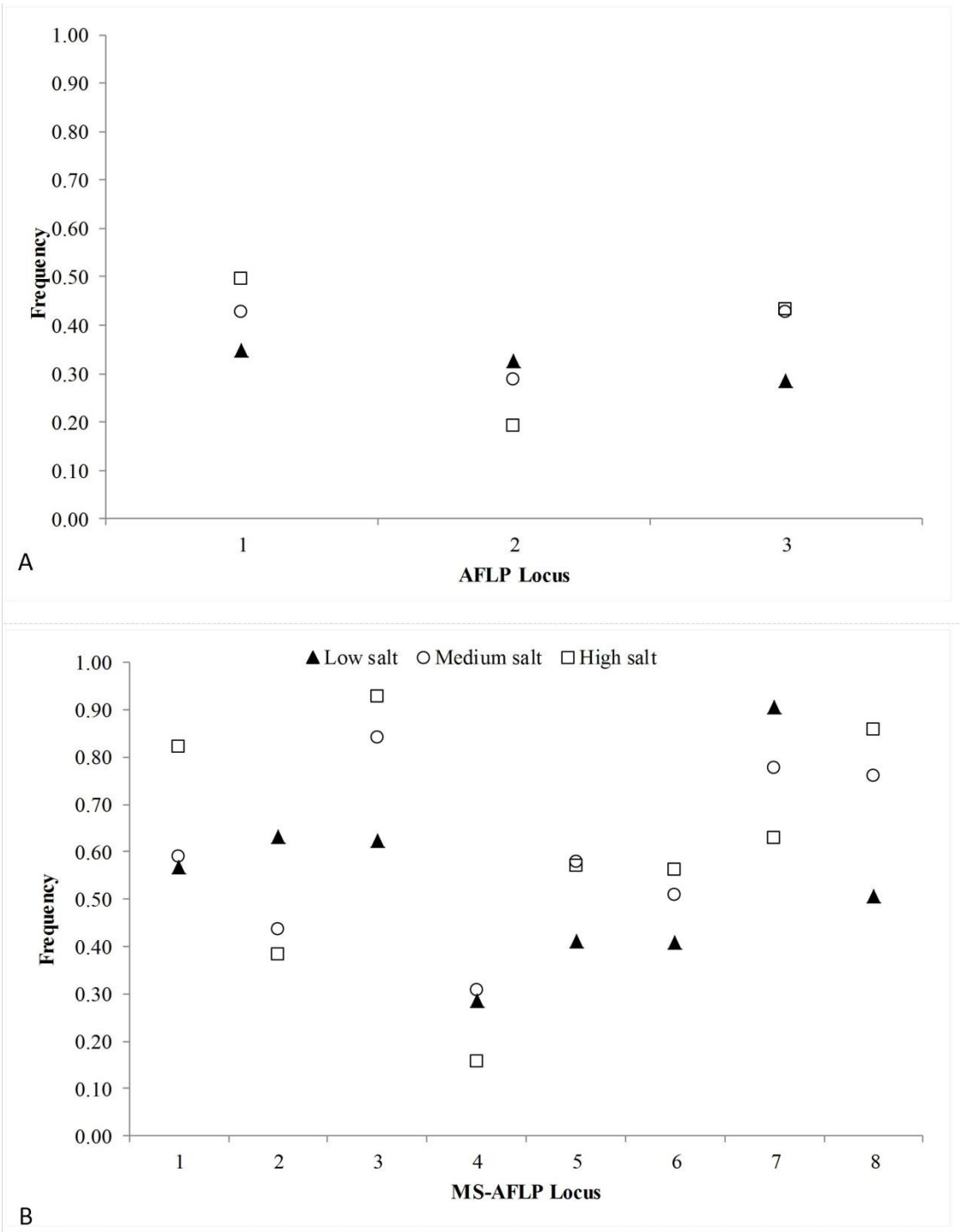

Figure 3.3. Frequencies of (A) AFLP and (B) MS-AFLP loci that were significantly correlated with habitat across five populations using a generalized linear model.

Chapter 9
Population Epigenetics

Christy M. Foust, Aaron W. Schrey and Christina L. Richards

Population Epigenetics

Population genetics examines the extent of genetic variation, and changes to genetic variation, in response to evolutionary processes in populations [1]. There is an enormous body of knowledge surrounding population genetics, extending from the 1930s with the introduction of the modern synthesis (MS) to the present. The MS encompasses population genetics questions that include the dynamics of alleles in populations as they pertain to the Hardy–Weinberg equilibrium (HWE; i.e., the assumptions of infinite population size, random mating, no migration, no mutation, and no selection) [2]. In addition to the assumptions of HWE, the MS is also bound by the confines of Mendelian genetics which calls for the random segregation and assortment of alleles [3].

Though the MS has been incredibly informative regarding evolutionary questions, limitations have been identified with advancements in molecular biology [4, 5]. For example, the MS does not consider the importance of non-Mendelian inheritance to evolution [4, 6]. Epigenetic inheritance is one type of non-Mendelian inheritance; which through mechanisms like DNA methylation or histone modification can account for the transmission of phenotypic variation that is independent from genotypic variation (i.e., DNA sequence) [7]. Since the MS is largely a theory of the dynamics of DNA sequence-based transfer through a population, and gene sequences are largely unaffected by environmental factors, environmental effects on differences in phenotype are not considered important factors within the MS [4]. The MS has been a revolutionary advancement to the study of evolution, because

C. M. Foust (✉) · C. L. Richards
Department of Integrative Biology, University of South Florida, 4202 E Fowler Avenue, Tampa, FL 33620, USA
e-mail: christyfoust@mail.usf.edu

A. W. Schrey
Department of Biology, Armstrong State University, 11935 Abercorn Street, Savannah, GA, 31419 USA

O. Pontes, H. Jin (eds.), *Nuclear Functions in Plant Transcription, Signaling and Development,* DOI 10.1007/978-1-4939-2386-1_9

it reconciles Mendelian inheritance with Darwinian gradual change in response to natural selection. Certainly, its ideas are still critical for evolutionary thinking, but the time has come to extend the MS to address many developments in the field.

Population epigenetics addresses some of the limitations of the MS. Similar to population genetics, population epigenetics examines the extent of epigenetic variation present in populations of organisms and the way this variation changes over time, or across the landscape with changing environmental conditions [8, 9]. However, in stark contrast to genetic variation, epigenetic variation may not be inherited via Mendelian processes [10–12], and epigenetic variation can be directly affected by the environment [13, 14]. Though the basic concepts defining population genetics are known, the body of knowledge concerning processes of change and mechanisms of inheritance of epigenetic variation remains largely unknown [15]. Theorists are developing models of epigenetic inheritance [16, 19], and the effect of selection on epigenetic marks [20]. These models provide a theoretical underpinning for understanding the behavior of epigenetic effects and are discussed later in this chapter.

History and Definition of Epigenetics

Conrad Waddington coined the term "epigenetics" while investigating genetic assimilation and the canalization of traits in the 1940s [21, 22]. Waddington [22] recognized that some characteristics that arise in response to environmental conditions (e.g., stressors) could be incorporated into the genome via the process of genetic assimilation. He also recognized that some phenotypes could be highly conserved regardless of genotype (i.e., canalized). His definition of epigenetics was broad and incorporated these processes and all interactions between genes and gene products that lead to an organism's final phenotype [23, 24]. The discovery of molecular mechanisms and processes that regulate gene activity has resulted in changes to Waddington's definition [23].

Epigenetics has more recently been defined as heritable, differential gene expression not based on changes in DNA sequence [8, 25–27], and the inclusion of "heritable" in this definition is debated [26]. Some biologists think that epigenetics should include all processes that result in differential gene expression in the absence of changes to DNA sequences, heritable or not [28]. Others think that heritability is a necessary component of epigenetics, because inheritance raises direct evolutionary questions that are consistent with population genetics expectations [8, 29–30]. Currently, little empirical data exist to address heritability of epigenetic traits. While it is relatively simple to screen for epigenetic variation, it is much harder to determine if this variation is heritable. We have proposed a definition of epigenetics as the study of molecular-level mechanisms that result in changes in phenotype that are not due to changes in DNA sequence and may lead to heritable change in phenotype [31, 32].

Measuring DNA Methylation

Several mechanisms contribute to epigenetic variation, but DNA methylation is the most studied in ecological epigenetics [33]. DNA methylation occurs when a methyl group attaches to a cytosine in several possible sequence motifs (CpG, CpCpG, CpHpHp, and CpNpG) [34]. DNA methylation can have variable effects on gene activity, but methylation of the promoter region often decreases gene activity, because it interferes with transcription enzymes [27, 35, 36]. Epigenetic variation is more labile than genetic variation and changes in DNA methylation can occur in direct response to environmental cues, and independently from DNA sequence [9, 13, 14]. Because of this, environmental conditions can cause differential methylation and ultimately differential gene expression in response to those environmental conditions.

Methylation-sensitive amplified fragment length polymorphism (MS-AFLP) has been the primary technique used to study DNA methylation in population-level epigenetic studies, though next-generation sequencing-based techniques are promising to become more common [33]. There are several benefits to using MS-AFLP to assess DNA methylation in population-level studies [33]. Like standard AFLP, MS-AFLP offers a genome-wide scan of DNA methylation, and patterns in DNA methylation can be compared among individuals within sites, habitats, or other groups of interest, even in nonmodel organisms that lack a sequenced genome. The technique is also relatively economical, which accommodates the large sample sizes necessary for population-level studies. MS-AFLP also utilizes the same equipment and methods as AFLP, which allows the techniques to be used in concert to address ecological questions and directly compare genetic and epigenetic variation.

One major area of research for population-level epigenetic variation is the relationship between genetic and epigenetic variation. How can the epigenetic contribution to an organism's response be teased apart from genetic variation? The correlation between epigenetic and genetic variation may be categorized as obligate, facilitated, or pure (Fig. 9.1) [8, 9]. Obligate epigenetic variation is directly correlated with genetic variation and appears to follow the rules of Mendelian genetics [9]. Facilitated epigenetic variation is partially correlated with genetic variation, and genetic code directs the epigenetic variation but the outcome is context dependent. Pure epigenetic variation is independent from genetic variation. Both facilitated and pure tend to exhibit non-Mendelian segregation and both can be affected by stochasticity in addition to environmental conditions.

Researchers have begun designing elegant studies that control for genetic differences among individuals [14, 37–40]. These studies have suggested that some epigenetic variation is correlated with environment and some environmentally induced epigenetic variation is passed on to the next generation. These studies have also identified correlations between epigenetic markers and environmental conditions (often stressors), even in the absence of genetic variation. Though directly connecting the effect of epigenetic variation on phenotype is generally lacking, these studies suggest that epigenetic effects could be important to evolution.

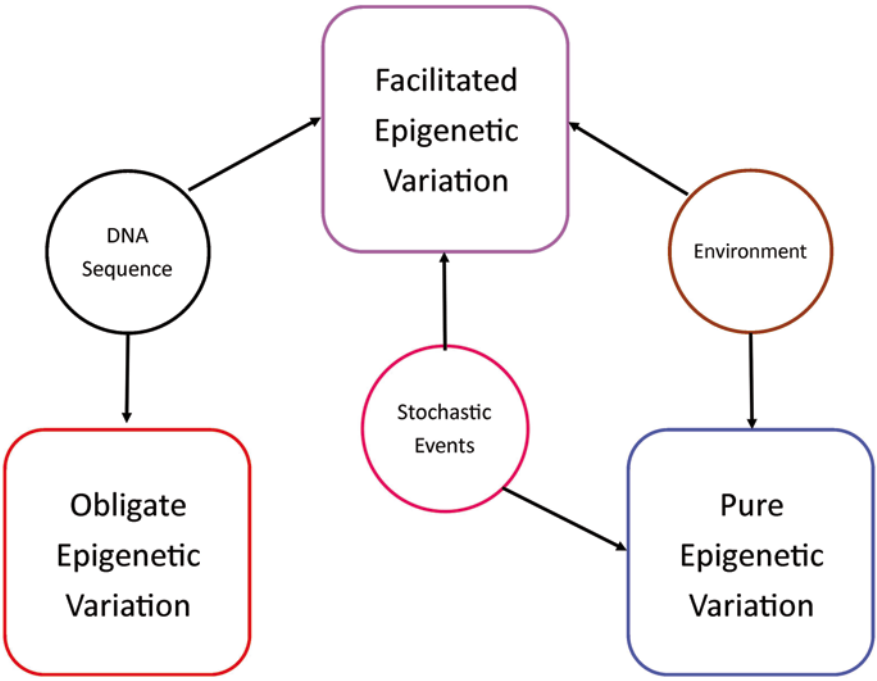

Fig. 9.1 Relationship among obligate, facilitated, and pure epigenetic variation with DNA sequence, stochastic events, and environmental conditions. (Adapted from [8, 9])

Questions We Should Be Asking

Richards [9] laid the foundation for population epigenetics by identifying four fundamental questions, and Bossdorf et al. [27] supplied a similar list of questions to ecologists. To date, with the exception of documenting epigenetic variation in natural populations, these questions remain largely unanswered.

1. How much epigenetic variation is present?
2. How independent is epigenetic variation from genetic variation?
3. What is the extent to which epigenetic states are inherited?
4. What is the importance of epigenetic variation in populations, whether inherited or not?

How Much Epigenetic Variation is Present?

In most epigenetics studies, epigenetic variation (i.e., DNA methylation) exceeds genetic variation [14, 37, 40–43]. This may be related to the much more rapid mutation rate in epigenetic than genetic contexts: replicate "mutation accumulation"

lines of a single *Arabidopsis thaliana* accession exhibited 10^3–10^4 more epigenetic mutations than genetic [44, 45].

Overall, "how much" epigenetic variation is present and the manner that the variation is generated may be critically important to understand the epigenetic response to environmental conditions. To address how much epigenetic variation there is, many ecologists have measured epigenetics in natural populations in the context of several ecological topics. These topics include identifying mechanisms for plasticity, rapid differentiation to habitat, and mechanisms which contribute to invasive species success.

Plasticity

Epigenetic diversity alone may be important for allowing organisms to respond to different environmental conditions. Using a classic phenotypic plasticity design, Bossdorf et al. [46] found that experimental alteration of DNA methylation altered important traits, and the plasticity of those traits, in response to nutrient addition in *A. thaliana*. Among 22 *A. thaliana* genotypes, there was also a difference in the *degree* to which trait means and plasticities were affected by the methylation inhibitor 5-azacytidine. Overall patterns of variability among the genotypes indicated that epigenetic changes can affect not only the short-term environmental responses of plants but also the evolutionary potential of important traits and their plasticities [46].

Epigenetic variation can also play an important role in the ability for clonal yeast (*Metschnikowia reukaufii*) to occupy different nectar habitats. Herrera et al. [37] grew clonal colonies of yeast in experimental media that mimicked naturally occurring differences in sugar combinations and concentrations of nectars to determine if the yeast exhibited epigenetic responses to the nectar variation. The clonal replicates controlled for genetic variation contributing to the response, and demonstrated that plasticity in resource use was correlated with changes in methylation (with MS-AFLP). The probability of an MS-AFLP marker changing from unmethylated to methylated was significantly correlated with sugar concentration, content, and their interaction. They also used 5-azacytidine to show that yeast growth response was significantly inhibited if epigenetic changes were prevented.

More recently, Herrera and Bazaga [39] examined the epigenetic signature between prickly and nonprickly leaves on *Ilex aquifolium,* English holly. *Ilex aquifolium* exhibits more prickly leaves in response to mammalian herbivory [47–49]. Both prickly and nonprickly leaves were sampled from five shrubs along a 450-m transect, and sampling the different leaf types from the same shrubs ensured that the genotypes were identical. Variation among MS-AFLP loci was significantly associated with leaf type, and the probability of methylation declined in nonprickly leaves compared to prickly. They found six MS-AFLP loci that distinguished prickly from nonprickly leaves, suggesting that the genes associated with these MS-AFLP loci could be important in mediating plasticity in leaf morphology in response to herbivory.

Habitat Differentiation

The studies exploring plasticity show that epigenetic diversity can contribute to response to environmental factors, suggesting that population epigenetic variation may be structured spatially based on environmental variables in natural populations. Differentiation of epigenetic marks among habitats could indicate that epigenetic effects allow organisms to adapt to local environmental conditions without a change in genetic code [27].

In white mangroves, both DNA methylation diversity and differentiation could be important for persistence in variable habitats. Lira-Medeiros et al. [50] examined epigenetic and genetic variation in *Laguncularia racemosa* from both riverine and salt marsh habitats in Brazil. Mangroves from both habitats exhibited higher levels of epigenetic variation than genetic variation as identified by comparing MS-AFLP and AFLP results. They also found epigenetic differentiation between mangroves from salt marsh and riverine habitats for *L. racemosa*, but no genetic differentiation. Plant phenotypes significantly differed between habitats for a number of ecologically relevant traits including height, diameter at breast height (DBH), leaf width, and leaf area. Though Lira-Medeiros et al. [50] found epigenetic population differentiation, it was not possible to determine if these differences were generated by stable or induced epigenetic effects, because they collected plant material from the field. Their findings suggest that epigenetics may play an important role in response to local habitat conditions; however, manipulative studies are required to determine the contribution of stable versus environmentally induced epigenetic marks.

Epigenetic diversity and differentiation could also be important in the response to herbivory of *Viola cazorlensis* [41]. Herrera and Bazaga [41] analyzed epigenetic and genetic diversity and differentiation among *V. cazorlensis* plants that had been exposed to 20 years of variable ungulate herbivory. As observed in white mangroves, *V. cazorlensis* also exhibited higher levels of epigenetic variation (e.g., DNA methylation) than genetic variation when comparing MS-AFLP and AFLP results. Additionally, *V. cazorlensis* exhibited both epigenetic and genetic population structure in response to herbivory. The epigenetic differences were correlated with genetic loci implicated with adaptive floral traits. These findings suggest that epigenetics could play an important role in flowering dynamics in the *V. cazorlensis* system that could ultimately result in the adaptive differentiation of populations over evolutionary time [41].

Population Bottlenecks

Epigenetic diversity may provide an additional source of variation to populations that have undergone reductions in genetic variation from demographic changes, like in invasive species. One consequence of population bottlenecks is inbreeding depression. Vergreer et al. [51] showed that epigenetics can play a role in inbreeding depression in the self-pollinating perennial plant *Scabiosa columbaria*. They inbred and outcrossed the plant and found that inbred plants exhibited decreased leaf number, biomass, and photosynthetic efficiency when compared with outcrossed plants.

They also found that genome-wide DNA methylation was 10 % higher in the inbred plants. Additionally, they treated some plants with 5-azacytidine, which decreased genome-wide methylation by 11 %. In the 5-azacytidine treatment, they found that biomass decreased in outcrossed plants and increased in inbred plants. Photosynthetic efficiency and leaf number were not affected in outcrossed plants, but inbred plants exhibited levels consistent with control, outcrossed plants. The biomass of inbred plants treated with 5-azacytidine was also significantly higher than control, inbred plants. The combination of these findings suggests that DNA methylation plays a role in mediating the effects of inbreeding depression for various traits.

These findings were supported in a study on house sparrows (*Passer domesticus*) introduced to Kenya in the 1950s [52]. Schrey et al. [53] showed that Kenyan house sparrows have lower levels of genetic variation than native populations, and Liebl et al. [54] showed that all house sparrows sampled from seven cities in Kenya exhibited different epigenotypes. Though the sparrows exhibited high levels of epigenetic variation, they did not exhibit epigenetic population structure among the sites. However, certain MS-AFLP loci were methylated more often in Kenyan populations when compared to native populations [53], which suggests that these loci could be involved in traits important to living in the new habitat. Liebl et al. [54] also discovered a trend suggesting the epigenetic variation may compensate for decreased genetic variation and increased inbreeding. Individuals exhibiting lower levels of genetic variation had higher inbreeding coefficients and exhibited higher levels of epigenetic variation. Overall, these findings suggest that epigenetic variation may compensate for decreased genetic variation in the early stages of invasion, thereby allowing the sparrows to occupy an expanded range of response that allows them to occupy many different habitats.

Different patterns of epigenetic variation in Japanese knotweed populations further support the hypothesis that epigenetic effects may contribute to response to local habitat. Richards et al. [40] sampled invasive Japanese knotweed (*Fallopia* spp.) individuals from roadside, beach, and salt marsh habitats across eastern New York state. Individuals were grown in a common garden to control for induced environmental effects prior to assessing genetic and epigenetic variation. Genetic diversity in these populations was low, with only four variable AFLP loci detected out of 200 loci scored. Epigenetic variation was significantly higher, with 19 variable loci detected out of 180 loci scored. Both genetic and epigenetic population structure was found among the different habitats, but habitat explained a significant proportion of the structure only for the epigenetic variation and not for genetic variation. Given the low genotypic diversity, these findings suggest that epigenetic effects may play an important role in knotweed's response to variable environmental conditions.

How Independent Is Epigenetic Variation from Genetic Variation?

The relationship between genetic and epigenetic variation can be complex [9], and determining how much epigenetic variation is obligate, facilitated, or pure has been challenging [27, 31]. Currently, the best way to address this question has been to

assess the importance of epigenetic variation in situations where genetic variation is lacking or minimal. Studies in clonal plants support the importance of pure or facilitated epigenetic effects by clonal species (i.e., dandelions, knotweed).

Verhoeven et al. [14] examined DNA methylation in response to five ecologically relevant treatments in replicates of a single dandelion (*Taraxacum officinale*) genotype. DNA methylation variation was significantly higher in response to each of three experimental treatments (i.e., low nutrients, jasmonic acid, and salicylic acid), than control, and the majority of the differences were inherited. Similarly, Richards et al. [40] found replicates of genetically identical individuals showed epigenetic differentiation to beach, roadside or salt marsh habitats. They grew the individuals in a common garden, and these patterns were persistent through clonal reproduction. The findings from these two clonal plant species suggest that epigenetic differences elicited by a single genotype in response to different environmental factors may persist into the next generation, and that the epigenetic effects are more than just a simple readout of the genotype.

Using clonal species is one way to control for the effect of genotype in population-level studies. In populations where it is challenging or impossible to control genetic variation among individuals with experimental design, researchers will have to find other ways to control for the effect of genotype. Adapting statistical tests to control for the genetic component of response is one way to do this. For example, we found both genetic and epigenetic population structure among habitat types in natural *Spartina alterniflora* populations (Foust et al. unpublished data). We used a partial Mantel test to correlate epigenetic distances with habitat (low, intermediate, and high-salt areas) while controlling for genetic distance. The partial Mantel test allowed us to find significant epigenetic population structure while controlling for genetic population structure. This is one example where statistics offered a way to tease apart the correlation between epigenetic and genetic variation.

What Is the Extent to Which Epigenetic States Are Inherited?

To be naturally selected, traits controlled by epigenetic variation must be transmitted to the next generation. Currently, there are few ecological epigenetic studies that directly connect heritable phenotypic traits to epigenetic marks. However, we know that epigenetic changes can be induced by environmental conditions, that epigenetic changes affect phenotype, and that these phenotypic changes can be passed to the next generation [7]. Epigenetic marks can also be highly conserved through transmission, with variability ranging from 1 % to 10^{-6} variations per generation [7, 8, 55], which means that induced epigenetic variation will likely persist in future generations.

Epigenetic recombinant inbred lines (epiRILs) are one valuable resource that have shed more light on this question [11, 56]. Two populations of epiRILs were developed from backcrosses of the methylation mutants *(ddm1* and *met1*) to Col-0 wild type in *A. thaliana*. Johannes et al. [11] showed that both flowering time and

plant height varied among epiRILs, and that these effects persisted for at least eight generations. While these findings were initially discovered in epiRILs created from one single genotype, Cortijo et al. [57] have confirmed that these epigenetic associations with phenotype are found across 138 natural accessions for which genome-wide methylation data and phenotype data were available [45, 58, 59].

Perhaps the most clearly defined example of epigenetic inheritance in a non-model species was discovered in *Linaria vulgaris* (toadflax) [12]. *Linaria vulgaris* exhibits radial flower symmetry when hypermethylation of the *Lcyc* gene, an epimutation, occurs. Individuals without the epimutation exhibit dorsoventral symmetry. This change in phenotype is ecologically important because it could affect pollination and overall fitness of the plant [60]. It could also be evolutionarily important because the epiallele can be directly transmitted to the next generation, but the mechanism is unclear because the epimutation is sometimes reset during somatic development [12]. More research in an ecological context is required to determine if this epimutation affects the evolutionary trajectory of the species.

Epigenetic inheritance also occurs in the perennial herbs, *Helleborus foetidus* [38] and *T. officinale* [14]. In both studies, MS-AFLP was used to identify patterns of epigenetic variation in parent plants and the next generation. *Helleborus foetidus* was sampled from three populations located on a latitudinal gradient in Spain. In addition to the parents, patterns of epigenetic variation were also assessed in the pollen produced by parent plants (i.e., the male gametophyte). Though there was some epigenetic resetting from the sporophyte to gametophyte generation, significant epigenetic population structure persisted among the gametophytes of the three populations, as predicted from the parental generation. Similarly, Verhoeven et al. [14] showed that between 74 and 92 % of epigenetic states present in the parent generation occurred in the offspring, even though the stressors (i.e., low nutrients, salt, jasmonic acid, salicylic acid, and control) were no longer present. These findings suggest that epigenetic population structure persists into future generations, which could help offspring respond to local environmental conditions [27].

What Is the Importance of Epigenetic Variation in Populations, Whether Inherited or Not?

Epigenetic studies of nonmodel organisms and studies at the population level typically do not directly identify the connection between epigenetic state and phenotype. This issue must be addressed to fully understand the importance of epigenetic variation. Theorists have developed models that show epigenetics can affect the evolutionary trajectories of organisms [16–19]. Bonduriansky and Day [18] recognize that epigenetics allows for the decoupling of genotype from phenotype, which releases some of the constraints on evolution as defined by Mendelian genetics. Day and Bonduriansky [61] developed a model that incorporates both genetic and epigenetic inheritance, and allows some genomic elements to accumulate more epigenetic variation than others [45]. Pál [16] and Pál and Miklós [17] found that epi-

genetic inheritance systems (e.g., transmission of DNA methylation patterns) can allow organisms to avoid valleys on an adaptive landscape. Ultimately, epigenetic inheritance can change the adaptive landscape. Thus, an organism that is trapped in an adaptive valley may be rescued by epigenetic variation. It is also possible that genetic inheritance may "take over" after epigenetic mechanisms have facilitated the transition across an adaptive valley (i.e., genetic assimilation [19, 21, 22, 62]).

Klironomos et al. [19] developed a model that showed epigenetics can affect the tempo and the overall outcome of evolution. They describe cases where adaptation occurs by epigenetic mechanisms, and epigenetic inheritance can ensure that the phenotype persists. Epigenetically derived phenotypes can "speed up" evolution and potentially change the trajectory of evolution. Geoghegan and Spencer [20] support this idea and show that incorporating epigenetic marks into selection models can also drastically alter trajectories. This is especially true considering that environmentally mediated epigenetic marks can regenerate depending on the scenario. This means that as the environment changes, epigenetic marks can revert and potentially take organisms back to the fitness peak that was pertinent to past environmental conditions. Each of these models demonstrates the potential importance of epigenetics to evolution. Incorporating empirical data to these theoretical advances will greatly expand our understanding of the importance of epigenetics.

Future Directions

The available epigenetics studies in natural populations and nonmodel organisms present compelling evidence that epigenetic mechanisms are important at the population level. However, there are major areas that need to be addressed by future studies. These include:

1. Understanding the relationship between genetic and epigenetic variation
2. Identifying stable versus environmentally induced epigenetic variation
3. Identifying the importance of inheritance of epigenetic variation

As epigenetic techniques and next-generation sequencing become more cost-effective, researchers should use these tools to address these issues in more detail [33]. Epigenetic sequencing coupled with next-generation sequencing will allow for cross comparisons with genomic information obtained from studies of model organisms on specific genes and gene networks associated with various environmental conditions.

Adapting statistical analyses to control for genetic response will be important in gaining understanding about the relationship between genetic and epigenetic variation. Adaptation of the partial Mantel test has been useful in uncovering epigenetic population structure associated with low, intermediate, and high-salt habitats, that is independent from genetic population structure in natural *S. alterniflora* populations (Foust et al. unpublished data). Other statistical tests could be modified to accomplish the same goal in systems where it is challenging or impossible to control

for genetic variation among individuals by experimental design. Using statistical approaches to control for genetic response will allow research to broaden to include more nonmodel organisms, thereby increasing our overall understanding of the relationship between genotype and epigenotype.

Another consideration that will be important in future studies of population epigenetics is the effect of nonstable epigenetic effects. which can be minimized in some cases with a modified experimental design. Most studies to date have collected samples in the field, thus confounding stable and induced DNA methylation. Richards et al. [40] controlled for these effects by collecting Japanese knotweed rhizomes in the field and growing them in common garden prior to assessing genetic and epigenetic variation. This allowed for any highly labile marks to "normalize," and stable marks that might contribute to habitat response to persist. Reciprocal crosses would help determine epigenetic response to local conditions, and address the potential "chicken or egg" scenario between the association of epigenetic marks and environmental conditions. For example, are the patterns of epigenetic variation present because the plants responded to environmental conditions? Or did plants that possess certain patterns of epigenetic variation thrive in specific habitats?

To address these questions, future studies should build upon current epigenetic surveys and begin including more manipulative experimental designs with nonmodel organisms in natural systems. Using multifaceted experimental designs will help to determine if patterns of epigenetic variation change in response to environmental conditions, which result in population structure, or if patterns of epigenetic variation that are already present enable an organism to live in a certain area [40].

Finally, to determine the importance of epigenetic variation to evolutionary questions, more multigenerational studies need to be performed. Ideally, multigenerational studies will be designed to address the questions brought forth in the current, proposed models. These questions include: what are the implications of phenotype being decoupled from genotype, how do epigenetic changes alter the adaptive landscape or allow populations to avoid adaptive valleys, does epigenetics really speed up evolutionary processes, and how does it change evolutionary trajectories? [16–20, 61]. To do this, researchers need to examine both short- and long-term epigenetic response to environmental conditions [19] and assess both genetic and epigenetic contributions to evolution.

Conclusions

Currently, the importance of epigenetics at the population level is often made via correlation. Manipulative field studies will need to be performed to determine if epigenetic population structure results from habitat response or if organisms with certain epigenetic signatures can more easily live in certain habitats [40]. Since patterns of epigenetic variation can be established in direct response to local environmental conditions, but can also be inherited from parents, it is important to address this causality problem to gain further understanding about how organisms respond

epigenetically to variable conditions. Understanding the different ways populations can respond to environmental conditions via epigenetics is also important, because population genetics ignores the effects of short-term and within-generation environmental variation on evolution.

Researchers have already begun addressing the questions Richards [9] set forth, and each of those papers further support the importance of epigenetics to ecologically-relevant traits in nonmodel and natural populations. The theoretical models presented in this chapter have established a good outline for the future direction of population epigenetics, and future studies should include multigenerational, population-level studies to test these models. Progress is being made in all of these areas; however, there is much that we do not know.

We do know that an extension of the MS that encompasses epigenetic mechanisms and inheritance is necessary [5]. It is becoming more clear, especially as more studies are performed at the population level, that epigenetics is important to short-term environmental response, and that DNA methylation changes in response to environmental conditions can persist into the future [63]. In some cases, these epigenetic responses provide an adaptive advantage to offspring that are subjected to those same environmental conditions [46]. To fully address these questions, laboratory-based studies on model organisms should be coupled with natural and manipulative studies on natural populations and nonmodel organisms to obtain a clearer picture of how epigenetics affects organisms [38].

References

1. Lewontin RC. The genetic basis of evolutionary change. West Sussex: Columbia University Press; 1974.
2. Pigliucci M. Do we need an extended evolutionary synthesis? Evolution 2007;61:2743–49.
3. Hartl DL, Jones EW. Essential genetics. 2nd ed. Sudbury: Jones and Bartlett; 1999.
4. Jablonka E, Lamb MJ. The epigenome in evolution: beyond the modern synthesis. VOGis Herald 2008;12:242–54.
5. Pigliucci M, Müller GB. Evolution – the extended synthesis. Cambridge: MIT Press; 2010.
6. Jablonka E, Lamb MJ. Evolution in four dimensions: genetic, epigenetic, behavioral, and symbolic. Cambridge: MIT Press; 2005.
7. Jablonka E, Raz G. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Q Rev Biol. 2009;84:131–76.
8. Richards EJ. Inherited epigenetic variation – revisiting soft inheritance. Nat Rev Genet. 2006;7:395–401.
9. Richards EJ. Population epigenetics. Curr Opin Genet Dev. 2008;18:221–6.
10. Grandjean V, Yaman R, Cuzin F, Rassoulzadegan M. Inheritance of an epigenetic mark: the CpG DNA methyltransferase 1 is required for de novo establishment of complex patterns of non-CpG methylation. PLoS One. 2007;11:e1136.
11. Johannes F, Porcher E, Teixeira FK, Saliba-Combani V, Simon M, Agier N, Bulski A, Albuisson J, Heredia F, Audigier P, Bouchez D, Dillman C, Guerche P, Hospital F, Colot V. Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS Genet. 2009;6:e1000530.
12. Cubas P, Vincent C, Coen E. An epigenetic mutation responsible for natural variation in floral symmetry. Nature. 1999;401:157–61.

13. Bollati V, Baccarelli A. Environmental epigenetics. Heredity. 2010;105:105–12.
14. Verhoeven KJF, Jansen JJ, van Dijk PJ, Biere A. Stress-induced DNA methylation changes and their heritability in asexual dandelions. New Phytol. 2010;185:1108–18.
15. Angers B, Castonguay E, Massicotte R. Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. Mol Ecol. 2010;19:1283–95.
16. Pál C. Plasticity, memory, and the adaptive landscape of the genotype. Proc R Soc Lond Biol. 1998;265:1319–23.
17. Pál C, Miklós I. Epigenetic inheritance, genetic assimilation, and speciation. J Theor Biol. 1999;200:19–37.
18. Bonduriansky R, Day T. Non-genetic inheritance and its evolutionary implications. Annu Rev Ecol Evol Syst. 2009;40:103–25.
19. Klironomos FD, Berg J, Collins S. How epigenetic mutations can affect genetic evolution: model and mechanism. BioEssays. 2013;35:571–8.
20. Geoghegan JL, Spencer HG. Population-epigenetic models of selection. Theor Pop Biol. 2012;81:232–42.
21. Waddington CH. Canalization of development and the inheritance of acquired characters. Nature. 1942;150:563–65.
22. Waddington CH. Genetic assimilation of an acquired character. Evolution. 1953;7:118–26.
23. Jablonka E, Lamb MJ. The changing concept of epigenetics. Ann NY Acad Sci. 2002;981:82–96.
24. Holliday R. Epigenetics: a historical review. Epigenetics. 2006;1:76–80.
25. Holliday R. Epigenetics: an overview. Dev Genet. 1994;15:453–7.
26. Bird A. Perceptions of epigenetics. Nature. 2007;447:396–8.
27. Bossdorf O, Richards CL, Pigliucci M. Epigenetics for ecologists. Ecol Lett. 2008;11:106–15.
28. Hallgrímsson B, Hall BK, editors. Epigenetics: linking genotype and phenotype in development and evolution. Berkeley: University of California Press; 2011.
29. Ho DH, Burggren WW. Epigenetics and transgenerational transfer: a physiological perspective. J Exp Biol. 2010;213:3–16.
30. Kovalchuk I. Transgenerational epigenetic inheritance in animals. Front Genet. 2012;3:1–2.
31. Ledón-Rettig CC, Richards CL, Martin LB. Epigenetics for behavioral ecologists. Behav Ecol. 2013;24:311–24.
32. Kilvitis H, Alvarez M, Foust CM, Robertson M, Schrey AW, Richards CL. Ecological epigenetics. In Landry C, Aubin-Horth N, editors. Ecological and evolutionary genomics. Dordrecht: Springer; 2014.
33. Schrey AW, Alvarez M, Foust CM, Kilvitis HJ, Lee JD, Liebl AL, Martin LB, Richards CL, Robertson M. Ecological epigenetics: beyond MS-AFLP. Integr Comp Biol. 2013;53:340–50.
34. Rapp RA, Wendel JF. Epigenetics and plant evolution. New Phytol. 2005;168:81–91.
35. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003;33:245–54.
36. Jablonka E, Lamb MJ. The evolution of information in the major transitions. J Theor Biol. 2006;239:236–46.
37. Herrera CM, Pozo MI, Bazaga P. Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. Mol Ecol. 2012;21:2602–16.
38. Herrera CM, Medrano M, Bazaga P. Epigenetic differentiation persists after male gametogenesis in natural populations of the perennial herb *Helleborus foetidus* (Ranunculaceae). PLoS One. 2013;7:e70730.
39. Herrera CM, Bazaga P. Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (Aquifoliaceae) trees. Bot J Linean Soc. 2013;171:441–52.
40. Richards CL, Schrey AW, Pigliucci M. Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. Ecol Lett. 2012;15:1016–25.
41. Herrera CM, Bazaga P. Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet *Viola cazorlensis*. New Phytol. 2010;187:867–76.

42. Massicotte R, Whitelaw E, Angers B. DNA methylation: a source of random variation in natural populations. Epigenetics. 2011;6:421–7.
43. Schrey AW, Coon CAC, Grispo MT, Awad M, Imboma T, McCoy ED, Mushinsky HR, Richards CL, Martin LB. Epigenetic variation may compensate for decreased genetic variation with introductions: a case study using house sparrows (*Passer domesticus*) on two continents. Genet Res Int. 2012;2012:1–7.
44. Becker C, Hagmann J, Müller J, Koenig D, Stegle O, Borgwardt K, Weigel D. Spontaneous epigenetic variation in the *Arabidopsis thaliana* methylome. Nature. 2011;480:245–9.
45. Schmitz RJ, Schultz MD, Urich MA, Nery JR, Pelizzola M, Libiger O, Alix A, McCosh RB, Chen H, Schork NJ, Ecker JR. Patterns of population epigenomic diversity. Nature. 2013;495:193–8.
46. Bossdorf O, Richards CL, Pigliucci M. Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. Evol Ecol. 2010;24:541–53.
47. Supnick M. On the function of leaf spines in *Ilex opaca*. Bull Torrey Bot Club. 1983;110:228–30.
48. Potter DA, Kimmerer TW. Do holly leaf spines really deter herbivory? Oecologia 1988;75:216–21.
49. Obeso JR. The induction of spinescence in European holly leaves by browsing ungulates. Plant Ecol 1997;129:149–56.
50. Lira-Medeiros, CF, Parisod C, Fernandes RA, Mata CS, Cardoso MA, Ferreira PCG. Epigenetic variation in mangrove plants occurring in contrasting natural environment. PLoS One. 2010;5:e10326.
51. Vergreer P, Wagemaker NCAM, Ouborg NJ. Evidence for an epigenetic role in inbreeding depression. Biol Lett. 2012;8:798–801.
52. Anderson TR. Biology of the ubiquitous house sparrow: from genes to populations. New York: Oxford University Press; 2006.
53. Schrey AW, Grispo M, Awad M, Cook MB, McCoy ED, Mushinsky HR, Albayrak T, Bensch S, Burke T, Butler LK, Dor R, Fokidis HB, Jensen H, Imboma T, Kessler-Rios MM, Marzal A, Stewart IRK, Westerdahl H, Westneat DF, Zehtindjiev P, Martin LB. Broad-scale latitudinal patterns of genetic diversity among native European and introduced house sparrow (*Passer domesticus*) populations. Mol Ecol. 2011;20:1133–43.
54. Liebl AL, Schrey AW, Richards CL, Martin LB. Patterns of DNA methylation throughout a range expansion of an introduced songbird. Integr Comp Biol. 2013;53:351–8.
55. Genereux DP, Miner BE, Bergstrom CT, Laird CD. A population-epigenetic model to infer site-specific methylation rates from double-stranded DNA methylation patterns. Proc Natl Acad Sci. 2005;16:5802–07.
56. Reinders J, Wulff BH, Mirouze M, Marí-Ordóñez A, Dapp M, Rozhon W, Bucher E, Theiler G, Paszkowski J. Compromised stability of DNA methylation and transposon immobilization in mosaic Arabidopsis epigenomes. Genes Dev. 2009;23:939–50.
57. Cortijo S, Wardenaar R, Tatché MC, Gilly A, Etcheverny M, Labaclie K, Caillieux E, Hospital F, Aury JM, Wincker P, Roudier F, Jansen RC, Colot V, Johannes F. Mapping the transgenerational epigenetic basis for complex traits. Science. 2014;343:1045–1048.
58. Brachi B, Faure N, Horton M, Flahauw E, Vazquez A, Nordborg M, Bergelson J, Cuguen J, Roux F. Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. PLoS Genet. 2010;6:e1000940.
59. Atwel, S, Huang YS, Vilhja´lmsson BJ, Willems G, Horton M, Li Y, Meng D, Platt A, Tarone AM, Hu TT, Jiang R, Muliyati NW, Zhang X, Amer MA, Baxter I, Brachi B, Chory J, Dean C, Debieu M, de Meaux J, Ecker JR, Faure N, Kniskern JM, Jones JDG, Michael T, Nemri A, Roux F, Salt DE, Tang C, Todesco M, Traw MB, Weigel D, Marjoram P, Borevitz JO, Bergelson J, Nordborg M. Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. Nature. 2010;465:627–31.

60. Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. Pollination syndromes and floral specialization. Annu Rev Ecol Evol Syst. 2004;35:375–403.
61. Day T, Bonduriansky R. A unified approach to the evolutionary consequences of genetic and non-genetic inheritance. Am Nat. 2011;178:E18–36.
62. West-Eberhard MJ. Developmental plasticity and the origin of species differences. Proc Natl Acad Sci. 2005;102:6543–9.
63. Duckworth RA. Epigenetic inheritance systems act as a bridge between ecological and evolutionary timescales. Behav Ecol. 2013;24:327–8.

SPRINGER
LICENSE TERMS
AND CONDITIONS

Sep 14, 2015

This is a License Agreement between Christy M. Foust ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3707860485380
License date	Sep 14, 2015
Licensed content publisher	Springer
Licensed content publication	Springer eBook
Licensed content title	Population Epigenetics
Licensed content author	Christy M. Foust MS
Licensed content date	Jan 1, 2015
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	1
Author of this Springer article	Yes and you are a contributor of the new work
Order reference number	None
Title of your thesis / dissertation	Population Genetic and Epigenetics of Two Salt Marsh Plant Species along an Environmental Gradient
Expected completion date	Dec 2015
Estimated size(pages)	60
Total	0.00 USD
Terms and Conditions	

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at

the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com.

Limited License
With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided its password protected or on the university's intranet or repository, including UMI (according to the definition at the Sherpa website: http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com).

The material can only be used for the purpose of defending your thesis limited to university-use only. If the thesis is going to be published, permission needs to be re-obtained (selecting "book/textbook" as the type of use).

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, subject to a courtesy information to the author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).

Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted
You may not alter or modify the material in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Sprinter Science + Business Media. (Please contact Springer at (permissions.dordrecht@sprinter.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCD's Billing and Payment terms and conditions.

Copyright Notice: Disclaimer
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Sprinter and the original publisher /journal title,

volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

Warranties: None

Example 1: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Example 2: Springer Science + Business Media makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.

Indemnity
Your hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing
This license may not be amended except in writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Sprinter Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgement, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction
All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in The Netherlands, in accordance with

Dutch law, and to be conducted under the Rules of the ‘Netherlands Arbitrage Instituut’ (Netherlands Institute of Arbitration). ***OR:***

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in the Federal Republich of Germany, in accordance with German law.

Other terms and conditions:

V1.3

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or =1-978-646-2777.