

# EVOLUTION IN CLOSELY ADJACENT SALT MARSH ENVIRONMENTS

by

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(Under the direction of Rodney Mauricio and Lisa A. Donovan)

## ABSTRACT

Environmental variation and its effect on phenotypic variation have long been of interest to evolutionary ecologists. Several hypotheses suggest how plants can live across steep environmental gradients. These include clonal integration, phenotypic plasticity and genetic differentiation. The main objective of this dissertation was to determine what strategies salt marsh plants use to live across the environmental gradients of the marsh. Field surveys of plant phenotypes, environmental parameters and allozyme patterns as well as a greenhouse study and a reciprocal transplant study were conducted to meet this objective.

Field surveys of 12 species indicated that plant phenotypic variation is correlated to environmental variation. In contrast, allozyme patterns showed no association between alleles or genotypes with microhabitats. Similarly, levels of diversity did not differ across microhabitats along the gradient. Much of the variation in the distribution of genetic diversity, however, was predictable based on the gradient. In addition, genetic diversity was surprisingly high and clone size was limited. In both the greenhouse and field reciprocal transplant studies there was evidence of phenotypic plasticity for all traits measured. The greenhouse study on outcrossed seedlings revealed genetic variation for only final height and concentrations of leaf elements Na, P, and Mg. Alternatively, the field experiment on clones of field collected plants, found genetic

variation in almost all salt tolerance traits. In high and low salt gardens, there was significant selection for increased total leaf area and water use efficiency (WUE). However, patterns of selection were significantly different in the two gardens only for stabilizing selection on WUE.

These studies suggest that salt marsh plants are highly plastic. Although there is a lot of genetic variation for salt tolerance traits, and some evidence for differentiation between the two habitats, there is little evidence that these habitats select on traits differently. Differential selection in the two habitats on WUE was the one exception, however there was no evidence of differentiation for this trait. These studies therefore reveal the importance of phenotypic plasticity as the predominant strategy for living across the environmental gradients of the salt marsh.

INDEX WORDS: Adaptation, *Borrchia frutescens*, Ecophysiology, Evolution, Halophytes, Natural selection, Phenotypic plasticity, Salt marsh plants, Sapelo Island, *Spartina alterniflora*

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DEDICATION

To my grandmother Dorothy Lee Leonard.

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## TABLE OF CONTENTS

|   | Page |
|---|------|
| ACKNOWLEDGMENTS.....  | v    |
| CHAPTER   |      |
| 1 INTRODUCTION AND LITERATURE REVIEW.....   | 1    |
| 2 HABITAT RANGE AND PHENOTYPIC VARIATION IN SALT MARSH<br>PLANTS.....   | 13   |
| 3 SEVERE ENVIRONMENTAL GRADIENT DETERMINES GENETIC<br>STRUCTURE OF TWO SALT MARSH PERENNIALS.....                     | 40   |
| 4 EVIDENCE FOR ADAPTIVE PLASTICITY ALONG A SALINITY<br>GRADIENT IN A SALT MARSH PERENNIAL.....                        | 72   |
| 5 THE RESPONSE TO DIFFERING SELECTION ON PLANT<br>PHYSIOLOGICAL TRAITS IN CONTRASTING SALT MARSH<br>ENVIRONMENTS..... | 100  |
| 6 CONCLUSIONS.....  | 128  |

## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

#### *Rationale for research*

Environmental variation and its effect on phenotypic variation have long been of interest to evolutionary ecologists. In particular, classic studies in evolutionary biology have focused on how steep environmental gradients are important in the process of differentiating populations to the point of speciation. Though many research programs have focused on this macroevolutionary effect of speciation in response to environmental gradients, these patterns result from the cumulative effect of processes occurring on a microevolutionary scale. Understanding these processes within populations lends important insight into how these macroevolutionary patterns develop over evolutionary time. Several different hypotheses have been suggested about how it is that plants can live across steep environmental gradients. These include clonal integration, phenotypic plasticity and genetic differentiation. The main objective of my research was therefore to determine what strategy or combination of strategies salt marsh plants use to live across the steep environmental gradients of the marsh.

#### *Background*

Variation between microhabitats can occur on a scale of centimeters and therefore has the potential to affect not only the distribution of species, but also patterns of clonal growth and the nature of phenotypic variation within species. Clonal plants have the ability to share resources across environmental gradients and therefore maintain growth under stressful conditions. Between genetically distinct individuals however, ecological theory posits that a large proportion

of phenotypic variation may represent adaptive matching of phenotypes to a variable environment (Clausen *et al.* 1948). This matching can occur either through natural selection producing genetically-differentiated ecotypes, or through phenotypic plasticity, in which different morphologies are produced from the same genotypes in different environments (Sultan 1995). It is well established that even under high levels of gene flow, natural selection can produce genetically differentiated ecotypes within species on a fine spatial scale (Antlfinger 1981, Antonovics and Bradshaw 1970, Nagy and Rice 1997, Silander 1984, 1985). Theoretical and empirical studies illustrate that genetic diversity can be maintained in populations in the face of natural selection by the maintenance of variants that survive different selection pressures in predictably different microhabitats (Caisse and Antonovics 1978, Feder *et al.* 1997, Hedrick 1976, Levene 1953, Schmidt and Rand 1999). Alternatively, phenotypic plasticity is thought to be favorable if the environment is variable and unpredictable and if there are costs to inappropriate specialized phenotypes (Dorn *et al.* 2000, Relyea 2002, van Tienderen 1991, but see Sultan 1995).

The predicted relationship between phenotypic variation and environmental variation can be complicated by clonal reproduction since genets can share resources across rhizomes and support ramets experiencing physical stress, competition or herbivory (Jonsdottir and Watson 1997, Pennings and Callaway 2000, Steuffer *et al.* 1996). In addition, the ability to discriminate between patches and proliferate in favorable habitat has been found to vary by genotype suggesting the potential for evolution of foraging behavior (Bazzaz 1991, Salzman 1985). These studies suggest that the strategy of clonal integration should lead to predictable patterns of clonal growth across environmental gradients.

In addition to the role of clonal integration, understanding adaptive evolution to differing environments requires studies of the nature of phenotypic variation in ecologically important traits. Phenotypic differences may result from phenotypic plasticity, in which different morphologies are produced from the same genotypes in different environments (Bradshaw 1965, Schmalhausen 1949) or from genetically based adaptation (specialization) to local conditions (Clausen *et al.* 1948, Turesson *et al.* 1922). By examining the norm of reaction of ecologically important traits across controlled environments, we can learn about the role of plasticity in adaptation to local conditions (Sultan 1993 a,b,c, 1995, 2001). Many studies of this nature have revealed genetically based differences in plastic response within and among natural plant populations for a variety of purportedly adaptive traits including response to light availability (Schmitt 1993, Sultan 1993a), water availability (Dudley 1996a, Sultan 1993b), nutrient availability (Crick and Grime 1987, Sultan 1993c) and salt tolerance (Hester *et al.* 1996, Smekens and van Tienderen 2001).

There have been many different approaches to revealing if plants are genetically different in response to differential selection in contrasting habitats. While the occurrence of genetic differentiation and local adaptation has been well documented (Linhart and Grant 1996), how selection acts in different environments to create these genetic differences has been little explored. Few studies have actually measured the strength and direction of selection in natural contrasting environments, despite the fact that estimating these selection parameters is essential for an understanding of how adaptation to local conditions is created and maintained (Kingsolver *et al.* 2001).

A thorough understanding of selection in contrasting environments requires examination of not only how selection affects overall performance, but also how selection acts on specific traits

and on suites of traits (Chapin et al. 1993, Donovan and Ehleringer 1994, Farris and Lechowicz 1990, Geber and Dawson 1990, 1997). By using multiple regression techniques (Lande and Arnold 1983), it is possible to evaluate a suite of potentially important traits to identify those that significantly relate to fitness and that are therefore likely to evolve. These analyses have identified that the adaptive value of traits changes across contrasting environments (reviewed in Ackerly et al. 2000, Arntz and Delph 2001, Kingsolver et al. 2001). For example, in the dune plant *Cakile edentula* (Brassicaceae), Dudley (1996a) demonstrated positive directional selection on water use efficiency (WUE) and stabilizing selection on leaf size for plants grown in dry environments while there was no significant selection on WUE or leaf size in adjacent wet environments.

By examining suites of traits, it is also possible to determine how traits are correlated and how these correlations may either constrain or enhance adaptation. Constraints to adaptation due to character correlations have been of considerable interest in evolutionary theory (Arnold 1992, Endler 1986), but there is little empirical work to test this idea. Dudley's work (1996a, 1996b) is among the few studies of the action of in situ selection on correlated characters in natural contrasting environments. She demonstrated that the correlation between WUE and leaf size might constrain adaptive evolution of these traits because changes in leaf size toward a greater adaptive value would lead to a lower adaptive value of WUE. This knowledge of trait correlations and selection under different conditions deepens our understanding of the mechanisms of adaptive evolution.

The salt marsh is an ideal system to evaluate the roles of clonal integration as well as plasticity and differentiation of important traits because the majority of salt marsh plant species are clonal and occupy habitats that vary widely in abiotic and biotic conditions. Southeastern

U.S. salt marsh plants regularly experience hypersaline, anaerobic, nutrient poor conditions, strong competition from neighboring plants and disturbance from wrack deposition (Mendelssohn and Morris 2000, Pennings and Bertness 2001). Despite a wealth of studies examining how these environmental gradients affect community level patterns in salt marshes (Pennings and Bertness 2001), little research has addressed how these gradients have shaped the genetic make-up and phenotypic response of salt marsh plant species.

A predominant feature of the salt marsh environmental gradient is the broad range in salinity (Pennings and Bertness 2001). A suite of physiological traits have been reported to be important for dealing with the toxic and osmotic effects of substrate salinity (Flowers et al. 1977), but few studies have investigated how selection varies on these traits in environments of contrasting salt content. In particular, halophytes can secrete, sequester or dilute (by increasing succulence) what would otherwise be toxic levels of Na. In addition, conservative water relations can reduce the uptake of Na and reduce the water demands of the plant (Flowers et al. 1977). These habitats are typically not only toxic because of sodium levels, but they are low in nitrogen (Smart and Barko 1980) and sodium can interfere with nutrient uptake (Flowers et al. 1977). Halophytes are able to maintain nutrient uptake in the presence of high Na and use Nitrogen rich compatible solutes and nutrient cations (K, Ca, Mg, Mn) for osmotic adjustment (Aerts and Chapin 2000, Antlfinger and Dunn 1983, Cavalieri and Huang 1979, Donovan et al. 1996, Donovan et al. 1997, Glenn and O'Leary 1984, Moon and Stiling 2000, Rosenthal et al. 2002).

## *Objectives*

The main objective of this dissertation was to determine what strategy or combination of strategies salt marsh plants use to live across the steep environmental gradients of the marsh. In chapter 2, I documented the extent of phenotypic variation for the twelve most common plant species that occur in Georgia salt marshes, and correlated the observed phenotypic variation with several environmental variables. After demonstrating that all of the species do in fact respond to the complex environmental gradients of the salt marsh, in chapter 3 I investigated how the environmental gradients structure fine-scale patterns of genetic differentiation and clonal growth of two dominant salt marsh perennials *B. frutescens* and *S. alterniflora*. The objectives of this study were to determine if microhabitat type significantly explains the distribution of clones and genetic variation for these species. Chapters 4 and 5 focus on the role of phenotypic plasticity and genetic differentiation in putative salt tolerance traits of *B. frutescens*. In chapter 4, I conducted a greenhouse study under controlled salt conditions on plants grown from outcrossed seed collected in two different habitats in the marsh. The objective of this study was to determine the degree of plasticity and genetic differentiation in salt tolerance traits in response to controlled salinity treatments. In chapter 5, I examined a similar suite of traits on clonal replicates from high salt and low salt source genets transplanted in to high salt and low salt marsh habitats. The main objective of this study was to determine if patterns of selection on morphological and physiological traits vary in contrasting environments and if there are trait correlations that constrain adaptive evolution.

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## CHAPTER 2

HABITAT RANGE AND PHENOTYPIC VARIATION IN SALT MARSH PLANTS<sup>1</sup>

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<sup>1</sup> Richards, C. L., S. C. Pennings and L. A. Donovan. Submitted to *Plant Ecology*, 7/11/2003.

## Abstract

Ecologists have long speculated that species with wider environmental ranges would have broader ranges in phenotype; however, most tests of this hypothesis have involved small numbers of species and/or closely related taxa. We related phenotypic variation in twelve salt marsh plant species from six families to variation in four environmental variables using multiple regression. Within species, plant phenotype was predictably related to environmental variation. Salinity was the most common predictor of plant traits, followed by organic content, water content and elevation. Across species, regressions of single plant trait CVs on range ( $2 \times \text{SD}$ ) of single environmental variables were not significant and did not support the hypothesis that species occupying broad environmental ranges would have broad ranges in phenotypes. However, regression of a composite phenotypic PCA1 on a composite environmental PCA1 showed a marginally significant ( $P = 0.054$ ) linear relationship for 10 species. Considering the different patterns of response across species, the lack of a relationship between variation in single phenotypic traits and single environmental variables is likely because the distantly-related taxa employed fundamentally different morphological and physiological strategies to respond to environmental stress gradients. The significant relationship between composite environmental and phenotypic variables reflects the complex nature of species phenotypic response to multivariate environmental gradients. Specifically, in this system, species increase variation in the number of leaves, but decrease variation in leaf size in response to an increase in range of salinity and decrease in range of water and organic content.

## Introduction

Individuals within a species typically differ in phenotype. Although some of this variation may be random, ecological theory posits that a large proportion of this variation may represent adaptive matching of phenotypes to a variable environment (Clausen *et al.* 1948). This matching can occur either through natural selection producing genetically-differentiated ecotypes, or through phenotypic plasticity, in which different morphologies are produced from the same genotypes in different environments (Sultan 1995). Regardless of the mechanism, a number of studies have demonstrated that environmentally-mediated variation in phenotype within a species can be adaptive (Reznick and Travis 1996, Briggs and Walters 1997).

If there is a relationship between environmental variation and phenotypic variation within species, it is reasonable to hypothesize that species that occupy a wider range of habitats (habitat generalists) will be more variable in phenotype than species that occupy a narrow range of habitats (habitat specialists) (Van Valen 1965, Baker 1974, Sultan 2001). Only a few tests of this hypothesis exist, and most have involved relatively few taxa and/or focused on closely related species (Rothstein 1973, Sultan *et al.* 1998, 2001, but see Van Buskirk 2002).

Here, we investigate the relationship between environmental and phenotypic variation in coastal salt marsh plants. Coastal salt marshes are ideal systems for examining this relationship because they contain severe environmental gradients that have been correlated to variation in plant phenotype (Valiela *et al.* 1978, Seliskar 1985a, 1985b, 1987, Bertness and Ellison 1987). While some salt marsh plant species occur across a broad range of environmental variables, others are more restricted in distribution (Bertness *et al.* 1992, Gough and Grace 1998, Sanchez *et al.* 1998, Pan *et al.* 1998, Rand 2000). Previous studies of intraspecific variation in salt marsh plant phenotypes have primarily focused on differences between extreme height forms,

especially for the grass *Spartina alterniflora* (Anderson and Treshow 1980, Gallagher *et al.* 1988, Trnka and Zedler 2000), or differences between isolated populations (Silander 1979, 1984, 1985, Silander and Antonovics 1979, Hester *et al.* 1996, Hamilton 1997). However, none of these studies investigated whether habitat breadth corresponded to phenotypic variability across a larger pool of species. Although this idea could be tested on many spatial scales ranging from the local to the geographic, we address it at the local scale of cross-marsh gradients, because local environmental gradients in salt marshes are so strong.

This study documents the extent of aboveground phenotypic variation for the twelve most common plant species that occur in Georgia salt marshes, and correlates the observed phenotypic variation with several environmental variables. We test the hypotheses that 1) within species, plant traits correlate with environmental variables, and 2) species with wider environmental ranges have more variable phenotypes.

## Methods

### *Study sites and species*

We studied twelve plant species that are common in southeastern USA salt marshes and represent six families (Asteraceae: *Aster tenuifolius* L., *Borrchia frutescens* L., *Iva frutescens* L.; Bataceae: *Batis maritima* L.; Chenopodiaceae: *Salicornia bigelovii* Torrey, *Salicornia virginica* L.; Juncaceae: *Juncus roemerianus* Scheele; Plumbaginaceae: *Limonium carolinianum* (Walt.) Britt.; Poaceae: *Distichlis spicata* (L.) Greene, *Spartina alterniflora* Loisel., *Spartina patens* (Aiton) Muhl., *Sporobolus virginicus* (L.) Kunth; all nomenclature follows Radford *et al.* 1968). We worked at seventeen sites on Sapelo Island, Georgia, USA (31° 28'N, 81° 14'W). The vegetation patterns in Sapelo Island marshes are typical of southeastern marshes in the

United States (Pomeroy and Wiegert 1981). Lower elevations of the marsh are subject to daily tidal submergence and are dominated by *Spartina alterniflora*. The higher elevations of the marsh are flooded irregularly and are often characterized by highly saline salt pans and associated salt-tolerant species such as *Salicornia virginica*, *Salicornia bigelovii*, *Batis maritima*, *Borrchia frutescens*, *Distichlis spicata* and *Sporobolus virginicus* (Antlfinger 1981). The terrestrial border of the marsh is typically dominated by *Juncus roemerianus*, *Spartina patens* or *Iva frutescens*. *Aster tenuifolius* and *Limonium carolinianum* occur at higher elevations mixed in with the zonal dominants. The details of the plant zonation patterns vary from site to site, and not every species occurs at every marsh site. The twelve species that we studied represent the vast majority of the species and the plant biomass present at all of our sites.

#### *Phenotypic and environmental sampling*

We sampled plant traits between 1 July and 16 August, 1999 ( $N = 1057$  plants). Within this time frame, each species was sampled after it had flowered and completed the majority of its vegetative growth across the marsh environmental gradients. Each species was sampled along one transect at each of eight or nine sites. Because the species composition of each site varied, the number of species sampled per site ranged from one to twelve. Individual transects ran from the upper to the lower elevational range of the target species at each site. Ten plants were selected along each transect using a stratified-random sampling scheme to ensure representation of the full extent of environmental breadth. Due to the broad horizontal and elevation range of *Spartina alterniflora*, we collected data on twenty individuals at each of the eight sites for this species.

Traits measured for each species included plant height, number of leaves, length, width and thickness of three fully emerged leaves, length of the third internode, and other traits as appropriate for the growth form of each plant species (Table 2.1). Height in the four grasses and most of the forbs and small shrubs was measured from the ground to the uppermost leaf node on the stem. The exceptions include *Iva* for which height was measured to the top of the canopy, the *Salicornia* species for which height was measured to the top of the uppermost appressed leaf, and *Juncus* for which height was measured to the end of each needle-like leaf. For flowering individuals of *Borrchia* and *Aster*, height was measured to the uppermost flower head. Leaf size was estimated as maximum length \* maximum width in all plants with the exception of the succulent *Batis* for which leaf volume was estimated by leaf size \* maximum thickness. Stem diameter was measured at the base of the stem with calipers. Base width was measured below the basal rosette (*Limonium*). Leaf serration was scored (*Iva*) on a scale of one to four with one indicating smooth leaves and four indicating highly serrated leaves.

After all plants were tagged and traits measured, we collected a soil sample adjacent to each plant within a three day period (21-23 August 1999). Soil was dried (60° C to constant weight) to determine relative water content, rehydrated in a known volume of distilled water to determine original pore water salinity (Pennings and Richards 1998), and ashed (550° C for 12 hours) to determine organic content. We surveyed the soil elevation at the base of each tagged plant to the nearest millimeter. Elevation was converted to a relative index for each species at each site by setting the lowest value for the species to 0 and the highest to 1. We chose these four environmental variables because 1) salinity is very important, 2) water content of soil, given the broad range of values obtained here, is correlated with redox and oxygen availability, which are much harder to measure, and 3) organic content should correlate with nutrient availability

and 4) elevation represents a combination of environmental factors. In addition, it was logistically feasible to sample these four environmental variables for large number of plants in a short time period that minimized temporal variation.

### *Statistical analysis*

*Intraspecific relationships.* Plant traits were regressed against environmental variables using simple linear and multiple regressions in SAS (SAS 2000). To simplify cross species comparisons, we report here the results for the three most-commonly measured traits (plant height, leaf size and number of leaves) and a composite variable (the first principal component axis obtained from a PC ordination of all of the traits measured for each species). Height and leaf number for all species were natural log transformed and leaf size was untransformed for most species with the exception of *Spartina alterniflora* and *Limonium* for which leaf size was Box Cox transformed (JMP 1999) to meet the assumptions of normality and homoscedasticity. Soil proportion water content and ash content were arcsine-square root transformed and relative elevation was Box Cox transformed (JMP 1999) to meet regression assumptions. This approach uses some of the data in more than one statistical test for each species. Given that the number of multiple tests is small, we did not adjust P-values.

We ran full multiple regression models with the four environmental variables for each dependent variable. After removing environmental variables that were not at least marginally significant ( $\alpha > 0.10$ ), we ran the models again to determine if marginally significant variables became significant in reduced models. Final reduced models include only those variables significant at the  $\alpha < 0.05$  level. For each species, we ran a separate principal components analysis (PCA) which included all of the traits measured for that species (Table 2.1) using proc

princomp in SAS (SAS 2000). All traits entered into the analysis were left unstandardized. The PCA1 accounted for between 34- 68% of the variation in each of the species (Table 2.1). PCA1 was consistently evenly loaded across three to five plant traits and no species showed a strong correlation between PCA1 and any one trait.

*Interspecific relationships.* To quantify the extent of phenotypic variation, we calculated the coefficient of variation, ( $CV = \text{standard deviation} / \text{mean}$ ). We used the CV of plant traits to account for the fact that, as is typical of many ecological data sets, the variance of traits rose with their mean. Thus, the variance in most traits of the larger plant species was greater than the variance of the smaller species. In this case, calculating the CV provides a measure of variation that is less affected by the mean. To quantify the range of each environmental variable, we calculated 2\* the standard deviation of that variable (using the CV of environmental variables in analyses did not alter our conclusions). To examine the relationship between phenotypic variation and habitat breadth among species ( $N = 12$ ), we regressed the CVs of height and leaf size on the range of each environmental variable (elevation was not used in these analyses because it was a relative index standardized to one). We also examined the relationship between a composite phenotypic variation variable (PCA1 of the three phenotypic CV values in Table 2.2) and a composite environmental variation variable (PCA1 of the three environmental ranges in Table 2.2) for the 10 species for which all of these variables were measured.

## **Results**

### *Extent of phenotypic and environmental variation*

Plant species differed considerably in phenotypic variability (Table 2.2). The coefficient of variation of plant height ranged from 0.24 (*Juncus*) to 0.64 (*Salicornia virginica*). The CV of

leaf size ranged from 0.22 (*Salicornia bigelovii*) to 0.82 (*Limonium*). There was a much broader range in the CV of the number of leaves, which ranged from 0.28 (*Spartina patens*) to 1.92 (*Salicornia bigelovii*).

Similarly, the range of environmental variables differed considerably among plant species (Table 2.2). The range (2\*SD) of salinity varied 4-fold among species, from 27.7 (*Iva*) to 120.8 (*Salicornia bigelovii*). The range of water content varied 3-fold, from 0.10 (*Salicornia bigelovii*) to 0.32 (*Juncus*). The range of organic content varied 3-fold, from 0.05 (*Limonium*) to 0.15 (*Iva*).

#### *Intraspecific relationships: environmental variation and plant phenotype*

For each plant species, variation in plant phenotype was correlated with variation in the environment, and over  $\frac{3}{4}$  of the relationships between height, leaf size or leaf number and environmental traits were significant (Table 2.3). There was variation among traits and species in the combination of environmental variables that predicted plant traits. However, salinity was the most common predictor variable, followed by organic content, water content and elevation. When multiple environmental variables were significant predictors of plant traits, salinity usually entered first into the step-wise regression model. As expected, the relationship between plant traits and 1) salinity was almost always (28/30) negative, 2) organic content was almost always (20/22) positive, 3) water content was usually (10/14) negative, and 4) elevation was usually (7/10) positive.

In all but three species, height correlated with environmental variables more strongly than did any other trait or PCA1. The model for leaf size showed the best fit for *Salicornia bigelovii* and the model for leaf number showed the best fit for *Limonium*. Surprisingly, the model for the

composite variable PCA1 showed the best fit in only one case (*Borrchia*), and in this case the  $R^2$  for PCA1 (0.24) was only slightly greater than the  $R^2$  for leaf size (0.22).

*Interspecific relationships: phenotypic variation and habitat breadth*

Linear regressions of plant trait CVs on range ( $2 \times SD$ ) of environmental variables were not significant (Fig. 1) and did not support the hypothesis that species with wider environmental ranges would have more variable phenotypes. However, quadratic regressions revealed that the relationship between CV height and range of salinity was significant ( $P = 0.006$ ,  $R^2 = 0.65$ ).

This relationship indicated that species inhabiting intermediate ranges of salinity exhibited the most variation in height whereas species inhabiting extremely small or extremely large ranges in salinity had less variation in height. Other quadratic regressions of plant trait CVs on range of environmental variables were not significant.

The PCA1 of the principal component analysis on the three phenotypic CVs accounted for 52.9% of the variation in the three traits with high loadings on leaf size (+) and leaf number (-). The PCA1 of the principal component analysis on the three environmental ranges accounted for 78.6% of the variation in the three variables with high loadings on all three variables: salinity (-), water content (+) and organic content (+). Regression of the composite phenotypic PCA1 on the composite environmental PCA1 yielded a marginally significant ( $P = 0.054$ ) negative relationship (Fig. 2).

## **Discussion**

Salt marshes contain steep environmental gradients: conditions are fairly mild near the terrestrial border of the marsh but become so severe in salt pans and extremely waterlogged areas

that even the most highly-adapted salt marsh plants cannot survive (Pennings and Bertness 2001). Across these strong environmental gradients, phenotypic variation of plants was correlated with environmental variables, as predicted by our first hypothesis. In contrast, our second hypothesis was not supported by linear comparisons of single plant traits with single environmental variables. We found instead that variation in height was maximized in species with intermediate ranges of salinity. In addition, a composite, complex phenotypic response (phenotypic PCA1) appeared to be related to a composite, complex environmental variable (environmental PCA1). This relationship suggests that species increase variation in the number of leaves, but decrease variation in leaf size in response to an increase in range of salinity and a decrease in range of water and organic content. Thus, there is a relationship between environmental and phenotypic variation, as we hypothesized, but the nature of this relationship is quite complex.

*Intraspecific relationships: environmental variation and plant phenotype*

All twelve of the salt marsh plant species that we studied displayed substantial variation in phenotype. Most of the previous attention paid to phenotypic variation in salt marsh plants has focused on intraspecific variation in *Spartina alterniflora*, the most abundant and widespread salt marsh plant on the Atlantic Coast of the United States (Valiela *et al.* 1978, Pomeroy and Wiegert 1981), although intraspecific variation has also been documented in some other salt marsh plant species (Antlfinger 1981, Seliskar 1985a, 1985b, 1987). Here, we show that intraspecific phenotypic variation is a general phenomenon of 12 common southeastern USA salt marsh plants. Given the strong environmental gradients present in salt marsh habitats, marked intraspecific variation in height and other phenotypic traits is probably the rule for all species of

salt marsh plants. For the few salt marsh plant species that have been studied, phenotypic variation is due largely to phenotypic plasticity (Valiela *et al.* 1978, Anderson and Treshow 1980, Antlfinger 1981, Seliskar 1985b, Richards *et al.* in prep.), although genetic differentiation can also play an important role (Antlfinger 1981, Silander 1985, Gallagher *et al.* 1988, Proffitt *et al.* 2003). Further studies are required to determine the relative contributions of plasticity and genetic differentiation to determining phenotypic variation in the particular species that we studied.

The majority of the relationships between plant traits and environmental variables were significant, indicating that variation in plant phenotype is predictable and correlated with environmental variation. For several plant species, some combination of the four environmental variables explained > 45 % of the variation in one or more of the traits (Table 2.3: *Aster*, *Distichlis*, *Juncus*, *Salicornia virginica*, *Spartina alterniflora*). In the worst case, environmental variables explained only 16 % of the variation in height of *Iva*, and did not predict *Iva* leaf size. However, we sampled environmental traits on only one date, and it is likely that an average of environment across seasons and tidal conditions would have explained more of the phenotypic variation in these species. Alternatively, phenotypic variation in these species may be better explained by other environmental variables that we did not measure.

The negative relationships that we observed between plant traits and salinity and waterlogging are consistent with the known physiological costs imposed on plants by these variables (Ponnampereuma 1972, Flowers 1977, Mendelssohn and Morris 2000). The high frequency with which organic content entered into the regressions suggests that, despite strong stress gradients in salt marshes, plant productivity may also be mediated by soil quality. Soils in these marshes had low organic content (averaging < 8 % for the species with positive

relationships with organic content), and under these conditions organic content may reflect the availability of a wide variety of nutrients (Lindau and Hossner 1981, Craft *et al.* 1991, Padgett and Brown 1999). Although salinity, waterlogging and organic content all vary across elevation, elevation *per se* was rarely a significant predictor of plant traits, likely because the stresses imposed on plants by these variables may interact in complex ways across the elevation gradient (Mendelsohn and Morris 2000, Proffitt *et al.* 2003).

Because many studies have argued that fitness is not related to any one single trait, but rather to a suite of traits and their interactions (Clausen, *et al.* 1948, Lechowicz 1984, Chapin *et al.* 1993), we expected that PCA1 would offer a composite “plant phenotype” that might give the best overall indication of plant response to environmental variables. However, we found that PCA1 correlated with environmental variables better than single plant traits for only one species (*Borrichia*). In most cases, plant height correlated with environmental variables better than any other plant trait or PCA1. Because height correlates with biomass for plants in general, and many of these species in particular (Bertness and Ellison 1987, Pennings and Callaway 2000), these results suggest plant fitness may also vary across salt marsh environmental gradients. Because most of these species are clonal perennials, however, documenting this variation in fitness will be a challenging task.

#### *Interspecific relationships: phenotypic variation and habitat breadth*

Ecologists have long speculated that species with wider environmental ranges would have broader ranges in phenotype (Van Valen 1965, Baker 1974, Sultan 2001). Most of the tests of this hypothesis, however, have involved small numbers of species and/or closely related taxa (Rothstein 1973, Sultan *et al.* 1998, Sultan 2001). In one study that did examine variation across

taxa, Van Buskirk (2002) found that frog species with the widest habitat ranges showed the largest morphological responses to predator variation. In contrast, we found that for plants from 6 families, linear regressions of plant trait CVs on ranges in soil variables (2\*standard deviations) were not significant in any case ( $P > 0.05$ , Fig. 1). Thus our data do not support the hypothesis that species occupying broad environmental ranges will have a linear response in breadth of phenotypes. We did, however, find a significant quadratic relationship between height CV and range of salinity suggesting that species that inhabit areas with intermediate ranges of salinity exhibit the most variation in height whereas species inhabiting extremely small or extremely large ranges of salinity have less variation in height. This trend is consistent with environmental canalization (Debat and David 2001, Wagner *et al.* 1997) for an optimum height in species that inhabit the most extreme range in salinities which is relaxed in species that inhabit intermediate ranges of salinities. However, the quadratic nature of this relationship is mostly influenced by the data for *Salicornia bigelovii* which inhabits the broadest range of salinity (2 SD = 120.8 ppt) and has very little phenotypic response to the salinity gradient (Table 2.3).

The lack of a linear relationship between phenotypic and environmental range in our study is likely due to distantly-related taxa responding to environmental challenges in different ways. For example, there are several different physiological and morphological solutions to the problem of coping with high salt environments (Hasegawa *et al.* 2000, Flowers *et al.* 1977). In contrast, closely-related taxa are likely to use the same mechanisms to respond to similar challenges. Thus, studies of closely-related taxa are more likely to observe a positive correlation between phenotypic and environmental range (Rothstein 1973, Sultan 1998, 2001, but see Van Buskirk 2002). Alternatively, it may be that, environmental gradients in the field, and the responses of organisms to these gradients, are inherently complex and multivariate. For this

group of species, we found that a composite, complex phenotypic response (phenotypic PCA1) explained 53% of the variance in the height, number and size of leaves. This composite variable was not significantly correlated with variation in height, but rather represented an increase in variation in the number of leaves and a corresponding decrease in variation in leaf size. The composite, complex environmental variable represented an increase in range of salinity inhabited with a corresponding decrease in range of water and organic content. The regression of these two composite variables suggests that the relationship between phenotypic variation and environmental variation is not simply linear. Instead, it appears that species increase variation in the number of leaves, but decrease variation in leaf size, in response to an increase in range of salinity and decrease in range of water and organic content.

Our results come with two caveats. First, our study was observational rather than experimental. It is possible that biotic interactions or other factors could have obscured additional relationships between phenotypic and environmental variation that might have been revealed by an experimental manipulation. In particular, some of the plant species are competitively excluded from habitats that they are physiologically capable of inhabiting (Pennings and Bertness 2001), thereby limiting the range of environmental variation that we observed. Second, given the modest number of species studied (12), the results of our species-level regressions may be influenced by single points. In particular, the linear relationship between the CV of height and the range of salinity (Fig. 1 top left) would be significant ( $R^2 = 0.48$ ,  $P = 0.03$ ) if one data point (*Salicornia bigelovii*) were removed.

In sum, we found that variability in phenotypes of all twelve salt marsh plants was correlated with variation in the physical environment; however, linear, univariate relationships between the range of environments occupied by a species and the range of variation in phenotype

did not occur. Rather, the relationship between variation in phenotype and variation in the environment was non-linear and/or multivariate in nature. We conclude that linear relationships between environmental and phenotypic variation are most likely to be found when comparing closely-related taxa and simple gradients than when comparing a broad range of taxa across complex gradients in the field. In the field, environmental gradients, and the responses of organisms to these gradients, are likely to be complex and multivariate.

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Table 2.1. Traits measured for each plant species. For each species, all traits indicated were used in a Principal Components Analysis (PCA). PCA1 indicates the amount of variation in all traits explained by the first principal component axis. Ht = height, LV = leaf volume, PD = plant depth, PW = plant width, LS = leaf serration, BD = base diameter, BP = number of primary branches, BS = number of secondary branches.

|                              | Traits measured |             |           |             |            |                |               |                  |                  | PCA1 |
|------------------------------|-----------------|-------------|-----------|-------------|------------|----------------|---------------|------------------|------------------|------|
|                              | Ht              | Leaf number | Leaf size | Leaf length | Leaf width | Leaf thickness | Stem diameter | Internode length | Other            |      |
| <i>Aster tenuifolius</i>     | X               | X           | X         | X           | X          |                | X             | X                |                  | 0.54 |
| <i>Batis maritima</i>        | X               | X           |           | X           | X          | X              |               |                  | LV               | 0.52 |
| <i>Borrichia frutescens</i>  | X               | X           | X         | X           | X          | X              |               |                  |                  | 0.40 |
| <i>Distichlis spicata</i>    | X               | X           | X         | X           | X          |                | X             | X                |                  | 0.47 |
| <i>Iva frutescens</i>        | X               |             | X         | X           | X          | X              |               |                  | PD, PW, LS (1-4) | 0.34 |
| <i>Juncus roemerianus</i>    | X               | X           | X         | X           | X          |                |               |                  |                  | 0.63 |
| <i>Limonium carolinianum</i> |                 | X           | X         | X           | X          |                | X             |                  | BD               | 0.68 |
| <i>Salicornia biglovii</i>   | X               | X           | X         | X           | X          |                | X             |                  | PW, BP, BS       | 0.49 |
| <i>Salicornia virginica</i>  | X               | X           | X         | X           | X          |                | X             |                  | BP, BS           | 0.64 |
| <i>Spartina alterniflora</i> | X               | X           | X         | X           | X          |                | X             | X                |                  | 0.62 |
| <i>Spartina patens</i>       | X               | X           | X         | X           | X          |                | X             | X                |                  | 0.58 |
| <i>Sporobolus virginicus</i> | X               | X           | X         | X           | X          |                | X             | X                |                  | 0.45 |

Table 2.2. Variation in 3 plant traits (CV) and 3 environmental variables (2\*SD) for the 12 plant species. NM = this variable was not measured for this species.

| <b>Species</b>               | <b>Height (CV)</b> | <b>Average leaf size (CV)</b> | <b>Number of leaves (CV)</b> | <b>Phenotypic PCA1</b> | <b>Salinity mean (2*SD) ppt</b> | <b>Proportion soil water content mean (2*SD) g/g</b> | <b>Proportion soil organic content mean (2*SD) g/g</b> | <b>Environmental PCA1</b> |
|------------------------------|--------------------|-------------------------------|------------------------------|------------------------|---------------------------------|--|--|---------------------------|
| <i>Aster tenuifolius</i>     | 0.53               | 0.80                          | 0.52                         | -1.15111               | 43.8 (44.9)                     | 0.26 (0.22)  | 0.04 (0.06)  | -0.2061                   |
| <i>Batis maritima</i>        | 0.53               | 0.23                          | 1.29                         | 1.638959               | 67.1 (68.2)                     | 0.27 (0.18)  | 0.05 (0.08)  | -0.67694                  |
| <i>Borrichia frutescens</i>  | 0.46               | 0.50                          | 0.86                         | 0.029147               | 54.6 (54.3)                     | 0.24 (0.17)  | 0.05 (0.08)  | -0.49835                  |
| <i>Distichlis spicata</i>    | 0.35               | 0.40                          | 0.39                         | -0.50632               | 64.6 (68.4)                     | 0.32 (0.26)  | 0.08 (0.13)  | 1.15901                   |
| <i>Iva frutescens</i>        | 0.29               | 0.36                          | NM                           |                        | 39.4 (27.7)                     | 0.24 (0.22)  | 0.08 (0.15)  |                           |
| <i>Juncus roemerianus</i>    | 0.24               | 0.46                          | 0.40                         | -0.97409               | 43.8 (28.7)                     | 0.36 (0.32)  | 0.08 (0.13)  | 2.574508                  |
| <i>Limonium carolinianum</i> | NM                 | 0.82                          | 0.33                         |                        | 44.9 (51.0)                     | 0.23 (0.19)  | 0.03 (0.05)  |                           |
| <i>Salicornia bigelovii</i>  | 0.33               | 0.22                          | 1.92                         | 1.854019               | 106.8 (120.8)                   | 0.20 (0.10)  | 0.03 (0.07)  | -2.76196                  |
| <i>Salicornia virginica</i>  | 0.64               | 0.52                          | 1.78                         | 1.555112               | 65.2 (72.8)                     | 0.30 (0.23)  | 0.07 (0.11)  | 0.354563                  |
| <i>Spartina alterniflora</i> | 0.47               | 0.56                          | 0.32                         | -0.77625               | 51.7 (48.3)                     | 0.59 (0.24)  | 0.15 (0.09)  | 0.542943                  |
| <i>Spartina patens</i>       | 0.29               | 0.65                          | 0.28                         | -1.61312               | 32.8 (39.7)                     | 0.28 (0.23)  | 0.08 (0.12)  | 1.227726                  |
| <i>Sporobolus virginicus</i> | 0.48               | 0.40                          | 0.47                         | -0.05635               | 58.3 (94.3)                     | 0.23 (0.15)  | 0.04 (0.07)  | -1.71539                  |

Table 2.3. Multiple regression models for 3 phenotypic traits and PCA1 for the 12 plant species. Environmental variables are listed in the order in which they loaded into a stepwise regression. The sign of the coefficient for each environmental variable is indicated.

The plant trait with the best fit for each species is indicated in bold. \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ . S = salinity, H = water content, O = organic content, E = elevation, NS = not significant, NM = this variable not measured for this species.

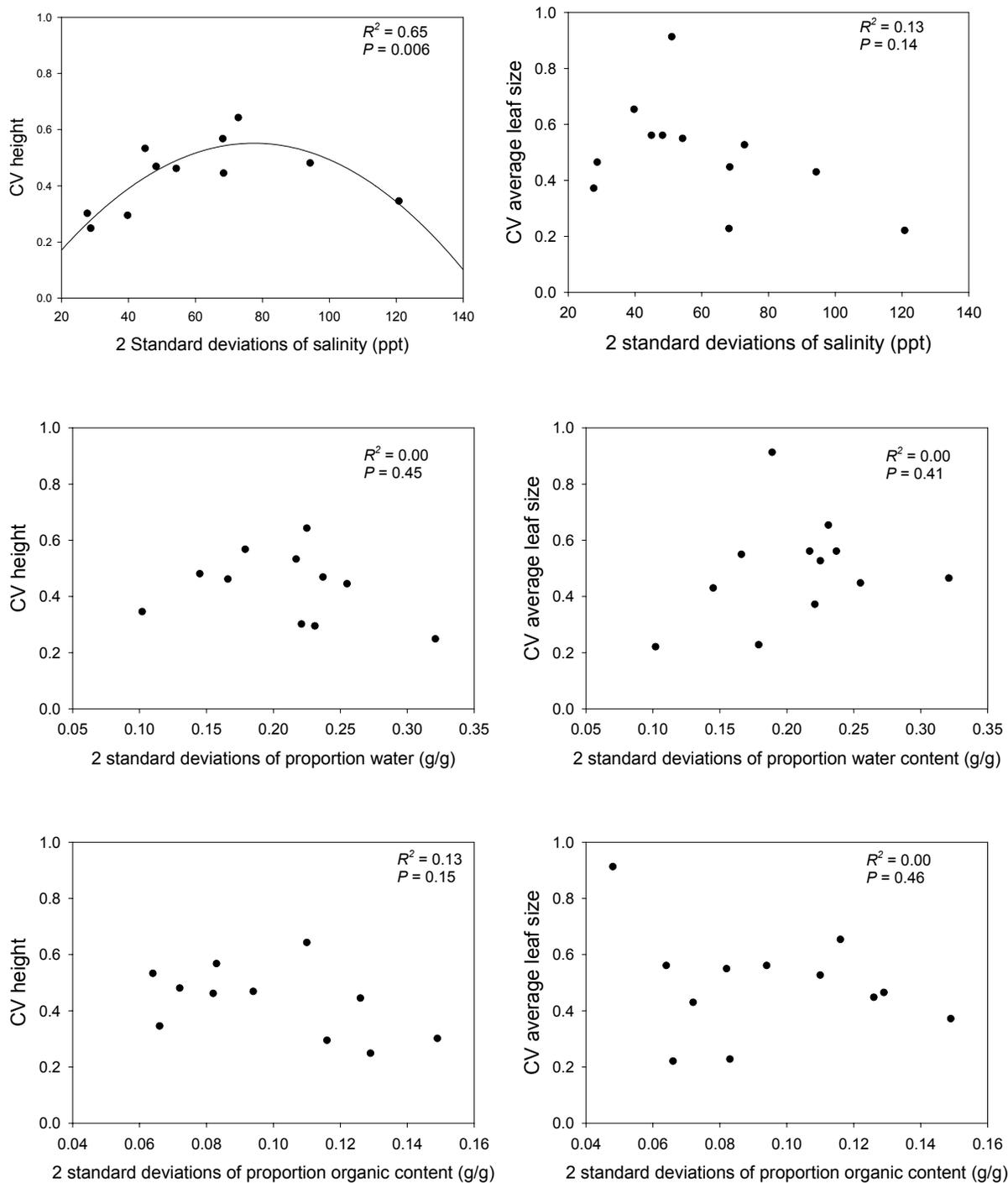
| Species                      | Ln height      |                    | Leaf area      |             | Ln leaf number |              | PCA 1          |              |
|------------------------------|----------------|--------------------|----------------|-------------|----------------|--------------|----------------|--------------|
|                              | $R^2$          | predictors         | $R^2$          | predictors  | $R^2$          | predictors   | $R^2$          | predictors   |
| <i>Aster tenuifolius</i>     | <b>0.49***</b> | <b>+O,-H,-S,-E</b> | NS             |             | 0.16***        | +O           | 0.22**         | -S,-H,+O     |
| <i>Batis maritima</i>        | <b>0.26***</b> | <b>-S</b>          | NS             |             | 0.17**         | -S           | NS             |              |
| <i>Borrchia frutescens</i>   | 0.20***        | -S,+O              | 0.22***        | -S,+E       | 0.19***        | -S,+O,-H     | <b>0.24***</b> | <b>-S,+E</b> |
| <i>Distichlis spicata</i>    | <b>0.65***</b> | <b>+H,-S</b>       | 0.32***        | +O,-H,-S    | 0.18***        | +O           | 0.47***        | +O           |
| <i>Iva frutescens</i>        | <b>0.17**</b>  | <b>+E</b>          | NS             |             | NM             |              | NS             |              |
| <i>Juncus roemerianus</i>    | <b>0.50***</b> | <b>-S</b>          | 0.45***        | +H,-S,+E,-O | NS             |              | 0.48***        | -S,+H,+E,-O  |
| <i>Limonium carolinianum</i> | NM             |                    | 0.25***        | -S,+O,-H    | <b>0.30***</b> | <b>-S,+O</b> | 0.23***        | -S,+O        |
| <i>Salicornia biglovii</i>   | 0.10**         | +O                 | <b>0.33***</b> | <b>+O</b>   | 0.17**         | +O,+S        | 0.30***        | +O           |
| <i>Salicornia virginica</i>  | <b>0.71***</b> | <b>-S,+O,-H</b>    | 0.32***        | -S,-H       | 0.41***        | -S,+O,-H     | 0.57***        | -S,+O,-H     |
| <i>Spartina alterniflora</i> | <b>0.53***</b> | <b>-S,-E</b>       | 0.31***        | -S,+E       | 0.17***        | -S,-E        | 0.34***        | -S           |
| <i>Spartina patens</i>       | <b>0.35***</b> | <b>-S,+H</b>       | 0.31***        | -S,+E       | 0.11**         | +S           | 0.26***        | -S           |
| <i>Sporobolus virginicus</i> | <b>0.33***</b> | <b>-S,+O</b>       | NS             |             | 0.13*          | +O,-H        | 0.06*          |              |

### Figure Legends

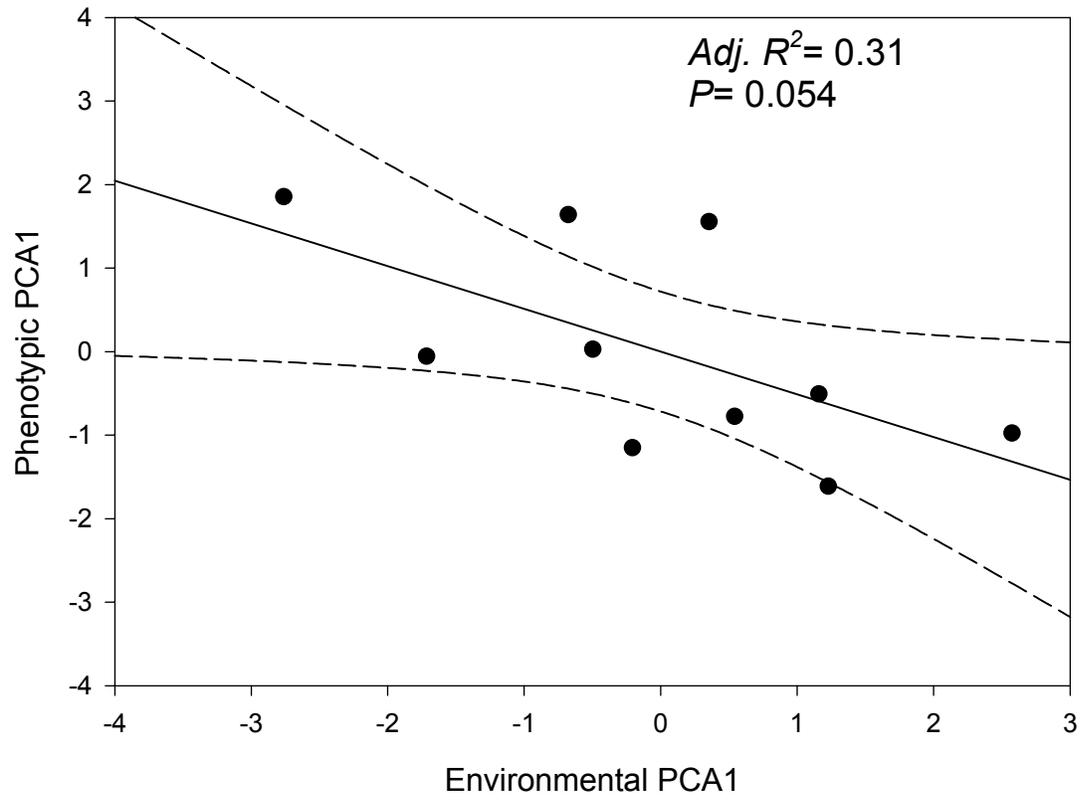
Figure 2.1. Relationship between CV of height or leaf size and range ( $2*SD$ ) of soil variables.

Each point represents a single plant species. Adjusted  $R^2$  and  $P$ -values are shown.

Figure 2.2. Relationship between phenotypic PCA1 (combining CV's for height, leaf size and number of leaves) and environmental PCA1 (combining  $2*standard$  deviation of soil salinity, proportion soil water content and proportion soil organic content). Data includes only those 10 species for which all three phenotypic traits were measured (excluding *Iva* and *Limonium*).



**Figure 2.1**



**Figure 2.2**

CHAPTER 3  
SEVERE ENVIRONMENTAL GRADIENT DETERMINES GENETIC STRUCTURE OF  
TWO SALT MARSH PERENNIALS<sup>1</sup>

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<sup>1</sup> Richards, C. L., L. A. Donovan, R. Mauricio and J. L. Hamrick. To be submitted to *Ecology*.

**Abstract**

Examining patterns of genetic structure across environmental gradients is a powerful tool in understanding how natural selection leads to local adaptation and subsequent genetic differentiation among plants living in different microhabitats. Current theory suggests that specific genotypes should cluster within microhabitats along environmental gradients, that this pattern should be stronger for plants that primarily reproduce clonally, and that genetic diversity should be lowest in habitats experiencing strong selection. We examined genetic diversity in two salt marsh plants across strong gradients in salinity. In contrast to current theory, there was no pattern of association between specific multilocus genotypes or alleles at any given locus with microhabitats along the gradient, and levels of diversity did not differ within microhabitats along the gradient. Much of the variation in the distribution of genetic diversity, however, was predictable based on the gradient. In addition, genetic diversity was surprisingly high and clone size was limited. These results suggest that current theory needs to be refined by considering that selection acts on ecologically important traits that are not necessarily represented by any one locus, but potentially overall patterns across loci, and indicate that sexual reproduction and recruitment from seeds are important factors maintaining diversity even in these highly stressful habitats.

**Key Words:** allozymes, *Borrchia frutescens*, clonal structure, environmental gradients, fine-scale differentiation, gene flow, population genetics, salt marsh plants, Sapelo Island, sea ox-eye daisy, smooth cord grass, *Spartina alterniflora*

## **Introduction**

Examining patterns of genetic structure across environmental gradients is a powerful tool in understanding how natural selection leads to local adaptation and subsequent genetic differentiation among plants living in different microhabitats. Variation between microhabitats can occur on a scale of centimeters and therefore has the potential to effect not only the distribution of species, but also the distribution of genetic variation within species. It is well established that even under high levels of gene flow, natural selection can produce genetically differentiated ecotypes within species on a fine spatial scale (Antonovics and Bradshaw 1970, Antlfinger 1981, Nagy and Rice 1997, Silander 1984, 1985). In addition to adaptive traits, studies often find an association between variation in environment and neutral genetic structure within species (Hamrick and Allard 1972, Hamrick and Holden 1979, Heywood 1991, Johnston, et al. 2001b, Salzman 1985, Schmidt and Rand 1999).

This association between microhabitat and adaptive as well as neutral variation is a direct result of natural selection eliminating variants that are less well fit and thereby reducing genetic variation. However, most species harbor a substantial amount of genetic diversity. Theoretical and empirical studies illustrate that genetic diversity can be maintained in populations in the face of natural selection by the maintenance of variants that survive different selection pressures in different microhabitats (Caisse and Antonovics 1978, Feder et al. 1997, Hedrick 1976, Levene 1953, Schmidt and Rand 1999). Considering the full distribution of genetic diversity of a species across habitats, we should expect not only genetic differences among subpopulations inhabiting contrasting microhabitats, but a reduction in diversity in more stressful microhabitats where plants experience more intense selection (Zangerl and Bazzaz 1984a and 1984b).

Although there is extensive evidence for the adaptation of specific genotypes to the local environment, clonal reproduction can complicate the predicted relationship between genotype and environment since genets can share resources across rhizomes and support ramets experiencing physical stress, competition or herbivory (Jonsdottir and Watson 1997, Pennings and Callaway 2000, Steuffer et al. 1996). Clonal plants also have the ability to forage for favorable habitats (Bazzaz 1991, Macek and Leps 2003). The ability to discriminate between patches and proliferate in favorable habitat has been found to vary by genotype suggesting the potential for evolution of foraging behavior (Bazzaz 1991, Salzman 1985). Studies have also found that populations of clonal species growing in contrasting habitats vary in growth rates (Karagatzides and Hutchinson 1991), biomass allocation patterns (Lovett Doust 1981) and allocation to sexual reproduction (Sun 2001).

The salt marsh is an ideal system to evaluate environmental sorting of genotypes as well as the effect of clonality on environmental sorting of genotypes because the majority of salt marsh plant species are clonal and occupy habitats that vary widely in abiotic and biotic conditions. Southeastern U. S. salt marsh plants regularly experience hypersaline, anaerobic, nutrient poor conditions, strong competition from neighboring plants and disturbance from wrack deposition (Mendelssohn and Morris 2000, Pennings and Bertness 2001). Despite a wealth of studies examining how these environmental gradients affect community level patterns in salt marshes (Pennings and Bertness 2001), little research has addressed how these gradients have shaped the genetic make-up of salt marsh plant species. We chose to investigate these issues in two contrasting dominant salt marsh perennials *Borrchia frutescens* (L) DC. (Asteraceae: sea oxeye daisy) and *Spartina alterniflora* Loisel. (Poaceae: smooth cord grass). Both species live across a broad range of environmental conditions. *Borrchia frutescens* is a C3 composite that

inhabits a spatially narrow range (a typical stand of *B. frutescens* covers 100-500 m<sup>2</sup> area) in the highly saline, high marsh areas. *Spartina alterniflora* is a C4 grass that lives in vast expanses across the middle and lower elevations of the marsh (at a given site, *S. alterniflora* covers a continuous area of 2,000-50,000 m<sup>2</sup>). Despite these differences, differential selection pressures of the salt marsh habitat should act to increase genetic structure on a fine spatial scale among microhabitats for both species. In addition, clonal reproduction is extensive for these two species, and rates of seedling emergence and establishment in the field are thought to be low in mature stands (Stiling 1994, Stiling and Rossi 1995, *Spartina* ref). Given enough time and extensive clonal growth, populations should be dominated by a few large, locally adapted, clones associated with a specific microhabitat type.

The objective of this study was to investigate how the environmental gradients of the salt marsh determine fine-scale patterns of genetic differentiation and clonal structure of two salt marsh perennials *B. frutescens* and *S. alterniflora*. We asked three major questions. First, does microhabitat type significantly explain the distribution of genetic variation for these species and do genetically similar individuals cluster within microhabitat type? Second, is genetic diversity lowest in the higher salinity microhabitats of the marsh? Third, do a few large clones dominate these sites and are clones associated with specific microhabitat type?

## **Methods**

### *Study Site and Study species*

The study was conducted on Sapelo Island, Georgia, USA (31° 28'N, 81° 14'W). Vegetation patterns in Sapelo Island marshes are typical of southeastern marshes in the United States

(Pomeroy & Wiegert 1981). Lower elevations of the marsh are subject to daily tidal submergence and are dominated by *S. alterniflora*. The middle elevations of the marsh are flooded irregularly and are often characterized by highly saline salt pans and associated salt-tolerant species such as *B. frutescens*, *Salicornia bigelovii* Torrey, *Salicornia virginica* L., *Batis maritima* L., *Distichlis spicata* (L.) Greene and *Sporobolus virginicus* (L.) Kunth (Antlfinger 1981). The terrestrial border of the marsh is typically dominated by *Juncus roemerianus* Scheele, *Spartina patens* (Aiton) Muhl. or *Iva frutescens* L. *Aster tenuifolius* L. and *Limonium carolinianum* (Walt.) Britt. occur at higher elevations mixed in with the zonal dominants (all nomenclature follows Radford et al. 1968). Details of the plant zonation patterns vary from site to site, and not every species occurs at every marsh site (Richards et al. in review).

*Borrchia frutescens* and *S. alterniflora* occur across a broad range of environments (for example, salinities range from 20 ppt to over 100 ppt, Richards et al. in review). Both species exhibit extreme phenotypic variation (heights from over 100 cm to less than 10 cm in *B. frutescens* and from over 200 cm to less than 20 cm in *S. alterniflora*, Richards et al. in review). Edaphic factors alone, significantly predict 20% and over 50% of the variation in height in natural populations of *B. frutescens* and *S. alterniflora* respectively (Richards et al. in review). For both species, salinity was the environmental factor most closely (negatively) correlated with change in height.

*Borrchia frutescens* is a C<sub>3</sub> composite and is abundant in the high marsh, which is marked by infrequent tidal flooding (Antlfinger 1981). This species tolerates high substrate salinities by increasing succulence, actively taking up sodium and other ions as well as manufacturing the nitrogen rich compatible solutes proline and glycine-betaine (Cavalieri and Huang 1979, Antlfinger and Dunn 1983, Moon and Stiling 2000). Although this native plant

occurs in salt marshes from Virginia south to Northern Florida and along the Gulf of Mexico coast (Duncan and Duncan 1987), the distribution is naturally patchy because highly saline areas of salt marsh occur on barrier islands or in isolated mainland salt marshes. Additional fragmentation occurs both by natural disturbances such as sediment movement and anthropogenic disturbances such as coastal housing developments and the formation of “spoil islands” (Antlfinger 1981, Rossi and Stiling 1998). Therefore, its naturally patchy distribution should limit gene flow and increase genetic structure between sites.

*Spartina alterniflora* is a C4 grass and is the dominant salt marsh plant of the Atlantic and Gulf coasts of North America. Like *B. frutescens*, *S. alterniflora* tolerates high substrate salinities by manufacturing the nitrogen rich compatible solutes proline and glycine-betaine (Cavaliere and Huang 1983). This species also compensates for an increase in salinity by closing stomata and thereby decreasing CO<sub>2</sub> assimilation (Mendelssohn and Morris 2000).

### *Sampling design*

We collected leaf tissue of *B. frutescens* and *S. alterniflora* in five marsh sites located 1.5-5 km apart around the island. At each site, we collected live leaf tissue from 96 individuals spanning the environmental gradient (from tall to short plants). For *B. frutescens*, within each of three microhabitats we collected tissue from approximately 32 plants at every one meter mark on grids of appropriate size at each site. Within each site, microhabitats were designated 1) adjacent to the salt pans, 2) approximately midway between salt pan and mid stand and 3) mid stand equally corresponding to high salt, intermediate salt and low salt microhabitats. We sampled larger populations with separate grids within each of the microhabitats. In smaller populations, the three microhabitats were contiguous. For *S. alterniflora*, we collected plant tissue from 32

individuals from three distinct microhabitats at each site as located adjacent to the salt pans, approximately midway between salt pan and creek bank and immediately adjacent to the creek bank. Within each *S. alterniflora* zone, we sampled plants with four sets of paired transects which ran perpendicular to the shoreline. The two transects within a pair were separated by one meter and plants were sampled every four meters.

### *Electrophoresis*

Leaf tissue samples were kept on ice and crushed within 24 hours for protein extraction using sea sand and cecropia buffer (cite) for *B. frutescens* and camellia buffer (Wendel and Parks 1982) for *S. alterniflora*. The extracted material was absorbed onto Whatman 3 MM filter paper wicks and then stored at -80° C until needed for electrophoresis. Extracted proteins were run on 9.5% starch gels (source). We used standard recipes for enzyme buffers and stains (Soltis et al. 1983, Cheliak and Pitel 1984). Seventeen allozyme loci were resolved for *B. frutescens*: ALD, IDH, 6-PGD and PGI (2) on buffer system 4; AAT and MNR on buffer system 7; FE (4), GDH, ME on buffer system 8-; F1,6 on buffer system 11 and CE, DIA, and PGM on buffer system 34. Twenty seven loci were resolved for *S. alterniflora*: MDH (3), 6-PGD and UGPP (4) on buffer system 4; AAT (3) on buffer system 7; ACO, AK, F1,6 (2), IDH, MPI, and SKDH on buffer system 11 and DIA(2), FE, LAP, PER, PGI, PGM, and TPI (2) on buffer system 34. (Need ACO, ALD, CE, LAP, PER, UGPP). Twenty-two of the 27 *Spartina* loci were scored as regular diploid loci. Two loci (IDH1 and PGI) displayed tetraploid patterns and were scored as 4 alleles per individual and analyzed as 2 loci each. Three (TPI2, PGM, and PER) loci displayed complex polyploid banding patterns and were scored as phenotypes. These three loci were excluded from

the analysis of general population genetics statistics, but they were used to identify multilocus genotypes and clonal structure.

### *Analyses*

Population genetic statistics including F-statistics, heterozygosity statistics, and gene flow estimates were calculated using the program LYNSPROG developed by M.D. Loveless (Dept. of Biology, College of Wooster, Wooster, OH) and A.F. Schnabel (Dept. of Biology, University of Indiana, South Bend, IN). At the species (subscript s) and population (subscript p) levels, we estimated percent polymorphic loci (P), mean number of alleles per polymorphic locus (AP), observed heterozygosity ( $H_o$ ), and expected heterozygosity ( $H_e = 1 - \sum p_i^2$ ), which estimates genetic diversity. Standard errors for within population parameters were obtained by averaging across all populations. For each species,  $G_{STS}$  was calculated following Nei (1987) across the five sites. We determined the significance of  $G_{ST}$  values by adding chi square values and degrees of freedom for allele frequency heterogeneity across all loci.

To partition genetic diversity into among site and among microhabitat within site components, we conducted a hierarchical analysis of population structure. For each species, we determined a total amount of genetic structure among the fifteen microhabitats across the five sites ( $G_{STM}$ ). This total  $G_{STM}$  was then partitioned into among site ( $G_{STS}$ ) and among microhabitats within site ( $G_{STAM}$ ) components by subtracting the  $G_{STS}$  from the total  $G_{STM}$ . The proportion of the overall total  $G_{STM}$  due to microhabitat within sites ( $G_{STAM}$ ) was determined as the  $G_{STAM}$  divided by the total  $G_{STM}$ .

We further examined fine scale genetic structure by investigating both clonal structure and patterns of relatedness among individuals within a patch. To assess clonal structure, we first

determined the multi-locus genotypes for each individual within each patch. We assessed genetic diversity first as the number of ramets per number of genets sampled ( $N/G$ ). Our second measure of genotypic diversity is Simpson's index:  $D = 1 - \sum((n_i(n_i-1))/(N(N-1)))$ ;  $n_i$  = number of ramets of the  $i^{\text{th}}$  genet and  $N$  = total number of ramets (Pielou, 1969). We also mapped multi-locus genotypes to examine spatial patterns of clonal structure.

## Results

### *Limited genetic structuring among sites*

Our measure of genetic diversity, expected heterozygosity ( $H_e$ ), was 0.089 for *B. frutescens*, which was much lower than average compared to the other groups with similar life history traits (Table 3.1). For *S. alterniflora*,  $H_e$  was about average compared to similar species. The proportion of genetic variation due to differences among all fifteen microhabitats ( $G_{\text{STAM}}$ ) was 0.101 for *B. frutescens* and 0.254 for *S. alterniflora*, which was comparable to values in related groups. Similarly, *B. frutescens* has typical levels of polymorphism (58.82% polymorphic loci), while *S. alterniflora* was well above average with 81% polymorphic loci

### *Microhabitat explains distribution, but not levels of genetic diversity*

Within sites pooled across microhabitats, observed and expected heterozygosities were not significantly different (G test,  $p > 0.05$ ), indicating allele frequencies were in Hardy-Weinberg equilibrium for both species (Table 3.2). However, when individual loci were examined within sites, five of the seventeen *B. frutescens* loci were significantly different (G test,  $p < 0.05$ ) from Hardy-Weinberg equilibrium in at least one site (average  $F_{\text{IS}} = -0.0599$ ). Of the loci significantly deviating from Hardy-Weinberg equilibrium, PGM and IDH had negative fixation indices ( $F_{\text{IS}}$ )

indicating an excess of heterozygotes, while MNR, GDH and 6PGD had positive fixation indices, indicating a lack of heterozygotes. Thirteen of the 27 *S. alterniflora* loci deviated from Hardy-Weinberg across sites. Of these, seven had mostly negative fixation indices ( $F_{IS}$ ), while six had mostly positive fixation indices across sites (average  $F_{IS} = -0.30$ ).

To further investigate fine-scale patterns of genetic structure, each of the five sites for each species was analyzed as three microhabitat zones, which corresponded to locations along environmental gradients as associated with low, intermediate and high salinity levels (Table 3.3). In both species, there was no change in genetic diversity (expected heterozygosity,  $H_e$ ), with increasing salinity content of the microhabitat (Figure 3.1). Similarly, there were no loci that demonstrated an association of allele frequencies with microhabitat type (Figure 3.2 PGM and MNR in *B. frutescens* and Figure 3.3 UGPP4 and PGI in *S. alterniflora* for examples). However, a hierarchical analysis showed that across all loci, 32% of the genetic structure ( $G_{ST}$ ) in *B. frutescens* and 51% of the  $G_{ST}$  in *S. alterniflora* was due to genetic differences among microhabitats within sites (Table 3.3). This level of structuring was significant at the  $p=0.05$  level because each separate  $G_{ST}$  calculated at all levels (15 microhabitat, 5 sites and 3 microhabitats within each separate site) were significant at the  $p=0.05$  level. The one exception was the marginal significance of among microhabitat  $G_{ST}$  in one *B. frutescens* site ( $p=0.07$ ).

#### *Clonal diversity is high and not correlated with microhabitat*

Seventy-four unique multi-locus genotypes out of 480 ramets were identified across the 5 *B. frutescens* sites. Of these, 25 genotypes were represented by only 1 ramet in 1 site, and 15 genotypes were represented by multiple ramets within only one site. Clonal structure and diversity varied among the five sites, though in general clonal diversity was high (Table 3.4a).

The average number of ramets/genet was 4.1, and average clonal diversity (measured as Simpson's diversity index) was 0.92 (Table 3.4a). The number of unique genotypes per site ranged from 16 at Shell Hammock to 31 at Marsh Landing. The majority of the 31 genotypes at Marsh Landing were represented by 1 to 6 ramets (Figure 3.4a). One obvious exception was genotype #22, which made up 18% of the population. Similarly, one genotype (#28) in the Shell Hammock site made up 38% of the ramets sampled (Figure 3.4b). Together, genotypes #28, #7, #24 and #31 made up over 60% of the ramets sampled at that site. Despite this dominance of specific genotypes at Shell Hammock, there was no obvious association of genotype with location along the height (and salinity) gradient. Similarly, we found no association of clones with microhabitat at any of the 5 sampling sites.

The *S. alterniflora* sites displayed an extremely high level of clonal diversity (Table 3.4b). Out of 480 ramets sampled, only 29 genets were represented more than one time for a total of 447 unique multi-locus genotypes. All of the 29 genets with multiple ramets were found in only one site and 26 of the 29 were represented by only 2 ramets. The average number of ramets/genet and average clonal diversity were therefore very close to 1 (Table 3.4b). The number of unique genotypes per site ranged from 84 at Lighthouse to 93 at Marsh Landing. Given this high level of genet diversity, there was no association of genotype or association of clones with location along the height (and salinity) gradient.

## **Discussion**

Despite the severe environmental gradients across which *B. frutescens* and *S. alterniflora* live in the salt marsh, we found no evidence for fine scale differentiation at any allozyme locus resolved for these species. In general, we found that there were small differences in genetic

make-up between sites. However, across loci, microhabitats explained a significant amount of the distribution of genetic diversity for both species. There were no differences in levels of genetic diversity associated with microhabitat type and although clonal diversity was high, it was also not associated with habitat type.

#### *Microhabitats explains distribution of genetic diversity*

Salt marshes vary widely in abiotic and biotic conditions which result in striking patterns of zonation across as well as within species. There has been some interest in whether species inhabiting these relatively harsh habitats are locally adapted, but the question has never been resolved. In controlled greenhouse studies, Antlfinger (1981) found that plants from opposite ends of the environmental gradient had genetically based differences in rhizome length and xylem water potential, but other morphological and physiological were highly plastic. Much work focused on the genetic basis of different height forms of *S. alterniflora* produced conflicting results (Anderson and Treshow 1980, Gallagher et al. 1988, Trnka and Zedler 2000). In particular, one reciprocal transplant study supports and two others refute genetic differentiation at the ends of gradients within populations (Gallagher et al. 1988, Shea et al. 1975, Valiela et al. 1978 respectively). Previous work has also shown no difference in germination or seedling growth response to salinity treatments from seed collected at opposite ends of the gradient (Mooring et al. 1971).

Although we found relatively low among site differences for *B. frutescens* ( $G_{ST} = 0.069$ ) and *S. alterniflora* ( $G_{ST} = 0.047$ ), a significant amount of the distribution of the genetic variation for both species can be attributed to among microhabitat within site differences. This finding is in keeping with a previous survey of *B. frutescens* which reported only one polymorphic locus

(GOT) and that allele frequencies were not different for plants from the ends of the salinity gradient (Antlfinger 1979). Similarly, Shea et al. (1975) reported no differences in banding patterns between tall and short form *S. alterniflora* at the MDH, LDH and IDH loci.

Our findings are therefore not as simple as other studies that report environmental sorting of genotypes or alleles at specific loci (Hamrick and Allard 1972, Hamrick and Holden 1979, Heywood 1991, Johnston, et al. 2001b, Salzman 1985, Schmidt and Rand 1999). For example, comparisons of hybrid genotypic classes formed between closely related species of *Iris* suggest that some genotypes are better suited to some habitat types (Johnston et al. 2001a). Field surveys of *Iris* found that not only are genotypic classes associated with environment, but a majority of the RAPD loci and cpDNA haplotypes displayed associations with environmental conditions (Cruzan and Arnold 1993, Johnston et al. 2001b). Johnston et al. (2001b) suggest that despite the fact that these are putatively neutral loci, their results are clearly the consequence of environment dependent selection. Although we did not find these patterns at specific allozyme loci, we did find significant structuring across all loci within each species. Selection may therefore be acting on ecologically important traits that are not simply represented by or linked to any one locus.

#### *Clonal diversity is high and not correlated with microhabitat*

The results indicate that patterns of clonal diversity and structure can vary from site to site in *B. frutescens* populations, but are consistently extremely high in *S. alterniflora*. In all sites, there were many genotypes, and overall, clonal diversity was much higher than expected for both species. *Borrchia frutescens* ramets spaced more than 2 m apart and *S. alterniflora* ramets spaced 1 m apart were unlikely to belong to the same clone, indicating that both clonal and sexual reproduction are important for these species.

The high clonal diversity in these species was somewhat surprising given that clonal reproduction is known to be extensive (Proffitt et al. 2003, Stiling 1994, Stiling and Rossi 1995), and seedling emergence in the field is also thought to be very low. Comparable results have been found for the clonal grass *Uniola paniculata*, another long-lived perennial with similar distribution (Franks et al. in review, Franks 2003). Many *Uniola* patches had a high diversity of clones even though clonal reproduction is extensive and seedling emergence in the field is very low. Occasional localized flushes of seedling establishment may explain these patterns of genetic structure (Franks et al. in review). In this case, rare long distance dispersal events reduce genetic structure, but clonal reproduction and recruitment of seedlings into localized patches maintain differences among sites. An analogous scenario may explain the patterns found in these *B. frutescens* and *S. alterniflora* populations.

Studies of clonal species have found that different genets vary in their ability to discriminate between patches and proliferate in favorable habitat (Bazzaz 1991, Salzman 1985). We therefore expected to find an association of clones and levels of clonal diversity with location along environmental gradients within the salt marsh. These populations of *B. frutescens* and *S. alterniflora* tended to be more diverse than we expected considering a survey of clonal structure in 45 plant species found an average of 26% multilocus genotypes (Widen et al. 1994). However, we did not find that overall levels of diversity ( $H_e$ ), or size or diversity of clones were related to location along environmental gradients. For example, there was no obvious association of a given multi-locus genotype or size of clones with either high, intermediate or low salt microhabitats. Likewise, visual inspection of the clonal maps suggest that instead, large *B. frutescens* clones tend to traverse the entire gradient through high, intermediate or low salt microhabitats (see Figure 3a. genets #44 at Marsh Landing and Figure 3b. genets #7 and #28 at

Shell Hammock). Other studies have shown that clonal structure can vary widely both among species and among populations within species (Kudoh et al., 1999; Reusch et al., 2000; Stehlik and Holderegger, 2000; Suyama et al., 2000). Populations of clonal species growing in contrasting habitats have been found to vary in growth rates (Karagatzides and Hutchinson 1991), biomass allocation patterns (Lovett Doust 1981) and allocation to sexual reproduction (Sun 2001). Clonal structure in the temperate woodland herb *Uvularia perfoliata*, was found to be strongly dependent on disturbance patterns, with patches in canopy gap habitats containing a few large clones but many different genotypes and patches in closed canopy habitats comprised of a single multilocus genotype (Kudoh et al. 1999). Disturbance may play a role in the clonal structure of *B. frutescens* and *S. alterniflora*, especially since erosion and storm generated disturbances are important factors determining salt marsh species distribution and population dynamics (Pennings and Bertness 2001).

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Table 3.1. Estimates of genetic diversity and structure for *B. frutescens* and *S. alterniflora* compared to species with similar breeding systems, distribution patterns and perenniality.  $P_s$  = percent polymorphic loci,  $H_e$  = mean Hardy-Weinberg expected heterozygosity,  $G_{ST}$  = proportion of total genetic diversity due to differences among populations. *Borrchia frutescens* and *S. alterniflora* statistics are from this study where total  $G_{ST} = G_{STM}$  (see methods). *U. paniculata* statistics from Franks et al., in review. Source for all others: Hamrick and Godt 1996 and Godt and Hamrick 1998.

| Taxa                         | No. of species | $P_s$ | $H_{es}$ | $G_{ST}$ |
|------------------------------|----------------|-------|----------|----------|
| <i>Borrchia frutescens</i>   | 1              | 58.8  | 0.089    | 0.101    |
| Asteraceae                   | 101            | 45.3  | 0.127    | 0.204    |
| Outcross/gravity             | 178            | 50.2  | 0.152    | 0.189    |
| Outcross/long-lived          | 241            | 65.5  | 0.180    | 0.094    |
| Narrow/long-lived            | 70             | 59.5  | 0.163    | 0.132    |
| Regional/long-lived          | 151            | 67.0  | 0.190    | 0.086    |
| <i>Spartina alterniflora</i> | 1              | 80.8  | 0.205    | 0.254    |
| <i>Uniola paniculata</i>     | 1              | 77.8  | 0.151    | 0.304    |
| Poaceae                      | 161            | 60.0  | 0.191    | 0.272    |
| Outcrossing grasses          | 69             | 69.1  | 0.212    | 0.112    |

Table 3.2. Within population estimates of genetic diversity and structure for a) *B. frutescens* and b) *S. alterniflora*.  $P_p$  (%) = percent polymorphic loci,  $AP_p$  = mean number of alleles per polymorphic locus,  $A_p$  = mean number of alleles per locus,  $A_{ep}$  = expected number of alleles per locus,  $H_{op}$  = observed heterozygosity,  $H_{ep}$  = mean Hardy-Weinberg expected heterozygosity.

a.) *B. frutescens*

| Site          | $P_p$ (%) | $AP_p$ | $A_p$ | $A_{ep}$ | $H_{op}$ | $H_{ep}$ |
|---------------|-----------|--------|-------|----------|----------|----------|
| Cabretta      | 53.85     | 2.14   | 1.62  | 1.21     | 0.114    | 0.126    |
| Hunt Camp     | 41.18     | 2.14   | 1.47  | 1.08     | 0.045    | 0.058    |
| Lighthouse    | 50.0      | 2.13   | 1.56  | 1.13     | 0.105    | 0.084    |
| Marsh Landing | 53.85     | 2.00   | 1.54  | 1.19     | 0.126    | 0.117    |
| Shell Hammock | 35.29     | 2.17   | 1.41  | 1.10     | 0.068    | 0.066    |
| Mean          | 46.83     | 2.12   | 1.52  | 1.14     | 0.092    | 0.090    |
| SD            | 5.71      | 0.07   | 0.08  | 0.06     | 0.011    | 0.018    |

b.) *S. alterniflora*

| Site          | $P_p$ (%) | $AP_p$ | $A_p$ | $A_{ep}$ | $H_{op}$ | $H_{ep}$ |
|---------------|-----------|--------|-------|----------|----------|----------|
| Cabretta      | 65.38     | 2.35   | 1.88  | 1.41     | 0.253    | 0.220    |
| Hunt Camp     | 65.38     | 2.35   | 1.88  | 1.42     | 0.270    | 0.209    |
| Island Apex   | 61.54     | 2.38   | 1.85  | 1.33     | 0.247    | 0.189    |
| Lighthouse    | 61.54     | 2.31   | 1.81  | 1.37     | 0.250    | 0.197    |
| Marsh Landing | 61.54     | 2.31   | 1.81  | 1.35     | 0.226    | 0.192    |
| Mean          | 63.08     | 2.34   | 1.85  | 1.37     | 0.249    | 0.201    |
| SD            | 4.23      | 0.03   | 0.04  | 0.04     | 0.014    | 0.019    |

Table 3.3. Hierarchical analysis of population structure for a.) *B. frutescens* and b.) *S. alterniflora*.

| a.) <i>B. frutescens</i>                  |     |         |            |
|---|-----|---------|------------|
| Grouping                                  | N   | $H_T^a$ | $G_{ST}^b$ |
| <u>Among microhabitats (within sites)</u> |     |         |            |
| Cabretta                                  | 96  | 0.247   | 0.073      |
| Hunt Camp                                 | 96  | 0.141   | 0.025      |
| Lighthouse                                | 96  | 0.160   | 0.020      |
| Marsh Landing                             | 96  | 0.210   | 0.025      |
| Shell Hammock                             | 96  | 0.183   | 0.016      |
| <u>Summary of <math>G_{ST}</math></u>     |     |         |            |
| Among 15 microhabitats                    | 480 | 0.151   | 0.101      |
| Among sites                               |     |         | 0.069      |
| Among microhabitats within sites          |     |         | 0.032      |
| b.) <i>S. alterniflora</i>                |     |         |            |
| Grouping                                  | N   | $H_T^a$ | $G_{ST}^b$ |
| <u>Within sites (among microhabitats)</u> |     |         |            |
| Cabretta                                  | 96  | 0.332   | 0.046      |
| Hunt Camp                                 | 96  | 0.311   | 0.037      |
| Island Apex                               | 96  | 0.296   | 0.051      |
| Lighthouse                                | 96  | 0.299   | 0.049      |
| Marsh Landing                             | 96  | 0.292   | 0.085      |
| <u>Summary of <math>G_{ST}</math></u>     |     |         |            |
| Among 15 microhabitats                    | 480 | 0.254   | 0.095      |
| Among 5 sites                             |     |         | 0.047      |
| Among microhabitats within sites          |     |         | 0.048      |

<sup>a</sup>  $H_T$  = total genetic diversity as measured by total heterozygosity computed by Nei's (1987) method using the software program Lynsprog.

<sup>b</sup>  $G_{ST}$  = proportion of total genetic diversity due to differences among populations, computed by Nei's (1987) method using the software program Lynsprog.

Table 3.4. Statistics of clonal structure and diversity a) *B. frutescens* sites and b) *S. alterniflora* sites. Each site is represented by 96 ramets. Average number of ramets per unique multi-locus genet and standard error between genets are presented. Clonal diversity = Complement of Simpsons diversity index ( $D = 1 - \sum [Ni(Ni-1)/N(N-1)]$ ). Probability of exclusion = 1- product of Probability of Identity /locus=  $\sum [xi^4 + xj^4 + xk^4 + 2ab^2 + 2ac^2 + 2bc^2]$ . See methods for details.

a). *B. frutescens*

| Site          | Ramets/<br>Genet (SE) | Clonal<br>Diversity | Probability<br>of exclusion |
|---------------|-----------------------|---------------------|-----------------------------|
| Cabretta      | 3.00 (0.49)           | 0.953               | 0.95                        |
| Hunt Camp     | 4.57 (1.07)           | 0.885               | 0.97                        |
| Lighthouse    | 3.69 (0.94)           | 0.896               | 0.93                        |
| Marsh Landing | 3.10 (0.62)           | 0.980               | 0.87                        |
| Shell Hammock | 6.00 (1.70)           | 0.872               | 0.90                        |
| Overall       | 5.9 (1.03)            | 0.96                |                             |
| Average       | 4.1 (0.21)            | 0.92                |                             |

b). *S. alterniflora*

| Site          | Ramets/<br>Genet (SE) | Clonal<br>Diversity | Probability<br>of exclusion |
|---------------|-----------------------|---------------------|-----------------------------|
| Cabretta      | 1.04 (0.02)           | 0.999               | >0.999                      |
| Hunt Camp     | 1.05 (0.03)           | 0.999               | >0.999                      |
| Island Apex   | 1.12 (0.05)           | 0.996               | >0.999                      |
| Lighthouse    | 1.14 (0.04)           | 0.997               | >0.999                      |
| Marsh Landing | 1.03 (0.02)           | 0.999               | >0.999                      |
| Overall       | 1.08 (0.02)           | 0.999               |                             |
| Average       | 1.08 (0.02)           | 0.998               |                             |

## Figure Legends

Figure 3.1. Genetic diversity, measured as expected heterozygosity ( $H_e$ ) for each of the three microhabitats within 5 sites of a.) *B. frutescens* and b.) *S. alterniflora* on Sapelo Island.

Figure 3.2. Allele frequencies at the PGM and MNR loci for each of the 5 populations by high, medium and low salt microhabitat type of *B. frutescens*.

Figure 3.3. Allele frequencies at the UGPP4 and PGI loci for each of the 5 populations by high, medium and low salt microhabitat type of *S. alterniflora*.

Figure 3.4. Maps of the clonal structure of *B. frutescens* at sampling sites a) Marsh Landing and b) Shell Hammock. Each number represents one plant. Different numbers symbolize unique multi-locus genotypes for allele combinations across 10 polymorphic loci. Genets with more than one ramet are represented in color.

Figure 3.5. Maps of the clonal structure of *S. alterniflora* at sampling sites a) Light House and b) Marsh Landing. Each number represents one plant. Different numbers symbolize unique multi-locus genotypes for allele combinations across 10 polymorphic loci. Genets with more than one ramet are represented in color.

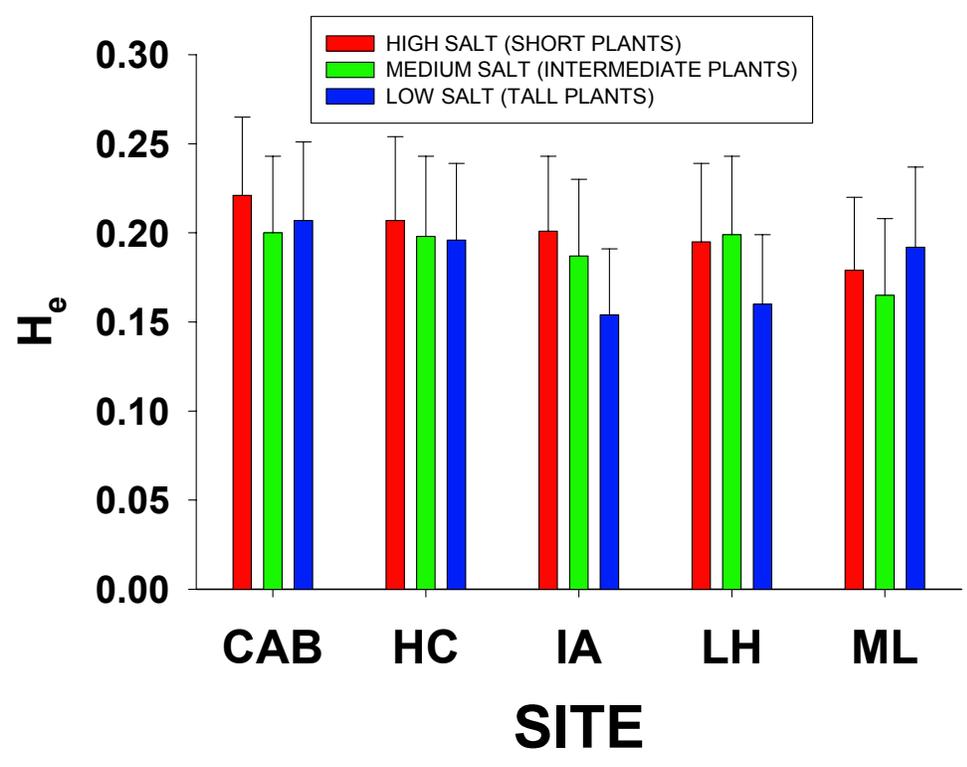
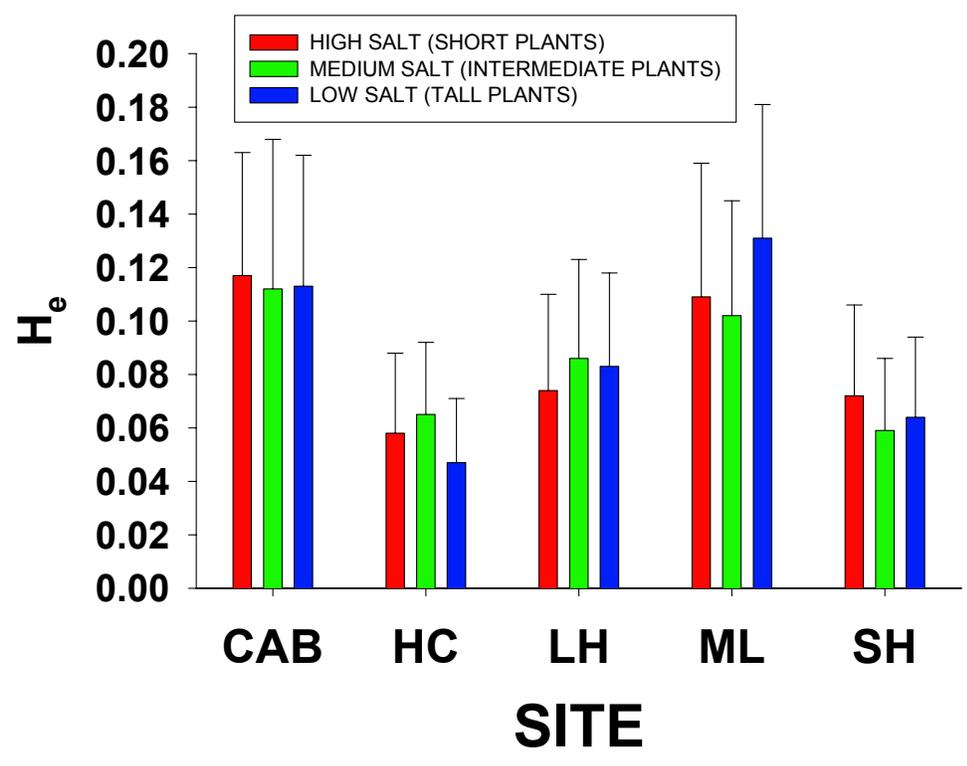


Figure 3.1.

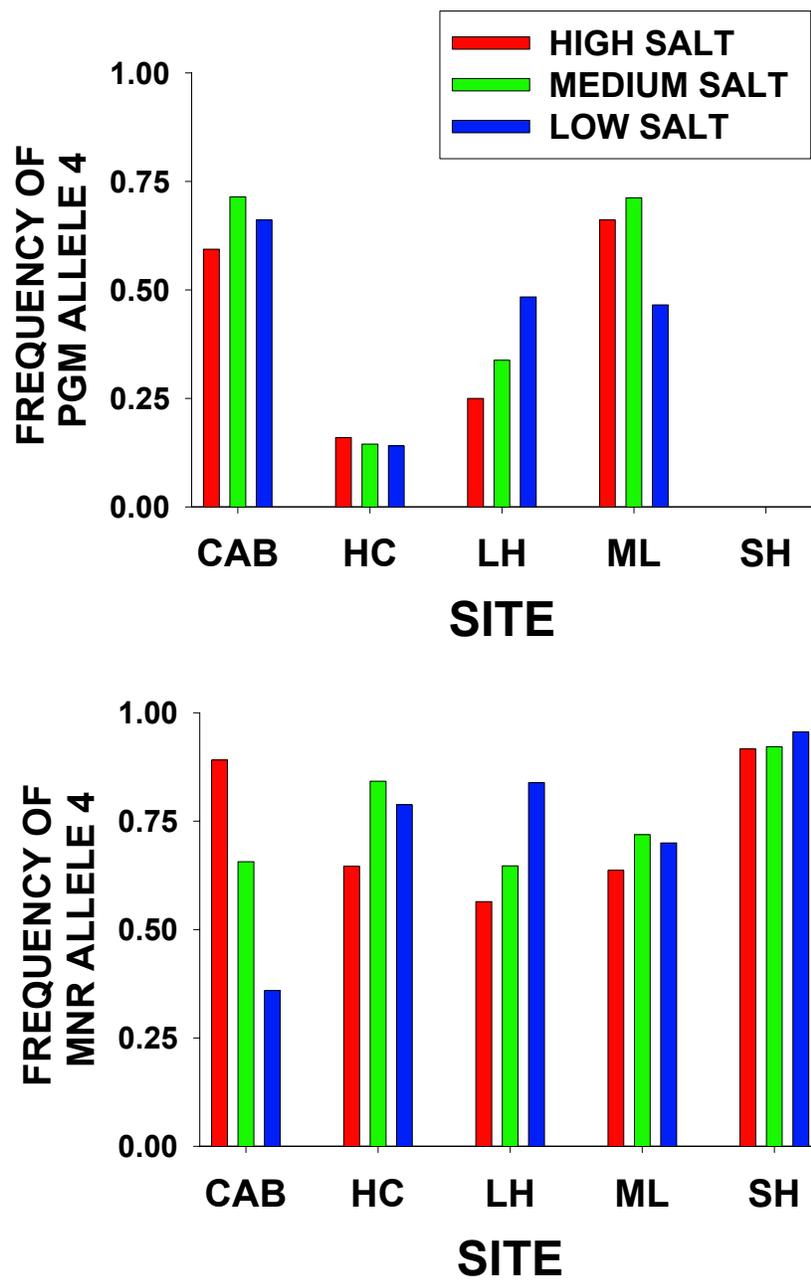


Figure 3.2.

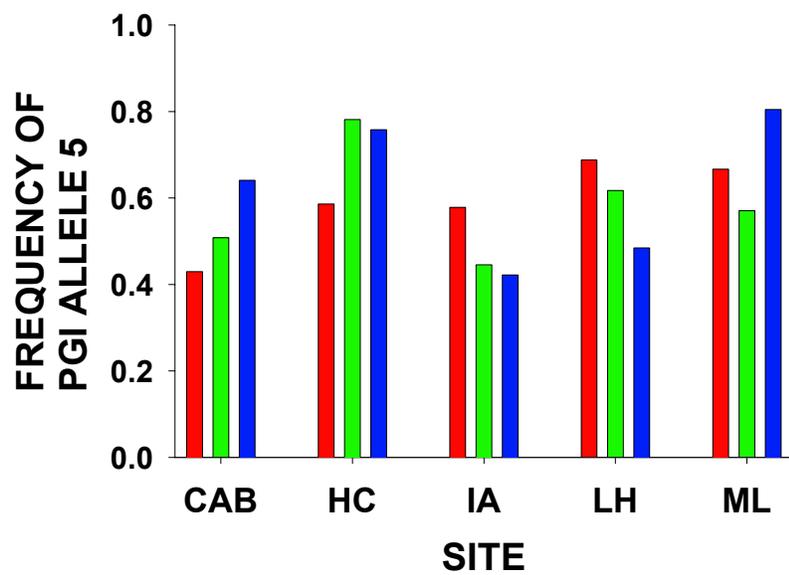
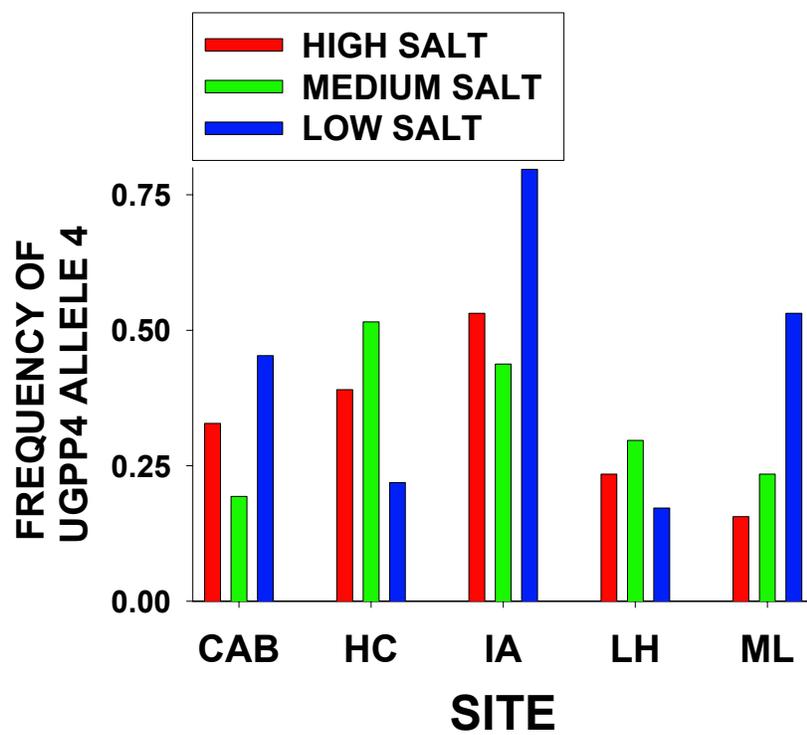


Figure 3.3.

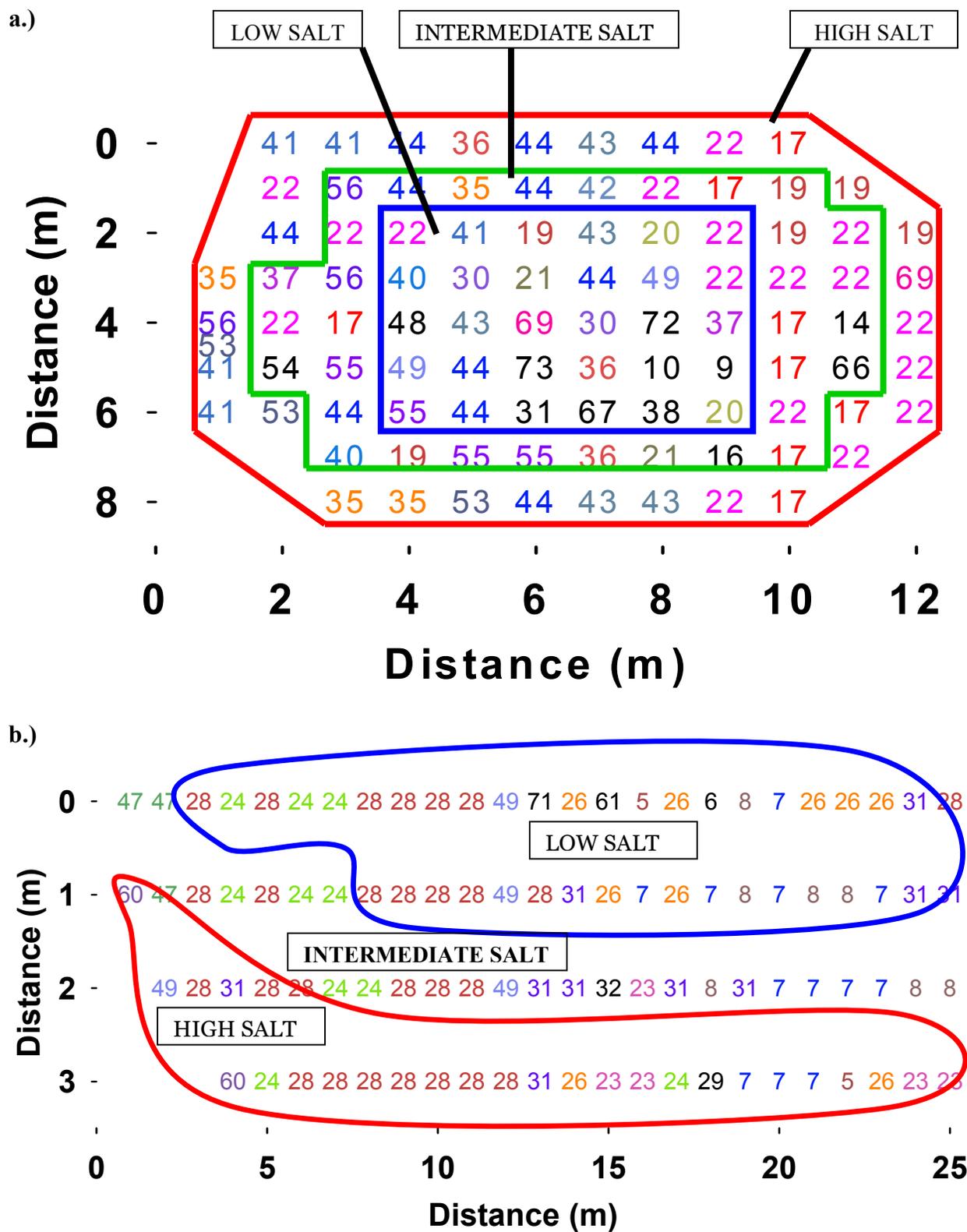


Figure 3.4.

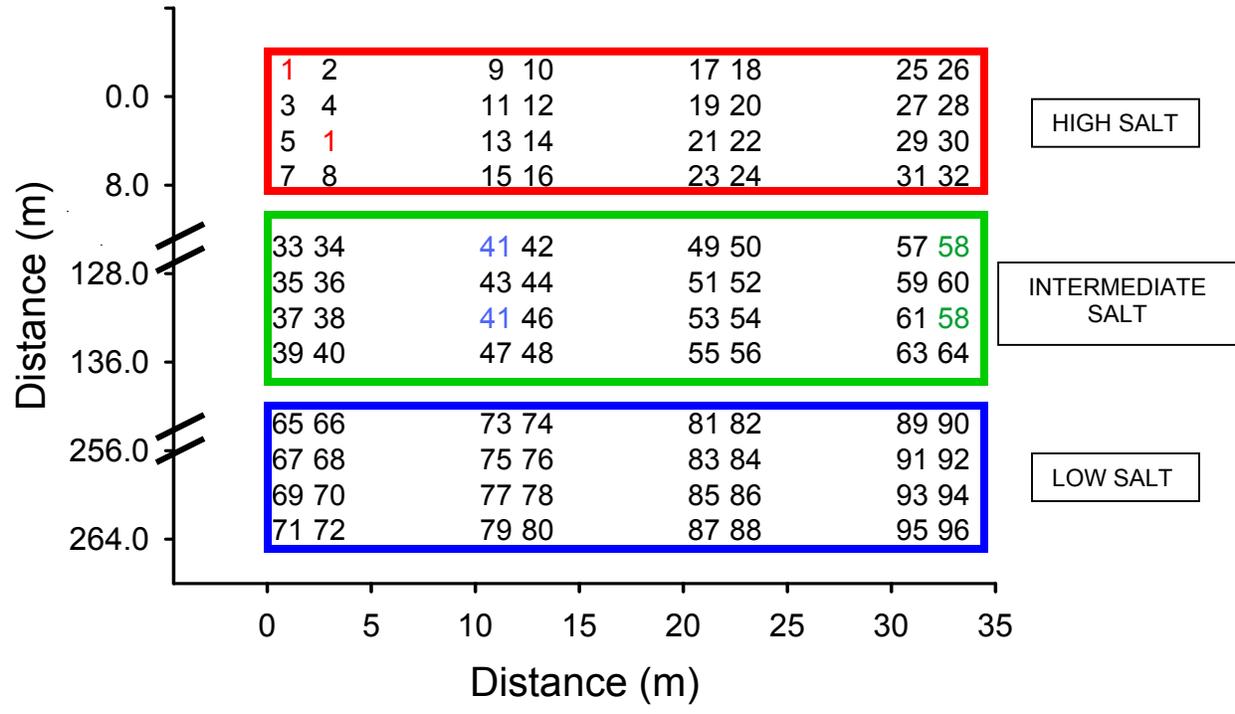
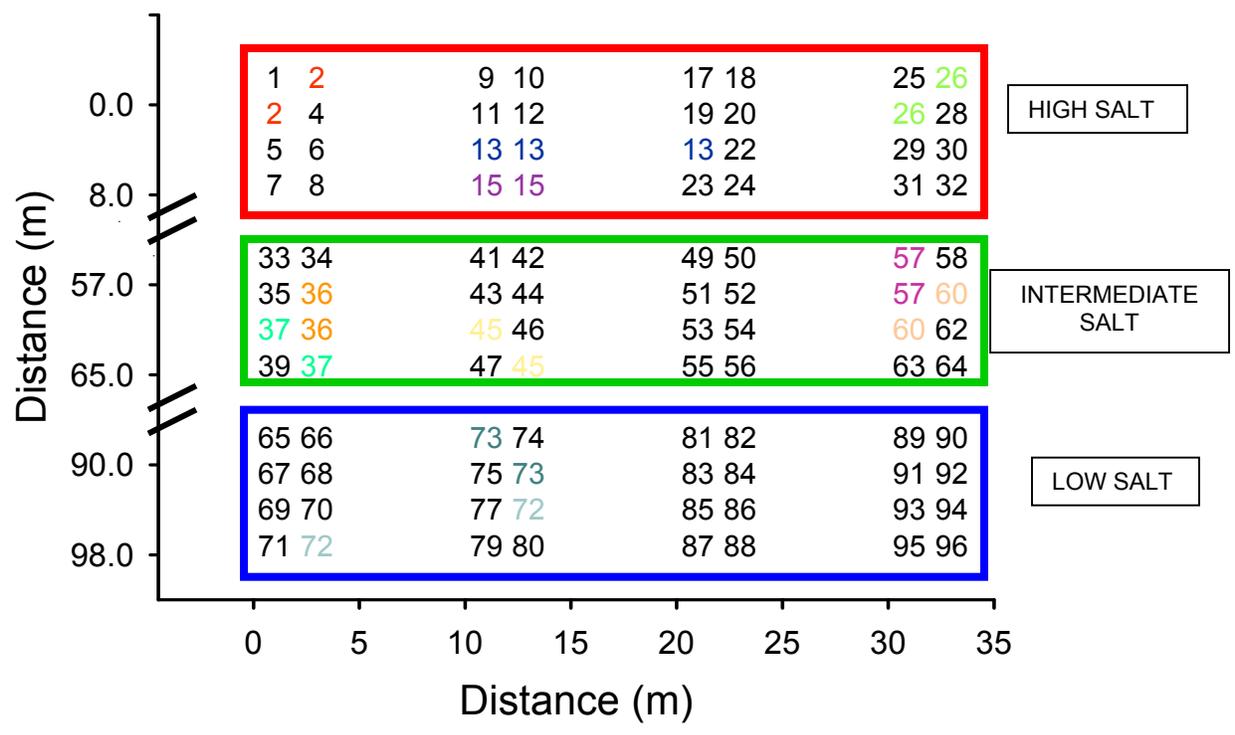


Figure 3.5.

CHAPTER 4  
EVIDENCE FOR ADAPTIVE PLASTICITY ALONG A SALINITY GRADIENT IN A SALT  
MARSH PERENNIAL<sup>1</sup>

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<sup>1</sup> Richards, C. L. S. N. White, M. A. McGuire, S. J. Franks, L. A. Donovan, R. Mauricio. Submitted to *New Phytologist*.

## Summary

- We investigated trait responses to controlled salinity treatments in the salt marsh plant, *Borrichia frutescens*, to examine if the species is responding to environmental variation through phenotypic plasticity or fine-scale genetic specialization.
- We collected *B. frutescens* fruits in high and low salt microhabitats on Sapelo Island, GA, USA. We assigned seedlings from high and low salt maternal parents to high and low salt treatments in the greenhouse and measured traits related to salt tolerance.
- All traits were highly plastic in response to salinity. There were significant genetic differences in final height and concentrations of leaf elements Na, P, and Mg. However, genetic variation in plasticity existed for only Na and B concentrations. Despite genetic variation for some traits, plants from high salinity areas did not respond differently than plants from low salt areas to the two salt treatments.
- The results support the hypothesis that *B. frutescens* lives across a steep environmental gradient in salinity through a high degree of phenotypic plasticity rather than by adaptation to local conditions through genetic specialization.

Key words: *Borrichia frutescens* (sea ox-eye daisy), genetic specialization, local adaptation, phenotypic plasticity, salt tolerance traits, salinity gradient, salt marsh plant

## Introduction

Plant species can exhibit dramatic differences in phenotype across environmental gradients. These phenotypic differences may be due to either genetically based adaptation (specialization) to local conditions (Turesson *et al.*, 1922; Clausen *et al.*, 1948), environmentally induced differences in phenotype (phenotypic plasticity) where different morphologies are produced from the same genotypes in different environments (Schmalhausen, 1949; Bradshaw, 1965), or some combination of both specialization and plasticity. Although many studies have found evidence for local adaptation (Linhart & Grant, 1996), even highly specialized individuals have a limited ability to accommodate to local conditions through the plasticity of morphological and physiological traits (Sultan *et al.*, 1998). A highly plastic trait, while not specialized to fit a specific environmental condition, can also be considered an adaptation if it allows an individual to occupy multiple habitats (Pigliucci 2001).

While trait response to environmental variation is likely a product of both local adaptation and plasticity, the degree to which specialists (locally adapted genotypes) are favored over generalists (plastic genotypes) depends on several factors, including the extent of environmental variation experienced at the scale of the individual (Bradshaw, 1965; Sultan, 1987; Sultan *et al.*, 1998). When environmental conditions are course-grained such that an individual experiences a uniform habitat, a single phenotype achieves the highest fitness, favoring specialists with this fixed phenotype. As conditions become increasingly fine-grained, selection favors higher levels of plasticity (Bradshaw, 1965; Sultan, 1987; Sultan *et al.*, 1998).

Carefully designed glass house studies have been essential in dissecting if species respond to environmental variation through phenotypic plasticity or fine-scale genetic specialization of traits (Sultan, 2000; Debat & David, 2001; Pigliucci, 2001). By manipulating

related individuals across treatments, researchers have used glass house studies to examine specific trait responses to isolated environmental parameters of putative ecological importance. In these studies, a trait is considered plastic if there is a significant treatment effect in an analysis of variance (ANOVA) for that trait. Individuals may vary in the degree or direction of plasticity for a given trait which is indicated if genotypes perform differently in treatments, (genotype by environment or G\*E interaction). This would be the case for instance if there were specialized genotypes that performed differently in two environments. An important consequence of significant G\*E for a trait is that it indicates the potential for the evolution of plasticity in this trait (Pigliucci, 2001).

In order to identify local adaptation, two conditions must be met to: (1) there must be genetic variation for a trait and (2) genetically based differences in response should be associated with habitat type. A significant genotype effect in an ANOVA indicates that there is genetic variation for a trait which may be distributed at random across habitats. A significant effect of habitat reveals that the genetic variation is associated with habitat type and that individuals from different habitats are genetically different. To demonstrate that these differences are adaptive requires that individuals from a given habitat outperform individuals from other habitats in response to the appropriate level of an environmental factor. For example, a plant adapted to a low light environment would outperform a plant from a high light environment under a controlled low light treatment in a glass house study. This differential performance is indicated by a significant interaction between habitat and treatment, but the adaptive nature of the response depends on trait values, revealed only through examination of the actual data. Interpreting the ANOVA in light of actual trait values therefore allows us to determine the relative roles of genetic specialization and phenotypic plasticity in trait response to environmental variation.

Salt marsh plants are ideal organisms in which to investigate the relative roles that local adaptation and plasticity play in determining phenotypic variation because these plants display dramatic differences in phenotype that are correlated to environmental conditions (Pennings & Bertness, 2001; Richards, 2004). For example, a predominant feature of the salt marsh environmental gradient is the broad range in salinity (Pennings & Bertness, 2001; Richards, 2004). A suite of physiological traits have been reported to be important for dealing with the toxic and osmotic effects of substrate salinity (Flowers *et al.*, 1977). In particular, plants specifically adapted to high salinity environments, called halophytes, can secrete, sequester, or dilute (by increasing succulence) what would otherwise be toxic levels of Sodium (Na). In addition, conservative use of water can reduce the uptake of Na and reduce the water demands of the plant (Flowers *et al.*, 1977). These habitats are typically not only stressful because of Na levels, but also because they are low in nitrogen (N) (Smart & Barko, 1980). Sodium can compound this nutrient limitation by interfering with nutrient uptake (Flowers *et al.*, 1977). Halophytes are able to maintain nutrient uptake in the presence of high Na and use N-rich compatible solutes and nutrient cations (Potassium, Calcium, Magnesium, Manganese) for osmotic adjustment (Cavaliere & Huang, 1979; Antlfinger & Dunn, 1983; Glenn & O'Leary, 1984; Donovan *et al.*, 1996, 1997; Aerts & Chapin, 2000; Moon & Stiling, 2000; Rosenthal *et al.*, 2002).

In this study, we investigate response of these traits to controlled salinity treatments in the dominant high marsh perennial, *Borrchia frutescens* (Asteraceae). This species exhibits extreme phenotypic variation which is correlated to the broad range of substrate salinity it inhabits (Richards, 2004). We expected that while plants from different microhabitats would have some degree of phenotypic plasticity to respond to different salt levels, differential selection pressures would lead to adaptation through genetic specialization to high and low salt habitats.

Specialization to microhabitats would therefore lead to high levels of genetically based variation in important traits. If genetic specialization has occurred between plants in different microhabitats with respect to substrate salt content, plants from high salt areas should perform better under controlled high salt conditions than plants from low salt areas, and the rankings should reverse under low salt conditions. To determine if *B. frutescens* is responding to environmental variation through phenotypic plasticity or fine-scale genetic specialization, we asked three major questions. First, how plastic are traits associated with salt tolerance? Second, is there genetic variation for salt tolerance traits? Three, is there evidence for genetic specialization in response to substrate salt content?

## Methods

### Study site and species

Sapelo Island is located on the Atlantic coast of Georgia, USA (31° 28'N, 81° 14'W). The vegetation patterns in Sapelo Island marshes are typical of southeastern marshes in the United States (Antlfinger, 1981; Pomeroy & Wiegert, 1981). Lower elevations of the marsh are subject to daily tidal submergence and are dominated by the clonal grass, *Spartina alterniflora*. The higher elevations of the marsh are flooded infrequently and are often characterized by highly saline salt pans and associated salt-tolerant species such as *Salicornia virginica*, *Salicornia bigelovii*, *Batis maritima*, *Borrchia frutescens*, *Distichlis spicata* and *Sporobolus virginicus*. The terrestrial border of the marsh is typically dominated by *Juncus roemerianus*, *Spartina patens* or *Iva frutescens*. *Aster tenuifolius* and *Limonium carolinianum* occur at higher elevations mixed in with the dominant species *J. roemerianus*, *S. patens* or *I. frutescens* (Antlfinger, 1981; Pomeroy & Wiegert, 1981).

The sea ox-eye daisy, *B. frutescens*, occurs across a broad range of salinities (ranging from 20 ppt to over 100 ppt) and exhibits extreme phenotypic variation (heights from over 100 cm to less than 10 cm; Richards, 2004) making this species ideal for studying the nature of phenotypic variation across contrasting environments. This C3 composite is abundant in the high marsh, which is marked by infrequent tidal flooding (Antlfinger, 1981). *Borrichia frutescens* tolerates high substrate salinities by increasing succulence, actively taking up Na and other ions as well as manufacturing the N rich compatible solutes proline and glycine-betaine (Cavalieri & Huang, 1979; Antlfinger & Dunn, 1983; Moon & Stiling, 2000). *Borrichia frutescens* reproduces both clonally and sexually, although the relative contribution of each type of reproduction is unknown (Richards, 2004). Selfing rates estimated from genetic studies of *B. frutescens* suggest that when the plant reproduces sexually, it is mainly by outcrossing (Antlfinger 1982).

### Sampling design

We collected a dry floral head (infructescence) from 10 plants in each of two microhabitats in the Cabretta Marsh on Sapelo Island. Each floral head constitutes a maternal family of seeds that, due to the outcrossing mating system, are at least half-sibs (Antlfinger, 1982). One microhabitat is characterized by low salt concentration (25-30 ppt) and the second microhabitat is characterized by high salt (100-120 ppt; Richards, 2004). The two microhabitats are separated by approximately 25 m. All seeds were removed from each floral head and seed mass was recorded to the nearest 0.01 mg.

Initial measurements of seeds from high salt and low salt microhabitats did not suggest that there were differences between the two sources. Average seed mass ( $0.871 \pm 0.025$  g and  $0.957 \pm 0.017$  g), average number of seeds ( $57.6 \pm 3.8$  and  $64.3 \pm 5.0$ ) and average germination

rates ( $37.6 \pm 5.5$  and  $46.4 \pm 5.5$ ) were not significantly different between families from high salt and low salt microhabitats. Linear regression showed no relationship between the total number of seeds produced and the mean seed mass ( $P= 0.822$ ,  $r^2= -0.05$ ). There was also no relationship between total number of seeds produced and percent germination ( $P= 0.139$ ,  $r^2= 0.069$ ). Mean seed mass was significantly related to percent germination ( $P= 0.029$ ,  $r^2= 0.195$ ).

Seeds were cold stratified for 7 days at 4° C. After stratification, seeds were planted in a completely randomized block design in individual 12 cm<sup>3</sup> wells in 72 well flats. We used a 1:1 mixture of sterilized sand and organic potting medium (Fafard #3B, Agawam, MA, USA). The flats were placed in a temperature and light controlled greenhouse under conditions approximating early summer conditions in the salt marsh. Photoperiod was controlled at 14 hours days and 10 hour nights. Day temperature was maintained at 30° C and night temperature at 25° C. Pots were watered daily and fertilized weekly with half-strength Hydro-Sol solution, containing a nutrient N:Phosphorus (P):potassium (K) ratio of 5:11:26 (Scotts-Sierra Horticultural Products Company, Maysville, OH, USA). Germination was recorded daily for the first 24 days and weekly thereafter.

### Experimental treatments

We selected seven maternal families (floral head) originating from low salt microhabitats and seven maternal families originating from high salt microhabitats. We randomly selected 16 seedlings from each family for the experiment. These seedlings were transplanted into 500 cm<sup>3</sup> plastic pots and placed on greenhouse benches in a randomized complete block design. Each spatial block consisted of 28 seedlings representing one of each family × salinity treatment

combination. Salinity treatments consisted of two levels of NaCl concentration in water: low salinity (L) at 4 ppt and high salinity (H) at 40 ppt. Although 40 ppt is less than the maximum salinity observed in field soils, it was chosen because preliminary studies indicated that this was close to the maximum limit for seedling survival in the greenhouse and we were interested in investigating the physiological limits of response to extremes in salt environment. We feel that this is an appropriate approach, particularly since comparing salinity between the field and plastic pots is not straightforward. We recorded individual seedling height at six weeks and initiated treatments. Salinity treatments were applied every other day and gradually increased from 1 ppt to 4 ppt (L) and 10 ppt to 40 ppt (H), reaching final salinity levels 2 weeks after treatment initiation. Thereafter, final salinity treatment levels were applied 3 times per week. Daily watering and weekly fertilization continued throughout the course of the experiment. However, on a daily basis, only enough water to saturate the soil was applied to minimize salinity loss. Live plants were harvested after five months.

#### Traits measured

We measured five morphological, four physiological, and eight leaf nutritional traits related to salt tolerance and overall performance for each plant. Morphological traits were height, shoot dry biomass, root dry biomass, total dry biomass and total leaf area (Li-Cor Model LI-3100 Leaf Area Meter: LiCor, Inc., Lincoln, NE, USA). Plants were dried in a forced air oven at 60° C for 72 h to determine total dry biomass. Physiological traits were succulence (g water in all leaves/ cm<sup>2</sup> total leaf area), photosynthetic rate (LICOR 6400: LiCor-Inc., Lincoln, NB, USA), midday xylem pressure potentials (PMS Inc. Corvallis OR, USA), and integrated photosynthetic water use efficiency (WUE) as determined from stable carbon isotope ratios ( $\delta^{13}\text{C}$ ).

Photosynthetic rate and midday xylem pressure potentials were measured between 10:30 am and 2:30 pm on clear sunny days.  $\delta^{13}\text{C}$  was measured on dried leaf material using continuous flow mass spectrometry (Farquhar *et al.* 1982; Donovan and Ehleringer 1994). More negative  $\delta^{13}\text{C}$  values are indicative of lower integrated WUE. Leaf nutritional traits were Na, N, P, K, boron (B), calcium (Ca), magnesium (Mg), and manganese (Mn) concentration. Leaf N was measured on dried leaf material using continuous flow mass spectrometry (Farquhar *et al.* 1982; Donovan and Ehleringer 1994). Leaf acid extracts (Sah & Miller, 1992) were analyzed for B, Ca, K, Mg, Mn, Na and P concentration on an inductively coupled plasma-atomic emission spectrophotometer (Thermo Jarrell-Ash Enviro 36 simultaneous inductively coupled argon plasma-emission spectrograph: Thermo Electron Corp. Woburn, MA, USA). A subset of all live leaf tissue for each individual was used for  $\delta^{13}\text{C}$  and leaf nutritional trait analyses. Leaves that were shed due to salt loading were excluded from all morphological, physiological, and nutritional measurements.

### *Data analysis*

We used the SAS statistical package (Version 8.02 for Windows) for all data analyses (SAS Institute, Cary, NC, USA). T-tests were used to test for differences between high salt and low salt microhabitats for maternal families average seed mass, average number of seeds and percent germination (n= 10 families for each microhabitat). Linear regressions were performed for mean seed mass on number of seeds, percent germination on number of seeds, and percent germination on mean seed mass. We used multivariate analysis of covariance (MANCOVA; Sokal & Rohlf, 1995) with initial height as a covariate to test for significance of differences in traits given the correlation structure. Because of limited degrees of freedom, responses were

analyzed in three groups for MANCOVA. Traits were analyzed in three groups: (1) morphological traits (2) physiological traits and (3) leaf nutritional traits. Height, total biomass, leaf area, shoot biomass and root biomass were loge-transformed to meet the assumptions of normality. We constructed MANCOVA models including the effects of microhabitat source, salinity treatment, family nested within microhabitat source and the interaction between microhabitat and salinity treatment (Table 4.1a.). We removed the effect of microhabitat source ( $P > 0.05$ ) for the final MANCOVA model which showed consistent significant effect of salinity treatment, family and interaction between salinity treatment and family (Table 4.1b) with the exception of the leaf nutritional traits ( $P = 0.06$ ). For each response variable, we also performed a separate univariate ANCOVA in which we included only independent variables that were determined to be significant in the MANCOVA: salinity treatment, family, the interaction between salinity treatment and family, block and initial height.

## Results

### Plasticity of salt tolerance traits

The response of seedlings to salinity treatment indicated that all of the phenotypic characters considered are highly plastic ( $P < 0.001$ , Table 4.2). All morphological characters: final height, total leaf area, shoot biomass, root biomass and total biomass decreased in response to high levels of salinity (Table 4.3). The magnitude of the change varied among traits. Total height was reduced by 53% and total leaf area by 90%. Total biomass was reduced by 89% in the high salt treatment, which reflected an 87% reduction in shoot biomass and a 91% reduction in root biomass. Photosynthesis decreased by 81% in the high salt treatment. Similarly, many of the physiological traits that are expected to increase with increasing salt stress did increase (Table

4.3). Leaf succulence increased by over 3500%, sodium levels increased by 163%, leaf N increased by 26%, midday xylem pressure potentials decreased by 115% and  $\delta^{13}\text{C}$  values decreased 10% indicating an increase in WUE. In keeping with predicted salt stress response, the leaf nutrient concentrations (B, Ca, K, Mg, Mn and P) were reduced in the high salt treatment.

#### Genotypic variation and local adaptation in *B. frutescens*

Across microhabitats, there was significant among family variation in only four traits as indicated by the significant family effect for final height, leaf Mg, Na and P (Table 4.2). Chi square contingency tables showed no significant difference in mortality between families ( $df= 13$   $\chi^2= 11.913$ ,  $P= 0.535$ ). We found significant G\*E (family\*salinity) for only Na and B (Table 4.2).

The performance of seedlings grown from low salt and high salt microhabitats gave no indication that genetic specialization has occurred because the seedlings from the two microhabitats are not genetically different from each other as indicated by the lack of significance of the microhabitat effect in the nested MANCOVA (Table 4.1a). In all cases, the general patterns of phenotypic response were similar in families from high salt and low salt microhabitats. In addition, flowering occurred in only six families (23 individuals): three from high salt and three from low salt source microhabitats. Plants from both microhabitats suffered mortality only in the high salinity treatment (15% and 21% from high salt and low salt microhabitats respectively). Chi square contingency tables showed no significant difference in mortality between families from the two microhabitats ( $df= 1$   $\chi^2= 0.811$ ,  $P = 0.368$ ).

### *Discussion*

*Borrchia frutescens* occurs along a steep environmental gradient and demonstrates substantial phenotypic variation across environments (Richards, 2004). We thus predicted that while plants from different microhabitats would have some degree of phenotypic plasticity in response to environmental variation, differential selection pressures would lead to high levels of genetic variation for important ecological traits and adaptation through genetic specialization to high and low salt microhabitats. In general, we found that traits were extremely plastic in response to controlled salinity treatments. However, across microhabitat type we found significant genetic variation for only 4 out of 17 traits and that families reacted differently to salt treatment (significant family by salinity interaction or G\*E) for only 2 traits. The results give no evidence for genetic specialization (local adaptation) in any of the 17 traits measured in this study, but suggest that the high degree of plasticity in these traits allows plants to live across a broad range in salinity.

### *Plasticity of ecologically important traits*

Plants that inhabit highly saline areas are characterized by traits that specifically ameliorate the toxic and osmotic effects of substrate salinity (Flowers *et al.*, 1977). In this study, salinity had a significant effect on all 17 traits measured, indicating that these traits are highly plastic. The pattern of response to high salt for all traits were as predicted: decreases in biomass and nutrient uptake (with the exception of N which is used in the production of compatible solutes), decreases in midday xylem pressure potentials, and increases in leaf succulence, WUE, N and Na. Although these trait responses are inevitable to some degree, we suggest that these

patterns of plasticity are adaptive because they allow plants to tolerate a broad range of salinity (Sultan, 1997; Pigliucci, 2001).

#### *Adaptive phenotypic plasticity*

These plastic responses include the inevitable effects of limitation of growth and physiology in the presence of salt. However, the plasticity of these traits allowed these plants to adjust and survive under different salt environments which suggests that the plasticity of these traits is adaptive. However, truly testing the adaptive value of plasticity is difficult because this would require comparing the effect of different trait values across environments. However, plasticity itself prevents expression of the “wrong” phenotype in an environment. Therefore, proof of adaptive plasticity has been limited to traits that can be induced to express the wrong phenotype either by an environmental cue or genetic modification (Schmitt *et al.*, 1995; Dudley & Schmitt, 1996; Sultan 2000).

#### *Genotypic differentiation in *B. frutescens**

Theoretical and empirical studies illustrate that genetic diversity can be maintained by habitat heterogeneity if genotypes differ in relative fitness under different conditions (Levene, 1953; Hedrick, 1976; Caisse & Antonovics, 1978; Feder *et al.*, 1997; Schmidt & Rand, 1999). We predicted that because of the high degree of environmental variation experienced by *B. frutescens* along the marsh gradient, differential selection should result in a substantial amount of genetic variation for salt tolerance traits. Instead we found that there was a significant effect of family for only 4 traits: height, leaf Mg, Na and P. This was true despite the fact that we used naturally outcrossed seeds, which should contain the maximum amount of genetically based

variation compared to mature individuals in the field, which have experienced selection. While these four traits are decidedly important for salt tolerance, it is surprising that there is no variation in the other 13 traits we measured.

#### Local adaptation in *B. frutescens*

If genotypes of *B. frutescens* were adapted to local conditions, genotypes from the high salt end of the gradient would have greater performance under high salt conditions than genotypes from the low salt end of the gradient. Similarly, low salt source genotypes should perform better than high salt source genotypes under low salt conditions. However, we found that genotypes from both microhabitats showed similar responses to variation in salinity (no effect of source microhabitat and no source microhabitat x salinity treatment interaction). Under high salt conditions, genotypes from the high salt areas did not have higher WUE, were not more succulent, did not have less negative midday xylem pressure potentials nor did they have higher photosynthetic rates compared to genotypes from the low salt areas. Thus, traits associated with salt tolerance did not show a pattern consistent with local adaptation.

After determining that these traits did not differ for families from the high salt microhabitat as compared to families from the low salt microhabitat, we looked for evidence of variation in plasticity (G\*E) at the family level across microhabitats. We found significant G\*E for only Na and B. This finding provides further evidence for the importance of plasticity for most traits in this species because as selection favors plasticity, it has been suggested that there should be less variation (G\*E) in plasticity (Pigliucci, 2001).

The significance of the G\*E for Na uptake is due to the fact that two families from the low salt microhabitat took up significantly more Na in the high salt treatment, which is the

opposite of our prediction for local adaptation. One interpretation might be that maintaining a lower level of leaf tissue Na instead of tolerating a high level of Na is adaptive, for example if *B. frutescens* relied more on compatible solutes instead of using Na ions to maintain osmotic adjustment (Cavaliere and Huang 1979). Alternatively, it may be that combining all of the live leaves at harvest might have underestimated the salt loading in the high salt treatment for families that previously shed salt-loaded leaves. Because the mechanisms employed by *B. frutescens* to tolerate high substrate salinity are not completely understood, we cannot say with certainty that maintaining lower levels of leaf Na are adaptive for this species. Indeed, field reciprocal transplants suggest that there is no local adaptation for this trait (Richards, 2004).

The significant interaction for B uptake is similarly driven by one of these same families, which had a pattern opposite to the rest of the families. The significance of B in the salt marsh system is not known, however it has been shown to cause toxicity in sunflower cultivation in low rainfall regions (Blamey *et al.*, 1997). Rosenthal *et al.* (2002) report that the desert adapted *Helianthus deserticola* had genetically based reduction of B uptake compared to a suite of closely related *Helianthus* species in controlled glass house experiments suggesting that this may be an important adaptation to the desert habitat.

A lack of local adaptation could be due to unpredictable environmental variation, a low cost of plasticity, high gene flow, or insufficient genetic variation. One or more of these factors may explain the lack of evidence for genetic specialization found in this study. Local adaptation is more likely to occur in constant than in variable environments. While regular patterns of tidal movement make conditions in southeastern United States salt marshes fairly predictable on short temporal scales (Pomeroy & Weigert, 1981), conditions vary on a daily, monthly and seasonal scale. In addition, storms and shifts in barrier island geomorphology

make marshes dynamic on longer temporal scales, and it is likely that these environmental changes reduce the benefits of adaptation to local conditions. This may be especially important for a long-lived perennial species like *B. frutescens*. The life span of a given genet is unknown, but probably longer than the time frame during which local conditions are predictable.

Likewise, genetic specialization is only expected to occur if plasticity is costly (DeWitt *et al.*, 1998; Pigliucci, 2001; Poulton & Winn, 2002). Although the construction and maintenance of organelles and the metabolism used in active ion transport and other processes involved in salt tolerance should be costly, it is possible that plasticity in these traits may not confer a cost. For example, Smekens & van Tienderen (2001) found no costs of plasticity of most traits related to saline conditions in the salt tolerant Buck's-horn plantain, *Plantago coronopus*.

Local adaptation is also less likely if gene flow is sufficiently high. In *Borrchia*, genetic neighborhood sizes appeared to be small enough to allow the possibility for local adaptation (Antlfinger, 1981, 1982) and many studies have demonstrated that genetically differentiated ecotypes occur even under high levels of gene flow (Antonovics & Bradshaw, 1970; Antlfinger, 1981; Silander, 1984, 1985; Nagy & Rice, 1997). However, gene flow in *B. frutescens* may be sufficiently high to prevent local adaptation. Lending support to this conclusion, a concurrent genetic study of this population of *B. frutescens* determined that there is relatively high genetic variation but no differentiation of specific loci across salinity gradients (Richards, 2004).

Finally, local adaptation would not be expected if there was insufficient genetic variation for important traits, since genetic variation is requisite for evolutionary change (Via

& Lande, 1985; Via, 1994). Although all of the traits we measured are highly plastic, there was little evidence that variation in responses were genetically based. Salt tolerance may have been under sufficiently strong selection that salt tolerance traits in *B. frutescens* have reached the limits of plant physiology, leaving insufficient variation for the evolution of locally adapted ecotypes.

*Borrchia frutescens* lives in salt marshes, which, because of their extreme edaphic conditions, might favor specialist inhabitants. Many studies of this nature however, have revealed evidence for adaptive plasticity for a variety of purportedly adaptive traits including response to light availability (Schmitt 1993; Dudley & Schmitt 1996; Sultan & Bazzaz, 1993a), water availability (Sultan & Bazzaz, 1993b; Dudley 1996), nutrient availability (Crick & Grime, 1987; Sultan & Bazzaz, 1993c), salt tolerance (Hester *et al.*, 1996; Smekens & van Tienderen, 2001), and antipredator morphology (van Buskirk & Ralyea, 1998; Lively 1986). Considering the pattern of trait response that we report, it appears that *B. frutescens* behaves more like a habitat generalists. Habitat generalists occur in habitats characterized by a wide range of conditions which they often accommodate by high levels of phenotypic plasticity (Baker, 1965; Bazzaz, 1986; Sultan *et al.*, 1998). Considering the broad range and temporal variation of environmental factors that typify salt marsh species habitat, perhaps phenotypic plasticity will be more the rule than the exception in this system.

Our results come with two caveats. First, our study was short term and performed using naturally outcrossed seed. This study would be informed by measuring the response of clonal replicates collected from individuals in the field. Mature plants in the field have survived generations of natural selection and therefore might reflect different patterns of adaptation (Jain & Bradshaw, 1966; Stanton & Galen, 1997). Second, this study was performed in the controlled

environment of the greenhouse allowing us to isolate the effects of one important environmental factor. However, environmental gradients, and the responses of *B. frutescens* to these gradients, are likely to be complex and multivariate. Reciprocal transplants of field collected *B. frutescens* reveal that there is genetic variation for most of the traits measured here and that some of these traits demonstrate patterns consistent with local adaptation (Richards, 2004).

In sum, we found that all 17 traits of *B. frutescens* had significant plastic responses to controlled salinity treatments suggesting adaptive plasticity. There was no association of microhabitat source and performance in two different levels of salinity. We also found that there was genetic variation for only 4 traits and a significant G\*E for only B and Na. We conclude that the broad range and temporal variation of environmental conditions that characterize the microhabitats of *B. frutescens* may select for adaptive phenotypic plasticity instead of genetic specialization in this species.

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Table 4.1. MANOVA's for a.) full model including main effect of microhabitat and b.) family model without the main effect of microhabitat. Traits are grouped as 1) biomass traits including height, total biomass, leaf area, shoot biomass, root biomass, 2) physiological traits including succulence, photosynthesis, midday xylem pressure potential, and water use efficiency and 3) leaf nutrition traits including leaf Nitrogen (N), Boron (B), Calcium (Ca), Potassium (K), Magnesium (Mg), Manganese (Mn), Sodium (Na) and Phosphorous (P). Multivariate F- statistics are presented with significance levels.

a.) Full model

|                       | Microhabitat<br>df = 1 | Salinity<br>treatment<br>df = 1 | Family<br>(microhabitat)<br>df = 12 | Microhabitat<br>x Salinity treatment<br>df = 1 |
|-----------------------|------------------------|---------------------------------|-------------------------------------|--|
| Multivariate <i>F</i> |                        |                                 |                                     |  |
| Biomass traits        | 2.060NS                | 316.480***                      | 1.570**                             | 0.980NS  |
| Physiological traits  | 0.480NS                | 55.770***                       | 1.230NS                             | 1.600NS  |
| Leaf Nutrition traits | 1.790NS                | 115.810***                      | 1.470**                             | 2.800*   |

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; NS  $P \geq 0.05$ .

b.) Family model

|                       | Salinity<br>treatment<br>df = 1 | Family<br>df = 12 | Family x<br>Salinity treatment<br>df = 1 |
|-----------------------|---------------------------------|-------------------|--|
| Multivariate <i>F</i> |                                 |                   |  |
| Biomass traits        | 302.290***                      | 1.650***          | 1.510**                                  |
| Physiological traits  | 40.110***                       | 1.690*            | 1.750*                                   |
| Leaf nutrition traits | 98.480***                       | 1.490**           | 1.360NS                                  |

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; NS  $P \geq 0.05$ .

Table 4.2. Two-way ANCOVA for biomass, gas exchange and elements. Mean square, F statistics and significance are presented for the main effects of salinity treatment, family and treatment by family interaction. Midday XPP= Midday xylem pressure potential, WUE= Water Use Efficiency, N= leaf Nitrogen, B= leaf Boron, Ca= leaf Calcium, K= leaf Potassium, Mg= leaf Magnesium, Mn= leaf Manganese, Na= leaf Sodium and P= leaf Phosphorous.

|                | Adj R <sup>2</sup> | Salinity       |             | Family      |          | Family x Salinity |          | Error |             |
|----------------|--------------------|----------------|-------------|-------------|----------|-------------------|----------|-------|-------------|
|                |                    | df = 1         |             | df = 13     |          | df = 1            |          | df    | MS          |
|                |                    | MS             | F           | MS          | F        | MS                | F        |       |             |
| Final height   | 0.80               | 25.01          | 444.58 ***  | 0.11        | 1.92 *   | 0.07              | 1.24 NS  | 153   | 0.06        |
| Total biomass  | 0.92               | 212.84         | 1377.77 *** | 0.20        | 1.29 NS  | 0.13              | 0.86 NS  | 149   | 0.15        |
| Leaf area      | 0.81               | 349.16         | 506.14 ***  | 1.13        | 1.64 NS  | 0.90              | 1.31 NS  | 147   | 0.69        |
| Shoot          | 0.88               | 192.47         | 905.83 ***  | 0.36        | 1.70 NS  | 0.26              | 1.23 NS  | 149   | 0.21        |
| Root           | 0.93               | 236.06         | 1685.72 *** | 0.17        | 1.18 NS  | 0.14              | 0.99 NS  | 149   | 0.14        |
| Succulence     | 0.39               | 0.32           | 39.37 ***   | 0.01        | 1.53 NS  | 0.01              | 1.59 NS  | 145   | 0.01        |
| Photosynthesis | 0.48               | 2293.47        | 55.13 ***   | 34.05       | 0.82 NS  | 43.69             | 1.05 NS  | 127   | 41.60       |
| Midday XPP     | 0.76               | 4767.96        | 56.53 ***   | 74.07       | 0.88 NS  | 101.65            | 1.21 NS  | 32    | 84.35       |
| WUE            | 0.87               | 214.79         | 408.48 ***  | 0.57        | 1.09 NS  | 0.68              | 1.29 NS  | 83    | 0.53        |
| N              | 0.49               | 4.16           | 45.10 ***   | 0.14        | 1.48 NS  | 0.08              | 0.90 NS  | 83    | 0.09        |
| B              | 0.54               | 2013.27        | 5.80 *      | 510.44      | 1.47 NS  | 884.19            | 2.55 *   | 71    | 346.84      |
| Ca             | 0.91               | 4968015626.00  | 445.57 ***  | 12353212.00 | 1.11 NS  | 6759316.00        | 0.61 NS  | 71    | 11149845.00 |
| K              | 0.52               | 378876221.90   | 20.85 ***   | 24619759.60 | 1.35 NS  | 17478386.10       | 0.96 NS  | 71    | 18175867.00 |
| Mg             | 0.86               | 93558958.70    | 259.09 ***  | 1109144.59  | 3.07 **  | 241245.64         | 0.67 NS  | 71    | 361105.50   |
| Mn             | 0.64               | 246422.18      | 60.24 ***   | 7736.78     | 1.89 NS  | 1957.70           | 0.48 NS  | 71    | 4090.92     |
| Na             | 0.94               | 11140305828.00 | 783.38 ***  | 51530275.00 | 3.62 *** | 91902061.00       | 6.46 *** | 71    | 14220812.00 |
| P              | 0.51               | 962358.13      | 6.28 *      | 342607.03   | 2.24 *   | 254468.59         | 1.66 NS  | 71    | 153224.46   |

Table 4.3. Total sample number (N), means (and standard errors) for 17 traits measured in high and low salt source plants under high and low salt treatments. Midday XPP= Midday xylem pressure potential, WUE= Water Use Efficiency, N= leaf Nitrogen, B= leaf Boron, Ca= leaf Calcium, K= leaf Potassium, Mg= leaf Magnesium, Mn= leaf Manganese, Na= leaf Sodium and P= leaf Phosphorous.

|   | Low salt treatment |                 |    |                  | High salt treatment |                 |    |                  |
|---|--------------------|-----------------|----|------------------|---------------------|-----------------|----|------------------|
|   | N                  | Low salt plants | N  | High salt plants | N                   | Low salt plants | N  | High salt plants |
| Final height (cm)                       | 56                 | 41.84(1.25)     | 48 | 39.50(1.13)      | 44                  | 18.23(0.82)     | 41 | 19.12(0.92)      |
| Total biomass (g)                       | 56                 | 22.89(0.55)     | 48 | 23.46(0.66)      | 41                  | 2.52(0.21)      | 40 | 2.71(0.25)       |
| Leaf area (cm <sup>2</sup> )            | 56                 | 352.64(10.52)   | 47 | 355.79(11.44)    | 41                  | 33.15(6.45)     | 39 | 34.08(5.26)      |
| Shoot (g)                               | 56                 | 11.65(0.35)     | 48 | 11.50(0.43)      | 41                  | 1.47(0.14)      | 40 | 1.57(0.17)       |
| Root (g)                                | 56                 | 11.24(0.42)     | 48 | 11.97(0.42)      | 41                  | 1.05(0.07)      | 40 | 1.14(0.09)       |
| Succulence (mg/ cm <sup>2</sup> )       | 56                 | 2.59(0.09)      | 47 | 2.56(0.09)       | 40                  | 108.78(26.14)   | 38 | 83.93(18.11)     |
| Photosynthesis (μmol/m <sup>2</sup> /s) | 55                 | 10.98(1.12)     | 46 | 10.96(1.21)      | 30                  | 1.81(0.40)      | 32 | 2.44(0.43)       |
| Midday XPP (MPa)                        | 21                 | -17.9(1.4)      | 15 | -1.54(1.3)       | 15                  | -36.6(3.3)      | 16 | -36.1(2.7)       |
| WUE (δ <sup>13</sup> C ppt)             | 28                 | -29.42(0.12)    | 28 | -29.16(0.10)     | 32                  | -26.30(0.16)    | 31 | -26.43(0.18)     |
| N (%)                                   | 28                 | 1.56(0.05)      | 28 | 1.49(0.05)       | 32                  | 1.91(0.06)      | 31 | 1.77(0.07)       |
| B (ppm)                                 | 46                 | 99(3.26)        | 40 | 104(3)           | 5                   | 104(9)          | 11 | 75(6.)           |
| Ca (ppm)                                | 46                 | 26339(555)      | 40 | 27413(648)       | 5                   | 2621(310)       | 11 | 3208(294)        |
| K (ppm)                                 | 46                 | 13811(687)      | 40 | 15356(714)       | 5                   | 5141(1211)      | 11 | 8597(1038)       |
| Mg (ppm)                                | 46                 | 4174(117)       | 40 | 4157(119)        | 5                   | 892(58)         | 11 | 1107(82)         |
| Mn (ppm)                                | 46                 | 215(11)         | 40 | 181(9)           | 5                   | 37(3)           | 11 | 37(3)            |
| Na (ppm)                                | 46                 | 21513(449)      | 40 | 20494(464)       | 5                   | 62704(6062)     | 11 | 51930(1698)      |
| P (ppm)                                 | 46                 | 1960(61)        | 40 | 2026(67)         | 5                   | 1640(419)       | 11 | 1576(114)        |

CHAPTER 5  
THE RESPONSE TO DIFFERING SELECTION ON PLANT PHYSIOLOGICAL TRAITS IN  
CONTRASTING SALT MARSH ENVIRONMENTS<sup>1</sup>

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## Abstract

Understanding adaptive evolution to differing environments requires studies of genetic variation for ecologically important traits, documenting variation in the patterns of natural selection in contrasting environments and measuring response to selection. A suite of physiological traits have been reported to be important for dealing with the stress of substrate salinity, but few studies have investigated how selection varies on these traits in environments of contrasting salt content. In this study, we performed a reciprocal transplant experiment with the perennial *Borrichia frutescens* collected from a high salt and a low salt habitat to gain insight into how differential patterns of selection vary in the salt marsh. We found genetic variation for thirteen putative salt tolerance and performance traits. At the genet level, we found selection for increased total leaf area and water use efficiency (WUE) in both high and low salt gardens. In addition, while there was no stabilizing or disruptive selection in the high salt garden, the low salt garden selected for intermediate levels of leaf area, N and WUE. Patterns of selection were significantly different in the two gardens only for stabilizing selection on WUE. We found negative genetic correlations between WUE and the four elements Na, N, Ca and P which could partly explain why these traits showed no response to selection. In contrast, positive genetic correlations between WUE and succulence could be reinforcing the patterns that we see for each of these traits.

Key words: adaptive differentiation, *Borrichia frutescens*, natural selection, partial regression analysis, salinity gradient, salt marsh plant, sea ox-eye daisy

## Introduction

Understanding adaptive evolution to differing environments requires studies of genetic variation for ecologically important traits, documenting variation in the patterns of natural selection in contrasting environments and measuring response to selection. While the occurrence of genetic differentiation and local adaptation has been well documented (Linhart and Grant 1996), how selection acts in different environments to create these genetic differences has been little explored. Few studies have actually measured the strength and direction of selection in natural contrasting environments, despite the fact that estimating these selection parameters is essential for an understanding of how adaptation to local conditions is created and maintained (Kingsolver et al. 2001).

A thorough understanding of selection in contrasting environments requires examination of not only how selection affects overall performance, but also how selection acts on specific traits and on suites of traits (Donovan and Ehleringer 1994, Chapin et al. 1993, Farris and Lechowicz 1990, Geber and Dawson 1990, 1997). By using multiple regression techniques (Lande and Arnold 1983), it is possible to evaluate a suite of potentially important traits to identify those that significantly relate to fitness and that are therefore likely to evolve. These analyses have identified that the adaptive value of traits changes across contrasting environments (reviewed in Kingsolver et al. 2001, Arntz and Delph 2001, Ackerly et al. 2000). These analyses also provide information on how traits are correlated and how these correlations may either constrain or enhance adaptation. Constraints to adaptation due to character correlations have been of considerable interest in evolutionary theory (Arnold 1992, Endler 1986).

Salt marshes are ideal systems in which to study the effects of differential selection on important traits because they exhibit strong gradients of environmental stresses and contain

plants with dramatic differences in phenotype that are correlated to these conditions (reviewed in Pennings and Bertness 2001, Richards et al. in review). Many studies have examined how community level patterns in salt marshes are affected by environmental gradients (Bertness and Ellison 1987, Seliskar 1985a & b, 1987, Bertness et al. 1992, Bertness and Hacker 1994, Pennings and Callaway 1992, Pennings and Richards 1998) however, little research has addressed genetic variation and variation in selection across these gradients. It remains unresolved, for instance, whether the extreme differences in plant phenotypes at the ends of the gradients are the result of genetically based response to selection (Anderson and Treshow 1980, Gallagher et al. 1988, Trnka and Zedler 2000). In one of the few studies examining genetic differentiation in marsh plants, Silander (1979, 1984, 1985) found that natural selection created genetically distinct populations of *Spartina patens* adapted to their microhabitats in dunes, swales and marshes in coastal North Carolina (nomenclature follows Radford et al. 1968 throughout). Silander did not however, identify which traits were responsible for the adaptive differentiation of these populations. In general, there is a lack of understanding of the nature of selection and potential for response to selection in salt marsh plant populations.

A predominant feature of the salt marsh environmental gradient is the broad range in salinity (Pennings and Bertness 2001). A suite of physiological traits have been reported to be important for dealing with the toxic and osmotic effects of substrate salinity (Flowers et al. 1977), but few studies have investigated how selection varies on these traits in environments of contrasting salt content. In particular, halophytes can secrete, sequester or dilute (by increasing succulence) what would otherwise be toxic levels of Na. In addition, conservative water relations can reduce the uptake of Na and reduce the water demands of the plant (Flowers et al. 1977). These habitats are typically not only toxic because of sodium levels, but they are low in

nitrogen (Smart and Barko 1980) and sodium can interfere with nutrient uptake (Flowers et al. 1977). Halophytes are able to maintain nutrient uptake in the presence of high Na and use Nitrogen rich compatible solutes and nutrient cations (K, Ca, Mg, Mn) for osmotic adjustment (Aerts and Chapin 2000, Antlfinger and Dunn 1983, Cavalieri and Huang 1979, Donovan et al. 1996, Donovan et al. 1997, Glenn and O'Leary 1984, Moon and Stiling 2000, Rosenthal et al. 2002).

In this study, we asked if these purported salt tolerance traits could evolve to different optima in response to contrasting salt marsh environments. Working with the dominant high marsh perennial *Borrchia frutescens* (Asteraceae: Sea Oxeye Daisy) on Sapelo Island, GA, we asked three major questions. First, is there genetic variation for ecologically important traits in *Borrchia frutescens*? Second, does selection on morphological and physiological traits in *B. frutescens* vary in contrasting environments? Third, are there trait correlations that constrain adaptive evolution in salt marsh environments?

## Methods

### *Study site and species*

Sapelo Island is located off the Atlantic coast of Georgia, USA (31° 28'N, 81° 14'W). The vegetation patterns in Sapelo Island marshes are typical of southeastern marshes in the United States (Pomeroy & Wiegert 1981). Lower elevations of the marsh are subject to daily tidal submergence and are dominated by the cord grass, *Spartina alterniflora*. The middle elevations of the marsh are flooded irregularly and are often characterized by highly saline salt pans and associated salt-tolerant species such as *Salicornia virginica*, *Salicornia biglovii*, *Batis maritima*, *Borrchia frutescens*, *Distichlis spicata* and *Sporobolus virginicus* (Antlfinger 1981).

The terrestrial border of the marsh is typically dominated by *Juncus roemerianus*, *Spartina patens* or *Iva frutescens*. *Aster tenuifolius* and *Limonium carolinianum* occur at higher elevations mixed in with the zonal dominants.

*Borrichia frutescens* occurs across a broad range of environments (for example, salinities range from 20 ppt to over 100 ppt) and exhibits extreme phenotypic variation (heights from over 100 cm to less than 10 cm, Richards et al. in review) making this species ideal for studying the mechanisms that create phenotypic variation across contrasting environments. This C<sub>3</sub> composite is abundant in the high marsh, which is marked by infrequent tidal flooding (Antlfinger 1981). *Borrichia frutescens* tolerates high substrate salinities by increasing succulence, actively taking up sodium and other ions as well as manufacturing the nitrogen rich compatible solutes proline and glycine-betaine (Cavaliere and Huang 1979, Antlfinger and Dunn 1983, Moon and Stiling 2000). *Borrichia frutescens* reproduces both clonally and sexually, although the relative contribution of each type of reproduction is currently unknown.

### *Experimental design*

In the fall of 2001, we collected 50 *B. frutescens* individuals separated by at least two meters from each of two microhabitats in the Cabretta Marsh on Sapelo Island. We collected individuals separated by at least two meters to minimize the possibility of collecting the same genet twice as per allozyme surveys across microhabitats (Richards et al. in prep). One source microhabitat is characterized by low salt concentration (25-30 ppt) and the second microhabitat is characterized by high salt (100-120 ppt Richards et al., in review). The two microhabitats are separated by approximately 25 m. We grew the field collected plants in the greenhouse for over a year to minimize environmental effects and clonally replicate genets. In June and July of 2002,

we propagated cuttings of each genet on a mist bench in the University of Georgia Plant Biology greenhouses for approximately 6-8 weeks. In early August of 2002, we chose 40 high salt and 40 low salt genets based on adequate establishment of at least 20 replicate cuttings (N=1600 cuttings). These cuttings were planted into 10 randomized complete blocks such that each block contained 2 replicates of each high salt and each low salt genet (block = 2 origin microhabitats \* 40 genets \* 2 replicates = 160 cuttings). We raised the cuttings in these blocks on the mist bench for 2 weeks and then in the greenhouse from mid August to late October 2002 under natural light conditions. Cuttings that did not survive any part of the transplant were replaced. On September 11, we began acclimating the plants to salt by applying a 5 ppt salt water (NaCl) solution. Salt water was applied twice a week and gradually increased from 5 ppt to 30 ppt on October 19.

On October 24 and 25 2002, we transplanted the ten blocks of *B. frutescens* back into the field: 5 blocks were transplanted into the high salt microhabitat and 5 blocks were transplanted into the low salt microhabitat. Therefore, we transplanted 10 replicates of each genet back into its "home microhabitat", and 10 replicates into the opposite microhabitat (N= 2 origin microhabitats \* 2 destination microhabitats \* 40 genets \* 10 replicates = 1600). Just prior to transplanting, we measured initial shoot height on all individuals. We replaced dead transplants monthly through February 2003 and recorded initial spring height on April 11, 2003. We recorded flowering and collected mature seed heads every 2 or 3 weeks throughout the summer.

On July 27, we collected 4-8 fully mature leaves for analysis of succulence (g water/ cm<sup>2</sup> leaf area), leaf elemental concentrations and integrated water use efficiency. Percent leaf nitrogen and integrated water use efficiency (WUE), as determined from stable carbon isotope ratios ( $\delta^{13}\text{C}$ ), were measured on dried leaf material using continuous flow mass spectrometry (Donovan and Ehleringer 1994, Farquar *et al.* 1982). More negative  $\delta^{13}\text{C}$  values are indicative of

lower integrative WUE. Acid extracts (Sah and Miller 1992) from leaf tissue were analyzed for Ca, K, Mg, Mn, Na and P on an inductively coupled plasma-atomic emission spectrophotometer (Thermo Jarrell-Ash Enviro 36 simultaneous inductively coupled argon plasma-emission spectrograph: Thermo Electron Corp., Woburn, MA). We measured final height and final number of leaves on all plants between 26 and 27 September. We harvested all plants between 4 and 6 October 2003 and separated individual plants into (1) leaves, (2) stems (3) roots and rhizomes. We measured area on the fresh leaves (Li-Cor Model LI-3100 Leaf Area Meter: LiCor, Inc., Lincoln, NE, USA) as well as the dry mass of each component. We also recorded the number of ramets as the number of stems originating from the roots and rhizomes.

We calculated the total leaf area and total number of leaves by adding the values collected in July to those collected in October. The total dry biomass included the leaf dry biomass from the July collection, leaf, stem, root and rhizome dry biomass from the October collection. We calculated succulence from the July leaf collections because unlike the leaves from the final October harvest, the July leaves were of uniform maturity across the experimental population. Succulence was calculated as the dry leaf biomass subtracted from the wet leaf biomass divided by the leaf area for each individual. We used two estimates of fitness in this study: total number of flowers and total dry biomass. Because less than one-third of the transplants flowered during the course of the experiment, we used total biomass for all selection analyses. Others have shown that for long lived clonal plants, total biomass is generally the best estimate of fitness (Watson et al. 1997, McLellan et al. 1997).

*Data analysis*

Our level of genetic analysis is the genet. Because each genet is an exact replicate of genotype, any among genet variance or covariance detected in this experiment represents total, rather than additive, genetic variance or covariance. Data were analyzed using the GLM procedure in SAS (SAS Institute 2000). After examination of residuals for normality and homoscedasticity, all variables were left untransformed.

We used the partial regression analyses of Lande and Arnold (1983) and of Rausher (1992). In these analyses, selection gradients are estimated as the partial regression coefficients of a trait regressed against relative fitness. Analyses were performed using both individual phenotypes (Lande and Arnold 1983) and genet means (Rausher 1992). The phenotypic analysis of Lande and Arnold (1983) has the advantage of providing more power than the family method of Rausher (1992). Rausher's approach is similar to that of the phenotypic analysis (Lande and Arnold 1983), but accounts for biases due to environmental correlations between traits and fitness by examining selection acting directly on genotypic means rather than on individual phenotypic values (Mauricio and Mojonier 1997). We measured selection acting on clonal replicates to estimate genotypic values.

In this regression analysis, partial regression coefficients are used to estimate selection gradients, which represent the direct action of selection on a trait. The partial regression coefficient of a linear regression of relative fitness on a trait estimates the directional selection gradient. The sign of the gradient indicates the direction of evolutionary change expected from selection acting directly on a trait. The second order coefficient of the quadratic regression of relative fitness on the trait estimates the stabilizing/disruptive selection gradient. The sign of the gradient indicates whether the fitness function is concave downward (negative = stabilizing

selection) or concave upward (positive = disruptive selection). The selective effect of destination microhabitat (high or low salt transplant) on plant traits was determined by comparing the slope or curvature of the fitness functions for the plant traits between the 2 types of microhabitats. If the slopes did not differ (no significant transplant by plant trait interaction) and the curvature is the same (no significant transplant by plant trait<sup>2</sup> interaction), then microhabitats are not exerting differential selection. Alternatively, a significant transplant by plant trait interaction for fitness implies that microhabitats impose different selection on plant traits (sensu Mauricio and Rausher 1997).

We ran a preliminary selection analysis on the phenotypic data of eleven of the fourteen traits measured (excluding height, total biomass and total flower number) to identify which traits were under selection. None of the micronutrients (Ca, K, Mg, Mn P), or PCA1 of a Principal Component Analysis (SAS 2000) of these 5 elements, showed a significant relationship with relative biomass. These elements along with final number of leaves (also not significantly correlated to fitness) were therefore dropped from the selection analysis. Results from genet and phenotypic level analyses are presented for the five traits that were significantly correlated to fitness in this preliminary phenotypic selection analysis: Total Leaf Area (TOTLA), Succulence, WUE ( $\delta^{13}\text{C}$ ), N and Na. We calculated Pearson correlation coefficients between all traits using PROC CORR (SAS 2000). Significance values were determined by the sequential Bonferroni technique (Rice 1989) using an experiment-wide significance value of 0.05.

## Results

### *Genetic variation for ecologically important traits*

We confirmed that the traits we measured were potentially ecologically important in these habitats because our transplant gardens had significantly different effects on trait values (TRANSPLANT effect, Table 5.1). Plants in the high salt garden had more than a 20% reduction in height, total number of leaves, total biomass, total N, total P, and total number of flowers per plant. Final concentration of K was reduced by 45% and total leaf area was reduced by 40%. The concentrations of Na and Ca were reduced by 7% and 3% in the high salt garden respectively while succulence increased by 10% and water use efficiency increased by 3%.

We found significant genetic variation in 13 of the 14 morphological and physiological traits that we analyzed in our experimental populations (Table 5.1) indicating that these traits can potentially evolve under natural selection. The one exception was the micro-nutrient Mn. Although there was genetic variation for almost all of the traits we measured, for most traits all genets appear to respond similarly to the two different gardens. The transplant by genet interaction (Table 5.1) indicated that genets respond differently to the two gardens for only two traits: leaf area and total biomass.

### *Selection in contrasting environments*

At the phenotypic level, we detected direct natural selection for an increase in succulence, total leaf area and WUE, and a reduction in N and Na in the high salt garden (Table 5.2a). There was no selection for N or Na in the low salt garden, but as in the high salt garden there was selection for an increase in succulence, total leaf area and WUE. In both gardens, there was directional selection at the genet level only for increased total leaf area and WUE (Table

5.2a). We found evidence at the phenotypic level of stabilizing selection for WUE and disruptive selection for total leaf area in the high salt garden, but only for stabilizing selection on WUE in the low salt garden (Table 5.2b). At the genet level however, the patterns were slightly different. There was no evidence of stabilizing or disruptive selection in the high salt garden. The low salt garden selected for intermediate levels of total leaf area, WUE and N (Table 5.2b).

We found that there were significant differences in the pattern of directional selection on succulence and total leaf area in the two different gardens, but only at the phenotypic level. This pattern did not hold at the genet level and there were no traits for which directional selection was significantly different in the two gardens (Table 5.3a). In addition, the two gardens differed significantly in patterns of stabilizing selection for both leaf area and WUE at the phenotypic level, but only for WUE at the genet level (Table 5.3b).

#### *Trait correlations*

There was a negative correlation between WUE and many of the elements purported to be important for salt tolerance. At both the phenotypic and genet levels, WUE was negatively correlated to Na, N, Ca, K, Mg and P (Table 5.4). In addition, WUE was positively and leaf area was negatively correlated to succulence (Table 5.4).

## **Discussion**

In this experiment, we found genetic variation for thirteen putative salt tolerance and performance traits. We also found selection for increased total leaf area and water use efficiency (WUE) in both high and low salt gardens. Patterns of selection varied in the two environments, however these differences were only significant for stabilizing selection on WUE. We found

significant genetic correlations between WUE and many traits which could be enhancing or constraining the evolution of those traits based on the patterns of selection we found.

*Genetic variation exists for ecologically important traits*

We found that our two common gardens significantly affected all of the traits that we measured. As predicted by physiological constraints to tolerating substrate salinity, plants were shorter, had less biomass, fewer leaves and smaller leaf area as well as reduced concentrations of essential nutrients. Similarly, succulence and WUE increased in response to high salt. Surprisingly, there was no increase in tissue Na, or the ions typically associated with osmotic adjustment (K, Ca, Mg, Mn). Instead, we found a decrease in these elements in response to high salt.

Many studies have demonstrated that even under high levels of gene flow, this type of environmental variation can result in genetic differentiation within species on a fine spatial scale (Antonovics and Bradshaw 1970, Antlfinger 1981, Nagy and Rice 1997, Silander 1984, 1985). The current study revealed a high degree of genetic variation for purportedly adaptive traits. Although our broad-scale genetic parameters included non-genetic components (*e.g.* maternal or environmental effects), we attempted to minimize those effects by growing all clones in the greenhouse for over a year. Our current findings are in contrast to our previous work in the greenhouse where we only found genetic variation for height, Mg, Na and P in response to controlled salinity treatments (Richards et al. in prep). That study however, was limited by small sample size. In addition, the greenhouse study manipulated only salt concentration holding plentiful and constant levels of water and nutrients. The discrepancy between the two studies could reflect that *B. frutescens* responds differently when it is only stressed by variation in salt

and has plenty of other nutrients available to cope with that salt (as was the case in the greenhouse). Numerous studies have reported that the salt marsh is a nutrient poor habitat (Mendelssohn and Morris 2000, Smart and Barko 1980) which could limit the ability of salt marsh plants to manufacture nitrogen rich compatible solutes or use nutrient ions for osmoregulation. Use of these elements would then not be important for adaptation to contrasting environments in the salt marsh, there would be no differential selection on the ability to uptake these elements and this would be reflected in no genetic variation for concentrations in our experimental plants.

The current study is similar to the previous greenhouse study in that we found surprisingly few G\*E interactions. In the greenhouse, we found G\*E for Na which was not evident in the current study. In the field, we revealed that genets respond differently to contrasting multivariate environments in only total leaf area and total biomass. In both studies, we predicted that genotypes from the areas of the marsh with predictably higher salt levels would demonstrate specialization to tolerate high salt concentrations in many if not all trait responses. Both studies however support the hypothesis that *B. frutescens* copes with substrate salinity through a high degree of plasticity. Although we would expect that the maintenance of machinery and physiology to process high sodium levels should be costly, and therefore lead to variation in plasticity depending on habitat, Smekens and Van Tienderen (2001) found no costs of plasticity to saline conditions for most traits in the salt tolerant Buck's-horn plantain, *Plantago coronopus*.

*Selection varies in contrasting environments*

By examining clonal replicates transplanted into separate microhabitats, we expected to find that the high salt environment would select for different trait optima than the low salt environment for *B. frutescens*. We expected that the high salt environment would select for increased tolerance of osmotic stress and nutrient stress. In particular, selection would act to increase succulence (g water/ cm<sup>2</sup> leaf area), uptake of macro and micro-nutrients (reflected in higher leaf tissue concentrations) and Na for osmotic adjustment as well as increase WUE and decrease leaf size to minimize the water requirements of the plant. At the genet level, we found evidence supporting these predictions for only WUE. Our study suggests that there is positive directional selection in both high and low salt gardens as well as stabilizing selection for WUE in the low salt garden. In contrast to our predictions about leaf area however, we found evidence for selection for increased total leaf area in both high and low salt gardens indicating that this is not an important component of limiting the water demands of the plant. Instead, the significance of this trait could reflect the unavoidable relationship between leaf area and our measure of fitness, total biomass. This does not however explain the significance of stabilizing selection on this trait in the low salt garden. This could be the result of balancing an increase in biomass with a decrease in surface area and therefore water demands of the plant. We were surprised to find no selection on other purportedly important salt tolerance traits, though this could be due to trait correlations which we discuss below.

Patterns of selection were significantly different in the two gardens only for stabilizing selection on WUE suggesting that the two gardens select for different optima in this trait. Our data therefore suggest that overwhelmingly WUE is one of the most important traits allowing *B. frutescens* to tolerate this habitat. However, three way ANCOVA examining the interaction

between the source of the genets (from high or low salt sources) and transplant garden does not support the hypothesis that high salt genets have higher WUE in the high salt garden ( $MS=0.28$ ,  $F=0.46$ ). Therefore we have no evidence for local adaptation in this trait.

In general, the patterns that we detected at the genet level were similar to those that we detected at the phenotypic level. Although the phenotypic analysis suggests that direct selection on WUE and stabilizing selection on total leaf area, succulence and WUE varies significantly between the two gardens, the genet level analysis refutes this pattern. Although our sample size is dramatically reduced in our genet level analysis, these data seem robust according to the assumptions of normality. We suggest that the patterns we found in the phenotypic analysis could very well be due to environmental correlations between traits and fitness (Mauricio and Mojonier 1997, Rausher 1992).

#### *Trait correlations potentially constrain adaptive evolution*

There are several reasons why selection might not be able to optimize all traits in all environments. Chief among those constraints on the evolution of traits is the existence of negative genetic correlations among traits such that selection to increase the value of one trait leads to a decrease in the value of another trait (Via and Lande 1985). In the salt marsh, a negative genetic correlation between maximizing water use efficiency and the efficient uptake of nutrients and sequestering of sodium might constrain the ability of a halophyte to live in the most saline environment. In the current study, we found that selection was significant for WUE and leaf area which are not correlated in this population of plants. We did however find negative genetic correlations between WUE and the four elements Na, N, Ca and P which could partly explain why these traits showed no response to selection. If WUE is the predominant trait that

allows for *B. frutescens* to survive the multivariate conditions of the salt marsh, strong selection to optimize this trait would inevitably constrain the evolution of traits that are negatively correlated with WUE.

In sum, we found that all 14 phenotypes of *B. frutescens* responded differently to the contrasting salt marsh environments. We found that there was genetic variation for 13 of these 14 traits indicating the potential for response to differential selection. However, genets responded differently to the two gardens in only two traits: total leaf area and total biomass. We found selection for increased total leaf area and water use efficiency (WUE) in both high and low salt gardens, but patterns of selection were significantly different between the two environments for only stabilizing selection on WUE. We conclude that WUE is an extremely important trait allowing *B. frutescens* to occupy these habitats and a high degree of trait correlations constrain the adaptive evolution of many purported salt tolerance traits in *B. frutescens*.

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Table 5.1. Three-way ANCOVA for putative salt tolerance and plant performance traits in *B. frutescens*. Mean square, F statistics and significance are presented for the main effects of transplant garden, genet source, genet and transplant garden by genet interaction.

|                               | R <sup>2</sup> | Transplant |        | Source      |        | Genet (Source) |        | Transplant *<br>Genet (source) |      | Error |            |
|-------------------------------|----------------|------------|--------|-------------|--------|----------------|--------|--------------------------------|------|-------|------------|
|                               |                | df = 1     |        | df = 1      |        | df = 78        |        | df = 1                         |      | df    | MS         |
|                               |                | MS         | F      | MS          | F      | MS             | F      | MS                             | F    |       |            |
| HEIGHT                        | 0.61           | 11694.4    | 320*** | 264.51      | 7.2**  | 59.5           | 1.6*** | 41.7                           | 1 ns | 857   | 36.6       |
| TOTLA                         | 0.52           | 36121.1    | 122*** | 2030.40     | 6.9**  | 485.8          | 1.6*** | 599.5                          | 2*** | 731   | 296.2      |
| TOTLVS                        | 0.39           | 7421.6     | 60***  | 2132.61     | 17***  | 174.2          | 1.4*   | 140.4                          | 1 ns | 777   | 124.0      |
| SUCCULENCE                    | 0.46           | 0.0        | 153*** | 0.01        | 40***  | 0.04           | 4.8*** | 0.0                            | 1 ns | 979   | 0.0        |
| TOTBIO                        | 0.54           | 41.9       | 47***  | 4.60        | 5.1*   | 1.4            | 1.6**  | 1.2                            | 1*   | 726   | 0.9        |
| NFLOWER                       | 0.31           | 0.7        | 3.4 ns | 4.55        | 22***  | 0.7            | 3.5*** | 0.3                            | 1 ns | 1322  | 0.2        |
| WUE ( $\delta^{13}\text{C}$ ) | 0.44           | 121.4      | 172*** | 15.22       | 22***  | 1.4            | 2.1*** | 0.6                            | 1 ns | 875   | 0.7        |
| N                             | 0.49           | 20.3       | 294*** | 0.0003      | 0 ns   | 0.1            | 1.5**  | 0.1                            | 1 ns | 872   | 0.1        |
| Na                            | 0.48           | 821685590  | 80***  | 59977931.00 | 5.8*   | 18631433       | 1.8*** | 11286166.0                     | 1 ns | 577   | 10302660.0 |
| Ca                            | 0.36           | 17858567.8 | 6.8**  | 23865643.80 | 9.1**  | 6633894.1      | 2.5*** | 2841412.8                      | 1 ns | 577   | 2628324.0  |
| K                             | 0.60           | 3627783556 | 360*** | 41734837.00 | 4.1*   | 28016091       | 2.8*** | 10398351.0                     | 1 ns | 577   | 10073949.0 |
| Mg                            | 0.51           | 15577846.1 | 17***  | 1927749.10  | 2.1 ns | 4438646.2      | 4.8*** | 1029945.5                      | 1 ns | 577   | 921940.0   |
| Mn                            | 0.58           | 38532.3    | 473*** | 306.72      | 3.8 ns | 104.8          | 1.3 ns | 97.3                           | 1 ns | 577   | 81.5       |
| P                             | 0.61           | 37952299.4 | 533*** | 1245343.48  | 17***  | 172074.4       | 2.4*** | 80159.3                        | 1 ns | 577   | 71226.7    |

\* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001; NS P > 0.05.

Table 5.2. Natural selection gradients for five putative salt tolerance traits of *B. frutescens* from selection analyses. (A) Directional ( $\beta$ ) and (B) stabilizing ( $\gamma$ ) were analyzed separately to avoid biases (Lande and Arnold 1983). Standard errors are in parentheses. Significance levels are indicated as \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; NS  $P > 0.05$ .

A. *Directional selection*

|                               | Low salt garden         |                           | High salt garden              |                           |                            |
|-------------------------------|-------------------------|---------------------------|-------------------------------|---------------------------|----------------------------|
|                               | $\beta$ genet means     | $\beta$ phenotypic        | $\beta$ genet means           | $\beta$ phenotypic        |                            |
| TOTLA                         | 0.023913 ***<br>(0.001) | 0.020793 ***<br>(0.001)   | TOTLA                         | 0.034761 ***<br>(0.00363) | 0.021934 ***<br>(0.002)    |
| SUCCULENCE                    | 2.685004 ns<br>(2.412)  | 4.988521 ***<br>(1.479)   | SUCCULENCE                    | 2.778555 ns<br>(2.29749)  | 8.787231 ***<br>(1.566)    |
| WUE ( $\delta^{13}\text{C}$ ) | 0.113944 *<br>(0.052)   | 0.102403 ***<br>(0.026)   | WUE ( $\delta^{13}\text{C}$ ) | 0.09235 **<br>(0.035128)  | 0.084137 ***<br>(0.024)    |
| N                             | -0.08468 ns<br>(0.171)  | -0.13377 ns<br>(0.087)    | N                             | -0.00474 ns<br>(0.140086) | -0.28106 *<br>(0.132)      |
| Na                            | -1E-05 ns<br>(1.07E-05) | -7.6E-06 ns<br>(6.11E-06) | Na                            | -8.1E-06 ns<br>(9.57E-06) | -3.2E-05 ***<br>(8.42E-06) |

B. *Stabilizing/disruptive selection*

|  | Low salt garden           |                           | High salt garden                           |                         |                           |
|--|---------------------------|---------------------------|--|-------------------------|---------------------------|
|  | $\gamma$ genet means      | $\gamma$ phenotypic       | $\gamma$ genet means                       | $\gamma$ phenotypic     |                           |
| TOTLA <sup>2</sup>                         | -0.00014 **<br>(4.48E-05) | -1.3E-05 ns<br>(2.43E-05) | TOTLA <sup>2</sup>                         | 0.000457 ns<br>(0.0007) | -0.0002 ***<br>(5.47E-05) |
| SUCCULENCE <sup>2</sup>                    | -245.245 ns<br>(148.407)  | -23.6601 ns<br>(65.831)   | SUCCULENCE <sup>2</sup>                    | 94.73084 ns<br>(222.39) | -81.5613 ns<br>(74.060)   |
| WUE ( $\delta^{13}\text{C}$ ) <sup>2</sup> | -0.15315 *<br>(0.065915)  | 0.078892 ***<br>(0.022)   | WUE ( $\delta^{13}\text{C}$ ) <sup>2</sup> | -0.02543 ns<br>(0.062)  | 0.072725 ***<br>(0.016)   |
| N <sup>2</sup>                             | -1.340 *<br>(0.684705)    | -0.04507 ns<br>(0.153)    | N <sup>2</sup>                             | -0.39767 ns<br>(0.720)  | 0.472987 ns<br>(0.339)    |
| Na <sup>2</sup>                            | 0 ns<br>(0)               | 0 ns<br>(0)               | Na <sup>2</sup>                            | 0 ns<br>(0)             | 0 ns<br>(0)               |

Table 5.3. Analysis of variance for relative fitness of *Borrchia frutescens* grown in the field. The transplant effect refers to the experimental gardens characterized by high and low salt. All effects are considered fixed. (A) Directional selection. (B) Stabilizing/selection. Only linear terms are included in the directional estimate to avoid biased estimates.

| <b>A. Directional selection</b>          |     |             |        |        | <b>B. Stabilizing/disruptive selection</b>             |     |             |       |        |
|--|-----|-------------|--------|--------|--|-----|-------------|-------|--------|
| Source of variation                      | df  | Type III SS | F      | p      | Source of variation                                    | Df  | Type III SS | F     | p      |
| <b>Genet means</b>                       |     |             |        |        | <b>Genet means</b>                                     |     |             |       |        |
| GENET(ORIGIN)                            | 78  | 2.30127646  | 1.43   | 0.0652 | GENET(ORIGIN)  | 78  | 1.9600262   | 1.36  | 0.1025 |
| ORIGIN                                   | 1   | 0.15025597  | 7.29   | 0.0087 | ORIGIN   | 1   | 0.0995566   | 5.39  | 0.0235 |
| TRANSPLANT                               | 1   | 0.00001786  | 0      | 0.9766 | TRANSPLANT   | 1   | 0.13049     | 7.06  | 0.0099 |
| TOTLA                                    | 1   | 1.54367561  | 74.86  | <.0001 | TOTLA  | 1   | 0.5749496   | 31.12 | <.0001 |
| SUCCULENCE                               | 1   | 0.00715722  | 0.35   | 0.5577 | SUCCULENCE   | 1   | 0.0019219   | 0.1   | 0.7481 |
| WUE ( $\delta^{13}\text{C}$ )            | 1   | 0.00019907  | 0.01   | 0.922  | WUE ( $\delta^{13}\text{C}$ )                          | 1   | 0.1334291   | 7.22  | 0.0092 |
| N  | 1   | 0.00870956  | 0.42   | 0.5179 | N  | 1   | 0.0054317   | 0.29  | 0.5896 |
| Na                                       | 1   | 0.0027588   | 0.13   | 0.7157 | Na   | 1   | 0.0135974   | 0.74  | 0.3942 |
| TOTLA*TRANSPLANT                         | 1   | 0.01268005  | 0.61   | 0.4356 | TOTLA <sup>2</sup> *TRANSPLANT                         | 2   | 0.0837032   | 2.26  | 0.1121 |
| SUCCULENCE*TRANSPLANT                    | 1   | 0.00033668  | 0.02   | 0.8987 | SUCCULENCE <sup>2</sup> *TRANSPLANT                    | 2   | 0.0032016   | 0.09  | 0.9171 |
| WUE ( $\delta^{13}\text{C}$ )*TRANSPLANT | 1   | 0.00004343  | 0      | 0.9635 | WUE ( $\delta^{13}\text{C}$ ) <sup>2</sup> *TRANSPLANT | 2   | 0.1377815   | 3.73  | 0.0294 |
| N*TRANSPLANT                             | 1   | 0.02127234  | 1.03   | 0.3133 | N <sup>2</sup> *TRANSPLANT                             | 2   | 0.0788388   | 2.13  | 0.1268 |
| Na*TRANSPLANT                            | 1   | 0.00370027  | 0.18   | 0.6732 | Na <sup>2</sup> *TRANSPLANT                            | 2   | 0.0142982   | 0.39  | 0.6807 |
| ERROR                                    | 69  | 1.42290984  |        |        | ERROR  | 64  | 1.1825629   |       |        |
| <b>Phenotypic selection</b>              |     |             |        |        | <b>Phenotypic selection</b>                            |     |             |       |        |
| GENET(ORIGIN)                            | 78  | 7.67901473  | 1.05   | 0.3822 | GENET(ORIGIN)  | 78  | 6.8965533   | 0.98  | 0.5369 |
| ORIGIN                                   | 1   | 1.53868183  | 16.35  | <.0001 | ORIGIN   | 1   | 1.2825446   | 14.17 | 0.0002 |
| TRANSPLANT                               | 1   | 0.02684453  | 0.29   | 0.5936 | TRANSPLANT   | 1   | 0.5064396   | 5.6   | 0.0184 |
| TOTLA                                    | 1   | 30.79182962 | 327.12 | <.0001 | TOTLA  | 1   | 5.9733852   | 66.01 | <.0001 |
| SUCCULENCE                               | 1   | 1.62747581  | 17.29  | <.0001 | SUCCULENCE   | 1   | 0.2579009   | 2.85  | 0.0921 |
| WUE ( $\delta^{13}\text{C}$ )            | 1   | 0.82381933  | 8.75   | 0.0033 | WUE ( $\delta^{13}\text{C}$ )                          | 1   | 1.8488179   | 20.43 | <.0001 |
| N  | 1   | 0.16913291  | 1.8    | 0.1808 | N  | 1   | 0.0668488   | 0.74  | 0.3906 |
| Na                                       | 1   | 0.63376859  | 6.73   | 0.0098 | Na   | 1   | 0.0038746   | 0.04  | 0.8362 |
| TOTLA*TRANSPLANT                         | 1   | 0.45642848  | 4.85   | 0.0282 | TOTLA <sup>2</sup> *TRANSPLANT                         | 2   | 0.518929    | 2.87  | 0.0579 |
| SUCCULENCE*TRANSPLANT                    | 1   | 0.388123    | 4.12   | 0.0429 | SUCCULENCE <sup>2</sup> *TRANSPLANT                    | 2   | 0.5466924   | 3.02  | 0.0498 |
| WUE ( $\delta^{13}\text{C}$ )*TRANSPLANT | 1   | 0.08642549  | 0.92   | 0.3385 | WUE ( $\delta^{13}\text{C}$ ) <sup>2</sup> *TRANSPLANT | 2   | 1.857188    | 10.26 | <.0001 |
| N*TRANSPLANT                             | 1   | 0.00010603  | 0      | 0.9732 | N <sup>2</sup> *TRANSPLANT                             | 2   | 0.0337501   | 0.19  | 0.8299 |
| Na*TRANSPLANT                            | 1   | 0.02275867  | 0.24   | 0.6232 | Na <sup>2</sup> *TRANSPLANT                            | 2   | 0.0038128   | 0.02  | 0.9792 |
| ERROR                                    | 441 | 41.511665   |        |        | ERROR  | 436 | 39.456425   |       |        |

Table 5.4. Genet correlations (upper diagonal) and phenotypic correlations (lower diagonal) over all 80 genets. Pearson correlation coefficients were calculated between all traits using PROC CORR (SAS 2000). Significance values were determined by the sequential Bonferroni technique (Rice 1989) using an experiment-wide significance value of 0.05.

|            | HEIGHT    | TOTLA     | TOTLVS    | SUCCULENCE | C13       | N         | Na        | Ca        | K         | Mg        | Mn        | P         |
|------------|-----------|-----------|-----------|------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| HEIGHT     |           | 0.71 ***  | 0.60 ***  | -0.31 ***  | -0.15 ns  | 0.46 ***  | 0.15 ns   | -0.07 ns  | 0.40 ***  | -0.29 *** | -0.59 *** | 0.44 ***  |
| TOTLA      | 0.63 ***  |           | 0.71 ***  | -0.20 *    | -0.10 ns  | 0.38 ***  | 0.12 ns   | -0.08 ns  | 0.35 ***  | -0.22 **  | -0.43 *** | 0.30 ***  |
| TOTLVS     | 0.56 ***  | 0.71 ***  |           | -0.18 ns   | -0.14 ns  | 0.28 ***  | 0.08 ns   | 0.11 ns   | 0.35 ***  | -0.05 ns  | -0.37 *** | 0.37 ***  |
| SUCCULENCE | -0.05 ns  | -0.04 ns  | 0.02 ns   |            | 0.22 **   | -0.46 *** | 0.00 ns   | -0.13 ns  | -0.55 *** | -0.04 ns  | 0.36 ***  | -0.43 *** |
| C13        | 0.13 ***  | 0.06 ns   | 0.09 *    | 0.18 ***   |           | -0.60 *** | -0.53 *** | -0.30 *** | -0.57 *** | -0.03 ns  | 0.48 ***  | -0.65 *** |
| N          | 0.17 ***  | 0.18 ***  | 0.14 ***  | -0.24 ***  | -0.30 *** |           | 0.30 ***  | 0.18 ns   | 0.65 ***  | -0.13 ns  | -0.66 *** | 0.77 ***  |
| Na         | -0.09 ns  | -0.14 *** | -0.22 *** | 0.09 **    | -0.34 *** | 0.02 ns   |           | 0.10 ns   | 0.26 **   | -0.06 ns  | -0.29 *** | 0.31 ***  |
| Ca         | -0.04 ns  | -0.04 ns  | 0.00 ns   | -0.11 *    | -0.10 ns  | 0.00 ns   | -0.12 *   |           | 0.22 *    | 0.65 ***  | -0.15 ns  | 0.33 ***  |
| K          | 0.10 ns   | 0.05 ns   | 0.06 ns   | -0.34 ***  | -0.30 *** | 0.43 ***  | 0.11 *    | 0.14 ***  |           | 0.03 ns   | -0.59 *** | 0.75 ***  |
| Mg         | -0.13 *** | -0.12 **  | -0.09 ns  | 0.00 ns    | -0.08 ns  | -0.21 *** | 0.12 ***  | 0.56 ***  | 0.11 *    |           | 0.21 *    | -0.05 ns  |
| Mn         | -0.42 *** | -0.32 *** | -0.26 *** | 0.20 ***   | 0.15 ***  | -0.37 *** | 0.05 ns   | -0.15 *** | -0.28 *** | 0.17 ***  |           | -0.65 *** |
| P          | 0.21 ***  | 0.15 ***  | 0.16 ***  | -0.29 ***  | -0.26 *** | 0.60 ***  | 0.09 ns   | 0.33 ***  | 0.55 ***  | 0.10 **   | -0.39 *** |           |

## CHAPTER 6

### CONCLUSIONS

Salt marshes contain steep environmental gradients: conditions are fairly mild near the terrestrial border of the marsh but become so severe in salt pans and extremely waterlogged areas that even the most highly-adapted salt marsh plants cannot survive (Pennings and Bertness 2001). Across these strong environmental gradients, phenotypic variation of all 12 salt marsh plant species was correlated with environmental variables, as predicted by the first hypothesis in chapter 2. In contrast, my second hypothesis which predicted that species with wider environmental ranges would have more variable phenotypes, was not supported by linear comparisons of single plant traits with single environmental variables. I found instead that variation in height was maximized in species with intermediate ranges of salinity. In addition, a composite, complex phenotypic response (phenotypic PCA1) appeared to be related to a composite, complex environmental variable (environmental PCA1). This relationship suggests that there is a relationship between environmental and phenotypic variation, as I hypothesized, but the nature of this relationship is complex.

Despite the severe environmental gradients across which all 12 species live, in chapter 3 I found no evidence for fine scale differentiation at any allozyme locus resolved for two contrasting dominant salt marsh perennials *B. frutescens* and *S. alterniflora*. In general, I found that there were small differences in genetic make-up between sites. However, across loci, microhabitats explained a significant amount of the distribution of genetic diversity for both

species. There were no differences in levels of genetic diversity associated with microhabitat type and although clonal diversity was high, it also did not vary with habitat type.

The results from the allozyme survey in chapter 3 suggest that current theory needs to be refined by considering that selection acts on ecologically important traits that are not necessarily represented by any one locus, but potentially overall patterns across loci, and indicate that sexual reproduction and recruitment from seeds are important factors maintaining diversity even in these highly stressful habitats. In addition, future studies in salt marsh ecology should reflect that although clonal integration may be of importance on a fine spatial scale for a given genet (Pennings and Callaway 2000), across the larger scale spanning the extent of the environmental gradients within populations, there is a great diversity of clones and therefore clonal integration does not fully explain how these species can live across these gradients.

After ruling out clonal integration as the main strategy that these plants use to live across environmental gradients, in chapters 4 and 5 I examined the role of phenotypic plasticity and genetic differentiation in *B. frutescens*. In particular, I focused on purported salt tolerance traits and their response to controlled salinity treatments in the greenhouse as well as response to reciprocal transplants in natural contrasting *B. frutescens* habitats.

In the greenhouse study described in chapter 4, salinity had a significant effect on all 17 traits measured, indicating that these traits are highly plastic. The pattern of response to high salt for all traits were as predicted: decreases in biomass and nutrient uptake (with the exception of N which is used in the production of compatible solutes), increases in Mid-day xylem pressure potentials, succulence, water use efficiency, N and Na (Aerts and Chapin 2000, Antlfinger and Dunn 1983, Cavalieri and Huang 1979, Donovan et al. 1996, Donovan et al. 1997, Flowers et al. 1977, Glenn and O'Leary 1984, Moon and Stiling 2000, Rosenthal et al. 2002). Across

microhabitats, however, I found genetic variation in plasticity (significant G\*E) for only Na and B. This finding was surprising considering the multivariate nature of salt tolerance. I predicted that genotypes from the areas of the marsh with predictably higher salt levels would demonstrate specialization to tolerate high salt concentrations and therefore display reduced levels of plasticity in many if not all trait responses. However, I found no evidence for local adaptation in any of the 17 traits that I measured.

In contrast to the greenhouse study, my field study revealed significant genetic variation in 13 of the 14 morphological and physiological traits that I analyzed in these experimental populations. The discrepancy between these two studies could reflect that the greenhouse study was limited by small sample size. In addition, the greenhouse study manipulated only salt concentration holding plentiful and constant levels of water and nutrients. *Borrichia frutescens* may respond differently when it is only stressed by variation in salt and has plenty of other nutrients available to cope with that salt (as was the case in the greenhouse). Given that the salt marsh is a nutrient poor habitat (Mendelsohn and Morris 2000, Smart and Barko 1980) *B. frutescens* could be limited in its ability to manufacture nitrogen rich compatible solutes or use nutrient ions for osmoregulation.

By examining clonal replicates transplanted into separate microhabitats in chapter 5, I expected to find that the high salt environment would select for different trait optima than the low salt environment for *B. frutescens*. I expected that the high salt environment would select for increased tolerance of osmotic stress and nutrient stress (Aerts and Chapin 2000, Antlfinger and Dunn 1983, Cavalieri and Huang 1979, Donovan et al. 1996, Donovan et al. 1997, Flowers et al. 1977, Glenn and O'Leary 1984, Moon and Stiling 2000, Rosenthal et al. 2002). At the genet level, I found evidence supporting my predictions for only WUE. Patterns of selection

were significantly different in the two gardens only for stabilizing selection on WUE suggesting that the two gardens select for different optima in this trait. Our data therefore suggest that overwhelmingly WUE is one of the most important traits allowing *B. frutescens* to tolerate this habitat. However, three way ANCOVA examining the interaction between the source of the genets (from high or low salt sources) and transplant garden does not support the hypothesis that high salt genets have higher WUE in the high salt garden (MS= 0.28, F=0.46). Therefore, I found no evidence for local adaptation in this trait. I also found negative genetic correlations between WUE and the four elements Na, N, Ca and P which could partly explain why these traits showed no response to selection.

Overall, these studies suggest that salt marsh plant populations consist of many more clones that are not as large as previously thought. Individuals are highly plastic for all traits measured in the controlled greenhouse environment as well as the field. Although there is a lot of genetic variation for these putative salt tolerance traits, and some evidence for differentiation between the two source habitats, there is no evidence that these habitats select on these traits differently. The one exception is that there was differential selection in the two habitats on WUE, however there was no evidence of differentiation for this trait. These studies therefore reveal the importance of phenotypic plasticity as the predominant strategy for living across the environmental gradients of the salt marsh.

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