The Molecular Ecology of *Vaccinium macrocarpon* Aiton, the American Cranberry

by

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Abstract

Cranberry (*Vaccinium macrocarpon*), a commercially grown evergreen dwarf shrub, is a dominant taxon in temperate bogs in North America. It spreads clonally by runners, and reproduces sexually predominantly by self-fertilization on upright stems. The objective of this project was to investigate genetic and clonal variation and phenotypic plasticity of *V. macrocarpon*. Specifically, I wanted to test whether there exists an inverse relationship between population genetic variation and the amount of overall phenotypic plasticity of vegetative characteristics.

As background information I assessed the vegetation and edaphic factors of marginal cranberry bogs found in the mid-to-southern Appalachians. A gradient of nutrient availabilities was found among bogs that was positively associated strongly with the dominance of the more generalist *Rubus hispidus* and negatively with ericaeous bog

shrubs such as *V. macrocarpon*. Eutrophication may lead to the replacement of endemic bog species with generalist plastic species.

Theoretically, it would be plausible for environmental heterogeneity or stress to allow selection for more phenotypically plastic clones within a species. A single adaptively plastic clone for growth strategy could sweep a site, excluding intraspecific competitors. That is, selection could favor clones with high plasticity that could subsequently lead to a loss of genetic variation within a population. The environmental and genetic conditions favoring this would more likely exist in distributionally marginal sites because of spatial and temporal heterogeneity and island-like biogeography. Field and common garden experiments in which nutrients were manipulated were performed to test for an inverse relationship between phenotypic plasticity and genetic heterogeneity. Random amplified polymorphic DNA (RAPD) profiling was coupled with ecological measurements of plant growth on the experimental clones and other clones from the experimental populations to estimate genetic heterogeneity.

Genetic heterogeneity was found to be significantly lower in marginal populations than in central populations. Phenotypic plasticity was somewhat higher in a more marginal population in the field sites, but direct statistical comparisons could not be made. The common garden study was inconclusive, possibly due to transplant shock, but a trend among natural populations was towards higher plasticity among marginal clones. Additional research on other species is needed to clarify the possible inverse relationship between phenotypic plasticity and genetic variation within populations.

Acknowledgements.

This dissertation is dedicated to my wife Susan and son Nathan. I would not have been able to accomplish this without my family's support. I am especially appreciative of Erik Nilsen for guidance and encouraging me to undertake an integrative project. He allowed me to hog the lab and stray away from ecophysiology enough to incorporate genetics into the project.

Nine members of my past and present graduate committee assisted me in the following ways: Bruce Turner suggested the usefulness of RAPDs for surveying clonal plant populations, and provided lab facilities. Karl Pedersen gave me a sound foundation in molecular biology and allowed lab usage as well. Duncan Porter provided summer funds that allowed further professional development. Joe Falkinham provided lab facilities; additionally, he and David West stepped into advisory roles during Dr. Nilsen's sabbatical. Thanks to Carrie Kroehler and David West for reading several grant proposals. I am appreciative of David Smith and the late Peter Feret for serving on my committee. The committee provided insightful conversations and advice.

Tom Wieboldt was very helpful in plant identifications. The following fellow graduate students provided essential support and comraderie: Suzanne Hill, Laura Via, Tom Laughlin, and Selester Bennett. There were many others too numerous to list. Financial support came from Sigma Xi, Ocean Spray Cranberries, Inc., and the Biology Department.

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Chapter One

Introduction

The purpose of this dissertation is to describe the phenotypic plasticity and genetic differentiation in American cranberry (*Vaccinium macrocarpon* Aiton) with special attention to southern, distributionally marginal populations. This chapter provides historical and operational background of research on phenotypic plasticity and genetic differentiation, as well as information on *V. macrocarpon* and clonal plants. In chapter 2, the vegetation of five Appalachian bogs is described. The correlation between soil nutrient levels and the importance of key bog species is addressed. In chapter 3, a new method of isolating DNA for random amplified polymorphic DNA (RAPD) profiling, and improved RAPD-PCR conditions are presented. Chapter 4 focuses on a method of estimating population genetic structure and variation using RAPD profile data. Specifically, distance-estimate algorithms and a permutational test of homogeneity of variance (HOMOVA) using a modified analysis of molecular variance (AMOVA) are introduced. In chapter 5, the results of a common garden fertilizer study using three central and three marginal clones are presented. Morphological, plastic and genetic variations are compared. Chapter 6 focuses on a field nutrient manipulation experiment in two natural Appalachian bogs. Clonal identity as determined by RAPD profiling and ecological responses of clones to nutrient manipulations are discussed. The research clarifies the relationship of genetic and environmental impacts on the phenotype of *V. macrocarpon* in particular, and clonal plants in general.

Since climates are forecast to change drastically during the next century, plant and conservation biologists are increasingly interested in the contributions of genetic and environmental factors to phenotypic expression. Early genecological investigations from the late 1800s to the 1950s focused on ecotypic variation and speciation. The central question in these studies revolved around the puzzle of differing phenotypes in altered environments. For example, upon noticing that populations of species A have a different appearance on a mountain top compared to valley populations, an observer may have pondered whether this variation was due to the nature of the individuals, or to the dissimilarity in environmental conditions. The approach taken by most researchers involved reciprocal transplants or common garden experiments. When mountain and valley representatives of species A were grown in a common environment, the effects of genetics and environment could be determined. If the two plants remained phenotypically distinct, then the overriding factor in this difference was deemed genetic. If they converged to a similar phenotype, then the primary effect was considered to be in response to the environment. This latter response has come to be known as phenotypic plasticity. These two effects are usually not distinctly dichotomous, thus genetic-environmental interactions are commonly found. Contemporary researchers (since the 1960s) have taken advantage of biotechnological and statistical advances, but they still employ reciprocal transplants and common garden experiments to investigate the effects of nature and nurture.

Common settings. Some of the earliest modern work about environmental influences on the phenotype was carried out by Bonnier (1890) and Kerner (1895). Bonnier primarily used reciprocal transplants in the Alps to test some Lamarckian principles. He reported that lowland species transmutated into alpine relatives when grown in alpine settings. Needless to say, these results have not been replicated. Kerner (1895) used common alpine gardens in Austria to examine environmental effects on phenotypic variation. He was among the first to show the dwarfing effect of severe alpine environments on non-alpine plants. These results have been reproduced by many others in additional taxa, most notably by Turesson (1922a,b) and Clausen et al. (1940). Within a species, Turesson found that some plants originating from extreme environments retained their distinctive dwarfed phenotype when grown in a lowland mesic common garden. He determined that these retentions were genetically-based, and thus he termed these local races "ecotypes" (1922a). In California, Clausen and his associates took a similar approach to studying ecotypic variation, but selected experimental gardens in three vastly different habitats: Mediterranean (Stanford), intermediate (Mather) and alpine (Timberline). Their work supported Turesson's ecotype concept that environmental factors act in the evolution of locally adapted races. However, the ecotype concept has not gone without criticism. Much of the ecotypic variation found has been shown to be a product of sampling strategy. If one surveys widely different populations that are isolated from one another, as did Turesson and Clausen et al., then the discovery of ecotypes is not unexpected. However, if clinal conditions exist then increased gene flow

among populations renders ecotypic variation less likely (Gregor 1939). Therefore, the resulting intraspecific classification would be based upon continuous versus discontinuous sampling in experiments (Gregor 1946). The term has also been criticized on a conceptual basis as not being useful as an ecological unit *sensu* Turesson (Quinn 1978). Quinn argues also that the basic evolutionary unit is the population, not the ecotype.

Although ecotypes were first found among allopatric populations, sympatric ecotypes have also been recently documented. Two examples are found in work by A.D. Bradshaw's lab in Britain (McNeilly 1968, McNeilly and Antonovics 1968). These researchers demonstrated that the clonal grass *Agrostis tenuis* showed genetic differentiation meters apart in response to copper in soil. On a site adjacent to a copper mine, where copper concentrations were lower, they found high genetic variation with regards to copper tolerance. Mine spoil populations were only copper tolerant. The distance between populations was a few meters. They showed that populations were interfertile but had differing flower phenologies, concluding that this could be sympatric speciation in progress. Silander (1984) also did research on sympatric ecotypes. Populations of clonal halophyte grass, *Spartina patens*, genetically correlated with habitats of differing heterogeneity and stresses. Stress followed dune > swale > marsh, with marsh having less salt and "unpredictability." *Spartina* genetic variation within subpopulations was inversely related to stress, and ecotypic difference related to salt tolerance was evident.

Phenotypic plasticity. Morphological or physiological characters which are phenotypically plastic will change in response to environmental stimuli (Bradshaw 1965). The plastic response may be adaptive, maladaptive, or neutral (Bradshaw 1965, Kuiper 1984, Sultan 1987). Reaction norms (Johanssen 1909) are the individual's phenotypic responses to environmental gradients, and are therefore useful in describing phenotypic plasticity (Fig. 1.1). If a reaction norm is flat, then a genotype is not phenotypically plastic. If the slope is not flat, then it is plastic to some degree. In a simple two clone example, non-parallel reaction norms indicate a genetic-environmental interaction. Reaction norms analysis will allow one to determine if a character is responding adaptively to an environmental change, thus giving a particular genet a possible competitive advantage (Stearns 1992).

Since phenotypic plasticity of a character is now considered to be a character itself (Schlichting and Levin 1986), it has been the focus of several recent studies in plants. Questions that are currently being asked by researchers are: 1) What specific plant traits are plastic and what environmental stimuli cause their plasticity (Schmalhausen 1949, Bradshaw 1965, 1974, Schlichting and Levin 1986)? 2) What is the relationship of phenotypic plasticity among congeners (Schlichting and Levin 1986)? 3) What is the molecular regulation of phenotypic plasticity (Shumney and Tokarev 1983, Kuiper and Kuiper 1988)? 4) What are the environmental characteristics that cause selection for phenotypic plasticity (Scheiner and Lyman 1991)?

All of the above questions concern the relationships among phenotypic plasticity,

heterogeneous and/or stressful environments, and genetic polymorphisms. Hypothetically, temporally or spatially fluctuating environments are thought to lead to strong directional selection for higher plasticity and lower genetic variation (Jain 1978, Lynch and Gabriel 1987, Sultan 1987). When an environment is temporally stable but spatially heterogeneous genetic differentiation is predicted to predominate. (Lynch and Gabriel 1987). The reverse case is predicted to select for plasticity (Lynch and Gabriel 1987). The organismal response to fluctuations depends upon whether the organism "views" the changes as "fine grained" or "coarse grained" *sensu* Levins (1968). If the fluctuations are fine grained, then an organism would experience many environments during its lifetime. These environmental patterns would select for plasticity in organisms that could not track environmental changes genetically.

Phenotypic plasticity was first formally hypothesized as an alternative to genetic variation by Gause (1947).[1] Studying protozoans, Gause concluded that many organisms displayed an "inverse relationship between phenotypic plasticity and genetic specialization among different races [populations] of the same species" (Gause 1947 p. 65). Thus, he

[1] In this context, authors have referred to genetic variation in a broad sense as heterozygosity, genetic variance, genetic diversity, and/or heterogeneity (Allard and Bradshaw 1964, Bradshaw 1965, Jain 1978, Scheiner and Goodnight 1984). In this dissertation, clonal diversity is defined as the number of different clones distinguishable (genets) per population, and molecular variation is the average difference of RAPD characters among those genets. Taken together in a broad sense, clonal diversity and molecular variation will be referred to as genetic heterogeneity, or genetic diversity.

saw phenotypic plasticity as an alternative to "genoadaptation." That is, plasticity is an acquired characteristic rather than genetically based. However, we know now that phenotypic plasticity is under genetic control (Bradshaw 1965, Schlichting 1986, Scheiner and Lyman 1991). A plastic character is merely one that is expressed differently in different environments, whereas a canalized character is not. Both are under genetic control. Plasticity is therefore a property of characters, not individuals (Sultan 1987). This fact does not preclude the possibility that the population biology and life history strategies of a species would favor many plastic traits rather than canalized traits.[2]

There has been conflicting evidence in plants of the inverse relationship between genetic heterogeneity and phenotypic plasticity (Table 1.1). There seems to be no definitive pattern among the limited species studied with regards to plasticity and genetic diversity, except that possibly annual and weedy species tend to have high genetic diversity and plasticity. Studies of this nature are very rare for non-graminoid clonal plants. There has been no research of this type on clonal shrubs, and none using DNA methods to assess molecular diversity. One of the primary needs for research on this topic is to tease apart the relative significance of genetic diversity and/or phenotypic plasticity to species' success in response to the predicted global climate changes (National Research Counsel 1990).

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[2]Plastic individuals or populations are entities that possess proportionately large numbers of plastic traits. Similarly, nonplastic individuals or populations possess proportionately large numbers of canalized traits.

Since phenotypic plasticity of a trait is a trait itself and is under genetic control, genetic diversity and plastic variation should not be mutually exclusive (Schlichting and Levin 1986, Scheiner and Lyman 1991, Scheiner et al. 1991). Theoretically, phenotypic plasticity in plants may be important for adaptation to environmentally stressful or unstable habitats, and may be controlled by selection (Grime et al. 1986, Scheiner and Lyman 1991).

Molecular controls of phenotypic plasticity. Little is known about the molecular genetics of phenotypic plasticity. However, differences in phenotypic plasticity of marginal and central populations of a species could result, for example, from differences in promoter or coding regions of structural or regulatory genes or differences in the sizes of gene families. Studies in other species support a model in which diversity at specific genetic loci is correlated with environmental factors. One example is the recent study in mole rats indicating that climatic selection plays a role in generating genetic diversity of urine deoxyribonuclease I isozyme genes (Nevo et al., 1990). Environmental factors have also been associated with rDNA gene family size (Saghai-Maroof et al. 1990). Nitrate reductase transcription rates have also been associated with nitrogen use efficiency and photosynthesis rates (Shumney and Tokarev 1983, Poorter et al. 1990).

Genetic diversity. Mendelian and most traditional genetics have been gene-centered. Individuals and populations are examined for allelic differences and amounts of

heterozygosity. Populations containing large numbers of polymorphic loci, are considered to have high genetic variation. The antithesis, populations with many monomorphic loci (fixed) or high homozygosity have low genetic variation. Factors such as mutation, outbreeding, large population size, gene flow among demes, heterosis, and disruptive or frequency-dependent selection encourage genetic variation. Inbreeding, random genetic drift, small population size, geographic isolation, and directional selection tend to decrease genetic variation.

High genetic variation or diversity in a population biology framework translates into population flexibility. That is, in a sexual population with high genetic variation, environmental changes may be tracked by individuals having an adaptive combination of alleles. So, genic combinations may change rendering high adaptability. This sex/gene-centered view of genetic variation has been the predominant line of reasoning behind many of the genetic variation and phenotypic plasticity studies during the last 30 years. I will explore an alternative viewpoint, acknowledging that in clonal plants genes are inherited modularly, with minimal recombination.

Clonal plants. Clonal plants are facultative or obligate asexual reproducers. This includes asexual seed production (apomixis) and vegetative spread. This dissertation focuses upon the latter.

Asexual reproduction-- *A priori*, one would expect that long-lived vegetatively spreading plants would have high plasticity for certain physiological traits such as

nutrient use efficiency or growth rate and form, if plasticity is adaptive. Selection time in these plants may range from hundreds to thousands of years (Mitton and Grant 1980). For example, some clonal plants are estimated to be 10,000 years old (Vasek 1980, Smith et al. 1992). Although the age of ericaceous genets[3] cannot be determined, a minimum age, based upon growth rates and vegetative spread, has been estimated at several thousand years for species of *Vaccinium* and *Erica* (Harberd 1961, and references therein). These slow-growing species would face short-and long-term environmental changes during their lifetimes, and the adaptively plastic genets would be the survivors. Members of the Ericaceae have been estimated to have spread vegetatively up to 800 m in diameter (Harberd 1961, and references therein), and possibly as high as 2000 m in diameter (Wherry 1972). Such expansive genets could experience large spatial environmental variation if the organism is experiencing this as fine-grained (Levins 1968). These features of clonal plants would suggest that phenotypic plasticity could be an important adaptive mechanism, yet phenotypic plasticity of clonal plants has been little studied (Silander 1985b, deKroon and Schieving 1990).

Sexual reproduction-- There is often little recruitment from seed in populations where large standing clones exist (Lovett Doust 1981, Cook 1985, Eriksson 1992). Many of the reasons for this are unknown, but many members of the Ericaceae must have their seeds scarified before germination. Scarification would occur when seeds pass

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[3]Genets are defined as genetically identical individuals, whereas ramets are defined as modular units of a particular genet (Harper 1977).

through a bird gut, for example. Sexual reproduction acts predominately as a long-range method to establish new populations (Ogle 1984, Eriksson 1992). Under global warming, large standing clones are believed to be less adaptive than seed banks (Eriksson 1992). Thus, plasticity, which allows clones to spread and track spatial and temporal environmental heterogeneity, could be maladaptive under long-term or rapid environmental change.

Phenotypic plasticity-- Plasticity has generally been described in clonal plants in terms of foraging strategy (Cook 1985, Grime et al. 1986). This analogy has been used to describe the manner by which clonal plants obtain resources. Simply put, clones produce runners, or other stems with long internodes when mineral resources are low (guerilla growth), or consolidate into closely packed ramets when nutrients are relatively high (phalanx growth) (Lovett Doust 1981). Most studies of clonal plant resource acquisition and growth have used early successional and weedy lawn plants as subjects. The few studies that have focused on plasticity in stress tolerators (Ohlson 1989, deKroon and Knops 1990, deKroon and Schieving 1990) and woody plants (Roberts and Struckmeyer 1942, Noble et al. 1979, Mitton and Grant 1980, Matlack et al. 1993) have found the converse (e.g., low resources result in phalanx growth, and high resources result in guerilla growth). However, much of the research on woody species had objectives other than studying phenotypic plasticity. deKroon and Schieving (1990) provide an excellent conceptual review of the growth strategies of clonal plants. They describe the strategy of "conservative growth" in stress-tolerating clonal plants.

Consistent with the views of Grime (1979) and Chapin (1980), they propose that these plants will conserve captured resources and exhibit guerilla-type growth only during resource pulses. Additionally, conservative growth plants will be subjected to high spatial and temporal patchiness, which will in turn select for high plasticity in life history traits over their long lives.

Genetic heterogeneity-- It has long been generally believed that central populations have greater genetic heterogeneity than marginal populations. In the northern hemisphere, plants at the northern margins of their distributions exist as large clones because the following are reduced: flowering, pollination (pollinators), fruit production (Callaghan 1974, Grant and Antonovics 1978, Jefferies et al. 1983, Parsons 1990), and gene flow due to isolating factors. Large, self-fertile clones have greater pollination between flowers of the same genet, resulting in high frequency of self-fertilization (Handel 1983). Plants at the southern limit of their natural distributions may be genetically isolated and possess traits that make them more tolerant of heat and associated stresses, compared to centrally distributed plants, if directional selection for those traits is operating (Antonovics 1976). Southern individuals may be adapted to the conditions that will likely occur (by imminent climatic change) in the geographic center of their present distributions. Genetic divergence of marginal populations that are adapted to warmer microsites is very likely due to isolation, drift, and/or selection. (Antonovics 1976). The comparison of marginal populations with central populations is vitally important if we are testing for specific adaptations, such as heat tolerance. Many of

these adaptations are likely to be in the form of phenotypic plasticity.

The life history strategy of perennial clonal plants encourages long distance seed dispersal, and very little sexual recruitment within populations (Eriksson 1992). The result is the colonization of few, and sometimes vastly genetically different, individuals that spread throughout a site. Thus, the traditional sex/gene-centered view of genetic variation within populations, and the models used to quantify it, are not appropriate for perennial clonal plants such as *V. macrocarpon*. Within any given population, there is little genetic recombination, since clones vegetatively spread throughout a site. So genes are inherited (ramet to ramet) modularly. Indeterminate growth allows for somatic mutations to be clonally inherited. So a vegetative lineage founded by one zygote may populate a site. It is conceivable that in isolating circumstances, any variation within a population may be due to somatic mutation. In sites with high gene flow, members of a clonal plant population may not be closely related. Therefore, I will not refer to genetic variation (heterozygosity) in the classical sense. Instead I will refer to genetic heterogeneity, which will compare the genetic similarities of individuals to one another. Populations consisting of a few clonal plants with diverse genomes have high genetic heterogeneity, whereas populations from a single vegetative lineage will have relatively low genetic heterogeneity. Algorithms used to measure population genetic heterogeneity reflecting these concepts will be described.

Synthesis-- Under changing environmental conditions (temporal heterogeneity), there may be fitness differentials among clonal individuals. Strong directional selection

will decrease the number of different clones and genetic heterogeneity. Also, spatial heterogeneity may simultaneously partition niche space among clones, acting to increase genetic heterogeneity. Alternatively, few phenotypically plastic individuals with wider niches could substitute for many narrow-niche individuals. Thus, phenotypic plasticity may be an adaptive mechanism by which single clones cope with spatial heterogeneity within a site.

***Vaccinium macrocarpon* and bog communities.** Peatlands are among the most prevalent and productive of high latitude ecosystems (Bliss and Matveyava 1992). North American peatlands range from Alaskan tundra to southeastern coastal pocosins and pinelands. These are typically treeless ecosystems dominated by dwarf shrubs in the family Ericaceae, most of which are clonal. The dominant bog species are typically stress- tolerators with sclerophyllous evergreen leaves (Small 1972a, Grime 1979). The most common stresses in bogs are nutrient limitations, cold temperatures, and also water stress when there are large differences in shoot and root temperatures (Small 1972b). Many studies have established that limiting nutrients in bogs are nitrogen and/or phosphorus (Chapin 1980). Stress tolerators have characteristically slow growth rates and high water and nutrient use efficiencies (Chapin 1980, 1987).

Vaccinium macrocarpon, the American cranberry, is self-fertile and rarely outcrosses (Eck 1990). It is the commercial cranberry species important in the small fruit industry that is not only popular during the traditional American holiday season, but

is also being used increasingly in processing (Eck 1990). Cranberry, a widely distributed North American clonal perennial trailing shrub, is usually important in the ground layer where it occurs. The majority of natural populations exist from Newfoundland to central Minnesota south to northern Illinois and New Jersey (Vander Kloet 1983). There are relict populations in the southern Appalachians and the North Carolina coastal plain (Ogle 1984, Fig. 1.2). Cranberry grows naturally in nutrient deficient peatlands, although it is commonly cultivated commercially in sand cultures (Eck 1990).

The primary cranberry producing states in the United States, in order of decreasing productivity, are Massachusetts, Wisconsin, New Jersey, and Washington (Eck 1990). These states are responsible for over 95% of the commercial cranberry crop in the U.S. The six most widely used commercial cultivars are simply isolates from natural populations, primarily from Massachusetts and Wisconsin. The isolates are vegetatively reproduced from cuttings, and thus a randomly sampled cranberry plant from a commercial bog will most likely be one of six genets. Therefore, barring somatic mutation during the last 100 or so years, there is an astounding amount of genetic homogeneity in the commercial cranberry crop.

V. macrocarpon, and high latitude plants in general, display a high incidence of self-fertility and exhibit clonal growth (Packer 1974, Reader 1977, Ogle 1984, Eck 1990). *V. macrocarpon* runners are more important in vegetative spread, and upright modules where flowers are borne are more important for fruit production (Fig.1.3.). It is thought that birds are primarily responsible for long-range seed-dispersal, whereas

reiterative clonal growth is more important in filling a site. This pattern seems to be characteristic of many clonal plants (Eriksson 1992). The combined syndromes of selfing and clonal reproduction tend to produce relatively low diversity within, and high differentiation among populations (Silander 1979, Eriksson 1992), but this is variable among species (Ellstrand and Roose 1987).

Toward the southern limit, cranberry exists as small, genetically isolated populations. These (and those of other boreal bog species) are thought to be relict populations from the last glacial maximum (Wieder et al. 1981, Ogle 1984). Although *V. macrocarpon* is important in the vegetation where it occurs, it is rarely found in most Appalachian bog and coastal pocosin communities. Therefore, these marginal populations are excellent places to: study clonal variation in ecologically peripheral sites, study clonal spread and ecology, and test some hypotheses about phenotypic plasticity.

Table 1.1. Summary of a selection of studies on phenotypic plasticity and genetic variation in plants.

Study	Species	Trade off?[4]	Pol-lina-tion	Life History	Method of genetic measurement
Non-clonal Plants					
Pederson 1968	*Arabidopsis thaliana*	yes	self	perennial weed	quantitative genetics
Wu & Jain 1978	*Bromus rubens*	yes	self	annual grass	allozymes
Jain 1978	*Bromus mollis*	yes	cross	annual grass	allozymes
Moran et al. 1981	*Xanthium strumarium*	no	self	annual weed	allozymes
Zangerl & Bazzaz 1983	*Polygonum*	yes	[5]		quantitative genetics
Wood & Degabriele 1985	*Echium plantagineum*	no	cross	annual weed	quantitative genetics
Schlichting & Levin 1986	*Phlox drummondii*	no	most cross	annual herb	[6]
MacDonald & Chinnappa 1989	*Stellaria longipes*	yes	cross	perennial herb	isozymes

[4] Is there an inverse relationship between phenotypic plasticity and genetic variation?

[5] P. pensylvaticum, a colonizing selfing annual, and P. virginicum, an understory outcrossing perennial are compared.

[6] Comparisons were made among selfing cultivars, which are rare, and more common outcrossers.

Rice & Mack 1991	*Bromus tectorum*	no	self	annual grass	quantitative genetics

Clonal Plants

Cook & Johnson 1968	*Ranunculus flammula*	yes	self	herb	quantitative genetics
Hume & Cavers 1982	*Rumex crispus*	no	self	weed	quantatative genetics
Scheiner & Goodnight 1984	*Danthonia spicata*	no	self	grass	quantitative genetics
Silander 1984, 1985a	*Spartina patens*	yes	cross	grass	allozymes
Taylor & Aarssen 1988	*Agropyron repens*	no	cross	grass	quantitative genetics
Thompson et al. 1991	*Spartina anglica*	yes	cross	grass	quantitative genetics

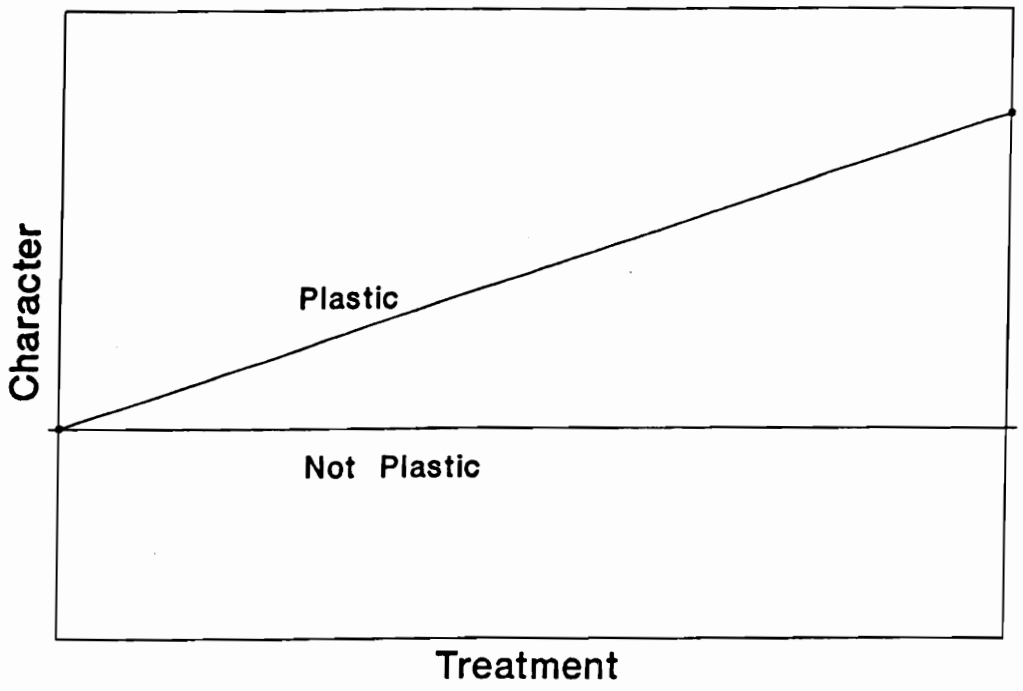

Figure 1.1. Reaction norms indicate the amount and direction of a plastic response. Regression analysis may be used to estimate the trait reaction norm of a genet against an environmental gradient.

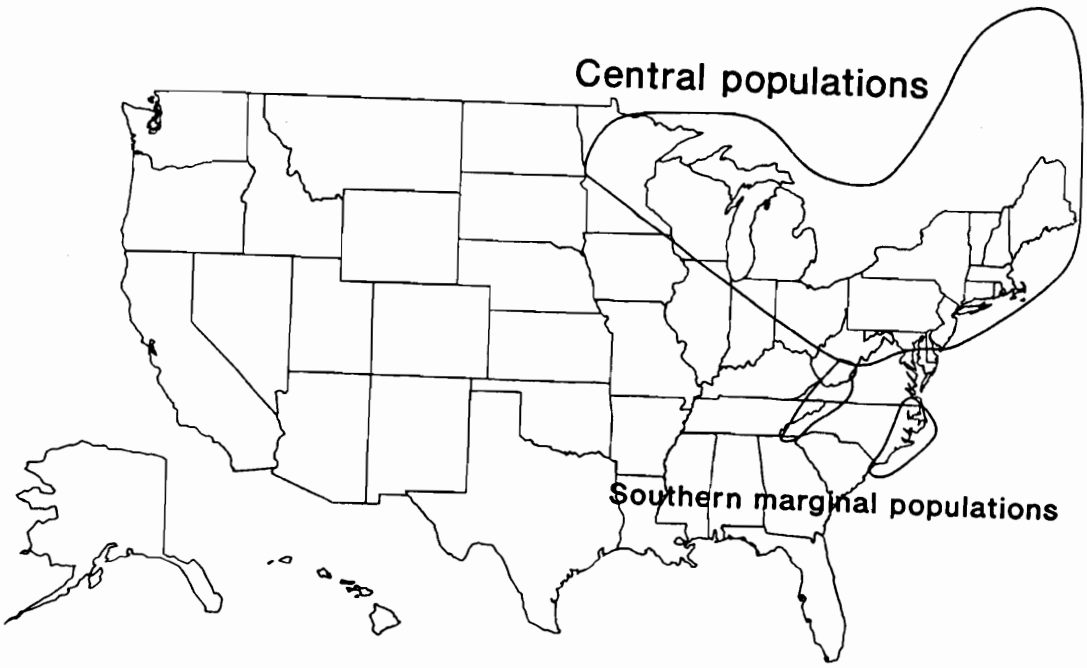

Figure 1.2. The contemporary distribution of natural *Vaccinium macrocarpon* populations.

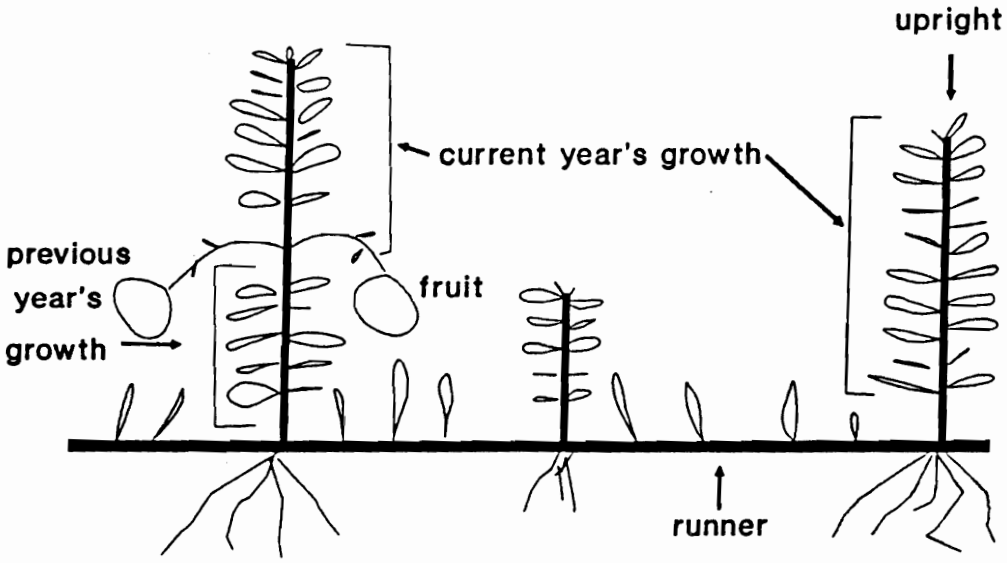

Figure 1.3. The modular morphology of *Vaccinium macrocarpon*.

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Chapter Two

Association of Edaphic Factors and Vegetation in Several Isolated Appalachian Peat Bogs[7]

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Introduction.

Peatlands are prominent ecosystems in the northern U.S. and Canada, most being located above 41° N latitude (Wieder and Lang 1983). They occupy vast areas, are ecologically important, and have been widely studied. Peatlands are generally characterized by cool wet conditions that encourage peat accumulation. Primary production and decomposition rates are low compared with those reported for other temperate freshwater wetland ecosystems (Schlesinger 1977, Bradbury and Grace 1983).

In the mid- to southern Appalachian Mountains (from Pennsylvania to Georgia), there are relict peatlands that are similar to northern peatlands, which have received little attention from the scientific community. These ecosystems, however, serve as genetic repositories for northern species and also serve vital hydrologic and ecological functions in the Appalachians, since they are a part of the headwaters of many rivers (Smith and Michael 1982).

Most Appalachian peatlands may be classified as small minerotrophic fens, as they are characteristically found in high valleys or synclines subject to cold air drainage and poor water drainage. They have a higher pH and receive significantly greater amounts of ions from surrounding soils than do northern bogs (Wieder et al. 1981, Wieder 1985). Ombrotrophic (rain-fed) bogs, receive most, if not all, ions from rain water, and have a low pH (<4.1). They also have higher peat accumulation rates and a convex surface profile compared to minerotrophic bogs (Heinselman 1970).

Aside from geochemistry, fens and bogs are differentiated by their vegetational

composition. Bogs are generally treeless and are dominated by dwarf shrubs, whereas fens have more variable vegetation including more trees, graminoids, and a higher overall species diversity (Gore 1983). The last is presumably a result of higher nutrient availability (Tallis 1983). Both types of wetlands typically are rich in bryophytes, especially Sphagnaceae, but this is especially true in ombrotrophic bogs.

I will use "bog" in a broad sense, to mean any relatively treeless, wet, sphagnous area, including fens. The sites used in this study fit this description and also contain members of the genus *Vaccinium* section *Oxycoccus* (cranberries). Cranberries are dwarf shrub bog endemics that are relatively rare in the Appalachians. For example, *V. macrocarpon* reaches its southern limit in Tennessee and North Carolina, and *V. oxycoccus* in West Virginia (Vander Kloet 1983; Ogle 1984).

The objectives of this study were to conduct a comparative quantitative analysis of the vegetation of dwarf shrub Appalachian bogs containing cranberries as important plants and to relate the composition of the vegetation to edaphic factors.

Study Sites.

Site 1 (Tennessee) is located in the Ridge and Valley physiographic province, 36°30' N 81° 57' W. The treeless area (bog) is about 0.16 ha in area. The site has an elevation of 1010 m and lies at the headwaters of Beaverdam Creek. It is located on Cross Mountain and lies adjacent to Shady Valley, a onetime large (>4000 ha) boggy area (Killebrew and Safford 1874). The site is bordered by forest on one side and by pasture on the other sides, and is situated on a cattle farm. The bog itself has been fenced for about 9 years to exclude cattle. The surrounding forest is an oak-hickory association. Because site 1 is on a farm, in a watershed impacted by agriculture, I predicted that it would have relatively high soil nutrient status.

Sites 2 and 3 are located on the Allegheny Plateau in West Virginia, 38° 5'N, 80° 17'W. The bogs are 0.25 ha (site 2) and 0.20 ha (site 3) in area. These sites have an elevation of 920 m and are perched on top of Droop Mountain. The bogs are separated by a 25 m wide strip of forest. The surrounding forest consists mainly of *Tsuga canadensis* and *Picea rubens*. Topography would suggest that these are ombrotrophic bogs, as they receive no mineral nutrients from runoff, and they have a deep peat layer (>1 m).

Sites 4 and 5 are also on the Allegheny Plateau in West Virginia, approximately 20 km from sites 2 and 3, 38° 12' 30" N, 80° 15' W. These sites are 3.2 ha (4: Flag Glade) and 11.3 ha (5: Round Glade) in area and have an elevation of 1030 m. They

are part of the Cranberry Glades, a wetland complex comprising about 300 ha. The two bogs are separated by approximately 40 m of scrub and forest. The surrounding forest is similar to that at sites 2 and 3. Because the bogs lie in a hanging valley and receive runoff from surrounding mountains, and the wetland system is dissected by streams, I hypothesized that the soil nutrient status would be intermediate between site 1 and sites 2 and 3. The vegetation at these two sites, unlike the others, has been previously studied (Darlington 1943, Edens 1973).

Materials and Methods.

Five soil cores of the surface layer (top 20 cm) of the substrate were taken from every second transect in each bog to determine soil nutrient status and pH. Macronutrients were determined by a double acid extraction procedure and measured with an inductively-coupled plasma spectrometer. Macronutrients, bulk density, and organic matter were determined at the VPI & SU soil laboratory by methods described by Donahue and Gettier (1988). Ammonium was determined by the indophenol blue method, a colorimetric procedure (Keeney and Nelson 1982). Soil nutrients were represented by mass per unit volume of soil to make valid comparisons among the soils since plants exploit soil nutrients on a volumetric basis.

The point-quarter method (Cottam and Curtis 1956) was used to determine importance values of the species surveyed in each bog. The standard algorithms associated with this technique were employed. However, this method was originally designed for tree surveys, where basal area is a measurement necessary to determine relative dominance, and, in turn, importance values. Since bog plants are of small stature, basal area is not a relevant measurement of size in this case. Instead, I used a relative size index based upon mean ramet volume occupied by each species, and standardized it to the largest species on the sites, *Osmunda cinnamomea*. *Osmunda* occupied ~ 1 m^3 and had a relative size of 10. *Symplocarpus foetidus* occupied ~ 0.6 m^3 and therefore had a relative size of 6, and so on (Table 2.1). Two species (*Drosera rotundifolia* and *Gaultheria procumbens*) that were relatively unimportant in the bogs I

surveyed and had extremely small ramet size they received an arbitrary relative size of 0.5, the lowest value given. Importance value = relative frequency + relative density + relative dominance. Relative frequency = frequency of each species / frequency of all species. Relative density = density of each species / density of all species. Relative dominance = size x relative frequency of each species x relative frequency of all species. The point-quarter method has been criticized by Risser and Zedler (1968) as an inappropriate technique for grassland vegetation quantification, because of a tendency to underestimate density of aggregated plant stands. Dwarf shrubs, which are by far the most important group in bogs, do not aggregate to the degree of grass ramets, but are more randomly and evenly distributed throughout a site. In comparing non-destructive techniques, the point-quarter method is probably at least as accurate at estimating vegetation importance as percentage cover in bog communities. I would contend that it would be superior because it takes into account the three dimensional structure of the vegetation.

The sampling strategy was thus: ten parallel, 20 m long transects were established 2 m apart across the bog. From these, three were randomly chosen. Every 2 m along the transect was a point, and for every point there were four quarters. A total of 120 quarters were sampled. If a species in the bog was not present within 200 cm of a point, it was not considered present for frequency, but was recorded arbitrarily as 400 cm from the point. Since the most important or dominant species tended to be very close to the point (<50 cm), any value over ~ 200 cm is negligible in the ultimate importance value

computations.

Regression analysis measured the relationships between important recumbent shrub/vine species and also between vegetation, and soil nutrient status (Zar 1984). Nomenclature follows Strausbaugh and Core (1978).

Results and Discussion.

Soil nutrients. Soil bulk density was much higher in site 1 (TN) than in the other sites (WV) (Table 2.2). This is a result of higher decomposition rates and minerotrophy at site 1, and is reflected by the lower organic matter fraction at site 1. The peat at site 1 was also more highly decomposed than at sites 2-5 (personal observation). All the surveyed bogs except site 1 were very acidic with low concentrations of plant macronutrients. Site 1 was more minerotrophic than the other sites, as was evident by higher soil pH and soil nutrient contents (Table 2.2). With the exception of site 1, phosphorus was especially low when compared with other wetlands (Schlesinger 1991).

Site 1 is a weakly minerotrophic (poor) fen based upon Heinselman's (1970) widely used criteria of peatland classification, which consider pH and cation concentrations. Site 1 is similar to the few bogs that have been studied in the southern Appalachians (Chappell 1972, Ogle 1982, Schafele and Weakley 1990). Based upon the same criteria, sites 2-5 (WV) are weakly ombrotrophic bogs. That is, these mid-Appalachian bogs have slightly higher pH and cation levels than the raised bogs of Minnesota (Heinselman 1970). These bogs seem to represent the maximum ombrotrophy found in the mid-Appalachians and are also similar to other WV bogs (Gibson 1982, Wieder 1985) and bogs in northern New Jersey and southern New York (Karlin and Lynn 1988). In general, soil fertility among sites had the relationship: 1 > 5 > 4 > 3 > 2, except for nitrate, in which there were no differences among sites 2-5 (Table 2.2).

Vegetational structure. The vegetation of site 1 was dominated by the recumbent *Rubus hispidus*, while sites 2-5 were dominated by the recumbent *Vaccinium oxycoccus*. In site 1, *Osmunda cinnamomea* and *V. macrocarpon* were also important. Sites 2-5 had similar co-dominant vegetation (*Pyrus melanocarpa*, *Rhynchospora alba*, *Rubus hispidus*, *Symplocarpus foetidus*) (Table 2.3). Notably, *R. alba* was very important at sites 4 and 5, and *R. hispidus* also at site 5 (Table 2.3). Sampling areas in sites 4 and 5 were located within Darlington's (1943) *Sphagnum*-cranberry-beaked rush association. The sampling was not intended to be a floristic survey, but a vegetational sampling of selected plots. Generally, all sites had one extremely important recumbent, and such species dominated the vegetational structure (Table 2.3).

Appalachian bog vegetation and northern bog vegetation. Anecdotally, Appalachian bogs have been compared to northern peat bogs because both are characteristically dominated by *Sphagnum* and dwarf shrubs and have similar geochemistry (Wieder et al. 1981). However, there are differences in species composition, the degree of minerotrophy, and modes of bog formation. The reasons for these differences have not been fully elucidated, but certainly climatic differences, topography, and island biogeographic effects are probably important factors (Wieder et al. 1981). Climate in the Appalachians can sometimes be cool enough for peat accumulation, which in turn supports acid-loving bog plants. Cool moist conditions for peat formation exist in hanging valleys and on level mountain tops, and there are few such sites in the mid- to

southern Appalachians conducive to bog formation. Therefore, the bogs in the Appalachians are characteristically isolated from one another. These factors contribute to differences in vegetation structures among Appalachian bogs and also to differences from northern bogs of similar geochemistry.

Several important species in the WV sites are absent in the TN site. Some examples are *Vaccinium oxycoccus*, which is at its southern limit in WV, *Rhynchospora alba*, *Eriophorum virginicum*, *Pyrus melanocarpa*, *Viburnum cassinoides*, and *Symplocarpus foetidus*. Similarly, the sites in this study are at or near the southern limits of dominant northern bog species. Among Appalachian bogs, however, there are several common dominant species: *Vaccinium oxycoccus*, *V. macrocarpon*, *Rhynchospora alba*, and *Eriophorum virginicum*. Likewise, some dominant Appalachian species are not important in northern bogs (*Rubus hispidus*, *Osmunda cinnamomea*, *Pyrus melanocarpa*, and *Symplocarpus foetidus*), although all are widely distributed throughout northern areas.

The bogs surveyed in this study had dominant vegetation similar to that of other Appalachian bogs. Cranberries, *R. hispidus*, *E. virginicum*, *R. alba*, and *Carex* are common elements in Appalachian bogs (Chappell 1972, Wieder et al. 1981, Gibson 1982, Ogle 1982, Schafele and Weakley 1990). However, the vegetational composition is widely divergent among Appalachian bogs for minor species.

Unlike northern ombrotrophic raised bogs, Appalachian bogs are difficult to define precisely by vegetation. Similarly, Appalachian bogs differ in species composition

from northern bogs. Nonetheless, both Appalachian and northern bogs are: 1) dominated by dwarf shrubs, 2) acidic and nutrient poor, 3) *Sphagnum* rich and have a peaty surface layer.

Edaphic factors and bog vegetation. Sites 2 and 3 are in the same wetland complex, and sites 4 and 5 also are proximate to each other. They were each treated singularly in the regression analysis because of their contrasting vegetation and soil nutrient status. In sites with high nutrient status, *Rubus hispidus* had increased importance as dwarf shrubs (Ericaceae) had decreased importance (Fig. 2.1). However, the recumbent shrub growth form remained dominant in all sites. There accordingly seems to be a tradeoff between *R. hispidus* and the other members of the growth form (Fig. 2.1). Importance value of *Rubus hispidus* was positively associated with macronutrients among sites (Fig. 2.2). Importance values of the remaining recumbent dwarf shrubs were negatively associated with macronutrients (Fig. 2.2). Results were statistically significant ($p < .05$; H_o: slope (b) = 0), with high r^2 values. The relationships between dominant vegetation and NH_4^+-N, P, K, as well as Mg and Ca (not shown), were much the same. Furthermore, the regression analysis was performed disregarding site 1, the results were still statistically significant, although the regression lines had slightly flatter slopes.

Substrate cation concentrations and pH are known to be important factors affecting bog vegetational composition (Heinselman 1970, Vitt and Slack 1975, Schwintzer 1978). There is extensive ecophysiological evidence that elevated levels of

soil nutrients have profound effects upon alpine and bog plants. Stress-tolerating evergreen species have a higher leaf turnover rate (Shaver 1981) and higher nutrients in litter (Chapin 1980, 1987). Higher nutrient status in leaf litter encourages higher soil nutrient mineralization rates (Schlesinger 1977, Vitousek 1982, Stewart and Nilsen 1992). The resulting fertilization effect, coupled with warmer climate, leads to higher primary productivity, which is observed in Appalachian peatlands compared with their northern counterparts (Wieder and Lang 1983; Wieder et al. 1989). Under global warming scenarios, peat could become a carbon source rather than its current status as a carbon sink (Billings et al. 1982).

Conclusions.

Bogs containing dwarf shrubs, such as *Vaccinium oxycoccus*, resemble northern ombrotrophic dwarf shrub bogs but have different species composition and higher minerotrophy. In the bogs I surveyed, recumbent shrubs dominated the vascular vegetation. There was a tradeoff in importance values between *Rubus hispidus* and dwarf ericaceous shrubs. This trend was correlated with soil macronutrient status, with *Rubus hispidus* gaining importance in nutritionally rich sites.

Similar studies of more sites are needed, not only in the Appalachians but in northern bogs as well. Further research is needed to determine if a causal relationship exists between soil nutrients and vegetation structure changes. This may be accomplished

by long-term monitoring of bog vegetation structure and also through long-term fertilization studies. If the levels of nutrients are kept chronically high at sites for a long period of time, we will better understand how nutrients induce changes in bog vegetation composition and which nutrients are most important in this role.

Table 2.1. Relative size index of species found in 5 Appalachian peat bogs.

Species	Relative size
Recumbent Shrubs/Vines	
Gaultheria procumbens	0.5
Rubus hispidus	0.5
Vaccinium macrocarpon	0.5
V. oxycoccus	0.5
Herbs	
Calopogon pulchellus	1
Drosera rotundifolia	0.5
Galium obtusum	0.5
Hypericum punctatum	1
Impatiens capensis	2
Osmunda cinnamomea	10
Polygonum sagittatum	2
Symplocarpus foetidus	6
Upright woody shrubs	
Acer rubrum	3
Alnus rugosa	3
Amelanchier bartramiana	2
Pyrus melanocarpa	2
Viburnum cassinoides	3
Graminoids	
Agrostis perennans	2
Carex incomperta	1.5
C. lurida	2
C. normalis	3

C. rostrata	3
Eleocharis tenuis	1
Eriophorum virginicum	2
Holcus lanatus	3
Juncus canadensis	3
Panicum dichotomum	2
Rhynchospora alba	1
R. capitellata	2

Table 2.2. Soil conditions of 5 Appalachian bogs. Statistical analysis used is ANOVA with multiple comparisons using Fisher's LSD (Zar 1984). Different letters in rows denote significant differences at the 0.05 level.

Site	1	2	3	4	5
Location	Johnson Co, TN	Pocahontas Co, WV: Droop Mtn		Pocahontas Co, WV: Cranberry Glades	
Elevation (m)	1010	920	920	1030	1030
Soil organic matter (%)	42 a	91 b	94 b	94 b	95 b
Soil bulk density (g/cm^3)	0.3 a	0.05 a	0.05 b	0.05 b	0.05 b
pH	4.7 a	4.0 b	3.7 c	3.6 c	3.9 b
Soil macronutrients ($\mu g/cm^3$)					
NH_4^+-N	7.16 a	1.60 b	0.78 c	2.52 d	5.02 e
NO_3^--N	0.90 a	0.15 b	0.15 b	0.15 b	0.15 b
P	1.20 a	0.06 b	0.08 b	0.17 c	0.22 c
K	14.3 a	0.36 b	1.18 bc	1.83 cd	2.45 d
Ca	97.2 a	3.45 b	5.25 b	7.40 b	19.0 c
Mg	17.7 a	1.04 b	1.66 b	1.35 b	3.07 c

Table 2.3. Importance values of vegetation of 5 Appalachian bogs.

Species	Importance values (Site)				
	2	3	5	4	1
Ground layer					
Gaultheria procumbens	-	-	15	-	-
Rubus hispidus	10	18	46	22	186
Vaccinium macrocarpon	17	2	-	-	17
V. oxycoccos	190	180	118	183	-
Herbs					
Calopogon pulchellus	5	7	-	-	-
Drosera rotundifolia	17	8	-	4	-
Galium obtusum	-	-	-	-	4
Hypericum punctatum	-	-	-	-	8
Impatiens capensis	-	-	-	-	6
Osmunda cinnamomea	2	8	-	4	22
Polygonum sagittatum	-	-	-	-	5
Symplocarpus foetidus	10	18	-	0.3	-
Upright woody shrubs					
Acer rubrum	1.3	7	-	-	-
Alnus rugosa	-	-	-	1.4	-
Amelanchier bartramiana	-	-	2	-	-
Pyrus melanocarpa	13	28	27	17	-
Viburnum cassinoides	0.8	4	15	1.9	-
Graminoid					
Agrostis perennans	-	-	-	-	8
Carex incomperta	-	-	-	3	10
C. lurida	-	-	-	-	6
C. normalis	-	-	-	-	5
C. rostrata	-	-	-	3	-
Eleocharis tenuis	-	-	-	-	7
Eriophorum virginicum	11	11	14	16	-
Holcus lanatus	-	-	-	-	1.1
Juncus canadensis	-	-	-	2	6
Panicum dichotomum	-	-	-	-	4
Rhynchospora alba	22	11	62	41	-
R. capitellata	-	-	-	-	5

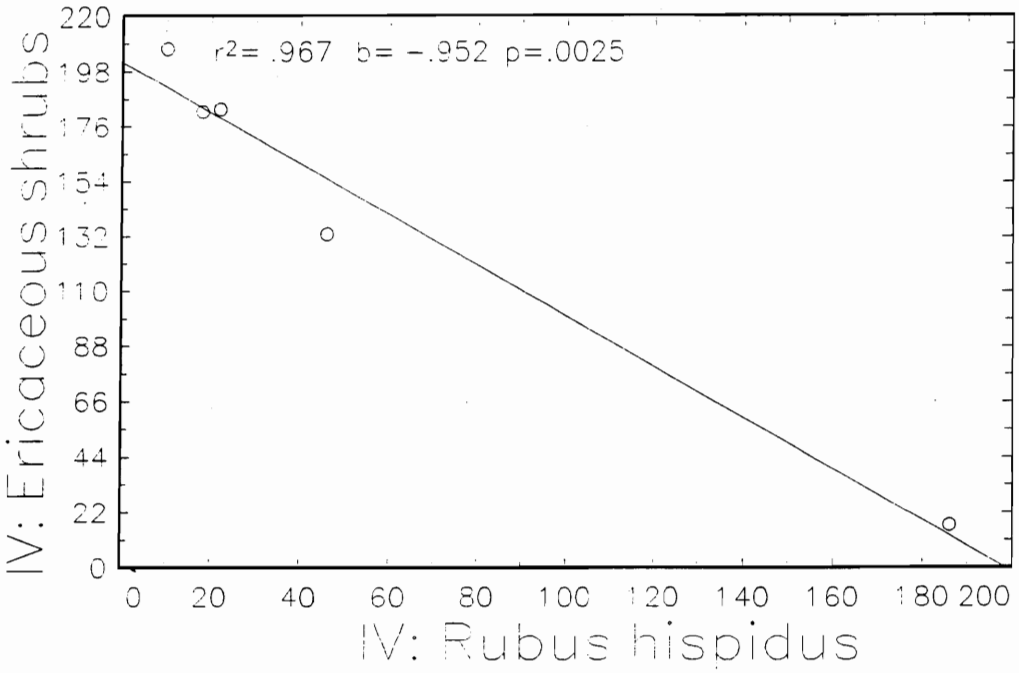

Figure 2.1. Regression analysis of importance values between *Rubus hispidus* and the remaining members of recumbent shrubs (all in the family Ericaceae) of 5 Appalachian bogs.

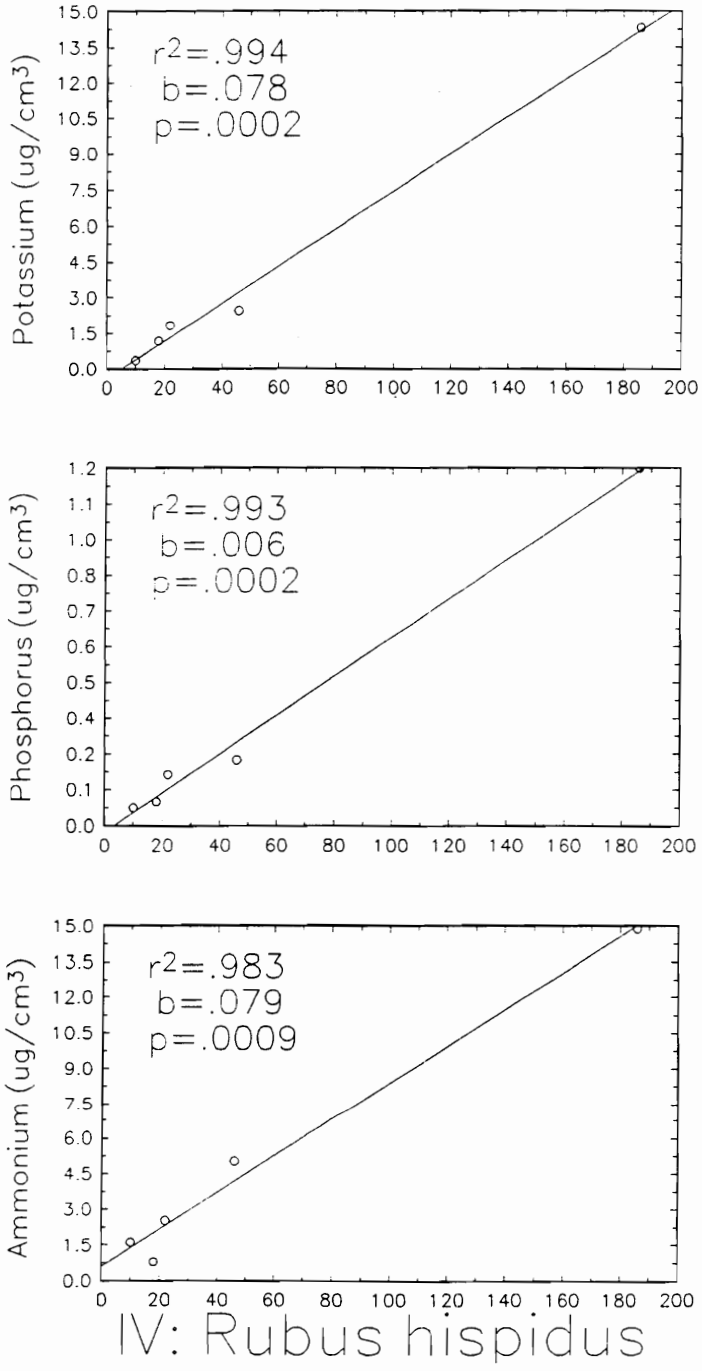

Figure 2.2. Regression analysis of NH_4^+-N, P, and K concentrations of soil and *Rubus hispidus* importance values.

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Chapter Three

A Rapid CTAB DNA Isolation Technique Useful for RAPD Fingerprinting and Other PCR Applications[8]

[8]This chapter was published in BioTechniques in 1993 (14: 748-751) with Laura Via.

Introduction.

Many DNA isolation techniques widely employed by plant molecular biologists use a CTAB (hexadecyltrimethylammonium bromide) extraction buffer coupled with reusable tissue homogenization systems such as a mortar and pestle (Saghai-Maroof et al. 1984, Doyle and Doyle 1987, 1990). These procedures, though simple, typically use large amounts of buffer (10 ml), utilize non-disposable homogenizers and require ethanol washes. The risk of cross contamination associated with reusing homogenizers and vessels is unacceptable if the DNA isolated will be amplified in PCR or RAPD (random amplified polymorphic DNA) (Williams et al. 1990) experiments. Recent DNA extraction methods developed to avoid potential contamination disrupt cells by biochemical means (Deragon and Landry 1992), leaf squashes (Landridge et al. 1991) or sodium dodecyl sulfate (SDS) mini-preps (Edwards et al. 1991). However, the biochemical lysis method and the leaf squash method are complicated and/or do not yield sufficient DNA for many replicate reactions. The SDS procedure is similar to the protocol described here, but the CTAB buffer should be more amenable to plant material containing polysaccharides (Doyle and Doyle 1990, Fang et al. 1992).

The procedure presented is a modification of the Doyle and Doyle CTAB method (Doyle and Doyle 1987) scaled to fit in microcentrifuge tubes with reagents (Fang et al. 1992) added to increase separation of polysaccharides from the DNA. Milligram amounts of leaf tissue are ground using a cordless drill-driven pipette tip (as devised by the author) in a microcentrifuge tube with hot CTAB buffer. A single chloroform-isoamyl alcohol (24:1) extraction is followed

by a single isopropanol precipitation. This simplified, quick, and inexpensive CTAB procedure yielded sufficient template for > 100 reactions using only disposable homogenizers and vessels, and requiring only one transfer of the DNA solution thereby reducing potential DNA cross contamination. The RAPD fingerprinting technique is thought to be sensitive to the quality of the DNA template (Williams et al. 1990). To show the success of the presented CTAB mini-prep method, DNA samples were isolated from 5 plant and 1 fungus species by both the Doyle and Doyle method (Doyle and Doyle 1987) followed by purification through a CsCl ethidium bromide gradient and the described mini-prep procedure. The DNA samples isolated by the two methods were used in RAPD reactions and the fingerprints compared. The DNA isolated by the described CTAB mini-prep method compared favorablely to the control CsCl-cleaned DNA for use in RAPD reactions.

Materials and Methods.

DNA was extracted from five plant species (American chestnut, *Castanea dentata*; American cranberry, *Vaccinium macrocarpon*; geranium, *Pelargonium x hortorum*; Peters Mountain mallow, *Iliamna corei*; and peanut, *Arachis hypogaea*), and one fungus (*Russula sp.*) by two methods. A standard CTAB genomic DNA isolation method (Doyle and Doyle 1987) followed by ultracentrifugation through a cesium chloride/ethidium bromide gradient (Maniatis et al. 1982) was used as a control isolation method against which the following method was judged. For the control method 0.33 grams of plant or fungal tissue was processed in 10 ml CTAB buffer and the resulting DNA was resuspended in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

For the CTAB mini-prep method, an Eppendorf 1000 μl plastic pipette tip that had been pushed onto a deburring tool mounted on a cordless drill served as the homogenizing pestle. The end of the pipette tip was crimped upward when pressed on the tool (the tip was pressed against the bottom of the pipette tip box), thereby creating a "blade" for homogenization (Fig. 3.1). Fresh leaf (or stipe) tissue ($\sim$ .025 g) was placed in 600 μl 70°C extraction buffer (2% w/v CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 2% w/v PVP-40 (polyvinylpyrrolidone) (Sigma Chemical Co., St. Louis, MO), 5 mM Ascorbic acid, 4.0 mM DIECA (diethyldithiocarbamic acid) (Sigma Chemical Co., St. Louis, MO) (Doyle and Doyle 1990) in a microcentrifuge tube. PVP-40, ascorbic acid, and DIECA additions were not used in the control. Just prior to homogenization, 3 μl of 2-mercaptoethanol was added to the tube. Immediately following homogenization (600 rpm for 20 s), the homogenate was extracted with

500 μl chloroform-isoamyl alcohol (24:1 v/v). This mixture was shaken for 5 min at 500 rpm (IKA-Vibrax-VXR, Cincinnati, OH) and centrifuged (1000 x g at 22°C) in a microcentrifuge for 5 min to separate phases. The upper, aqueous, DNA-containing phase was transferred to a fresh microcentrifuge tube, precipitated with 0.7 volume isopropanol for 5 min at 22°C and centrifuged (14,000 x g) for 20 min. The pellet was air-dried and resuspended in 100 μl of TE.

DNA from both procedures was quantified using a mini-fluorometer (Hoefer TKO 100). The RAPD protocol of Williams et al. (1990) was modified as follows: each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.1 mM of each deoxynucleotide, 25 pmoles of a single 10 base primer OPA-04 (5'AGTCAGCCAC) (Operon Tech. Inc., Alameda, CA), 2% (v/v) glycerol, 1 unit *Taq* DNA polymerase and 5 ng of template DNA. The 25 μl volume reactions were overlaid with 50 μl light mineral oil and denatured at 95°C for 5 min. The reactions were processed through 75 cycles of 94°C for 10 s, 36°C for 10 s, and 72°C for 2 min. Either a PTC-100 thermocycler (MJ Research) or a Perkin-Elmer-Cetus cycler was used.

The amplification products were separated by gel electrophoresis (3 V/cm) through a 1.6% gel (0.8% agarose and 0.8% Synergel^R (Diversified BioTech, Newton Centre, CT))(Perlman et al. 1987) in recirculating TAE buffer. The DNA bands were visualized by ethidium bromide staining.

Results and Discussion.

Yields from the control isolation procedure (Doyle and Doyle 1987) were 50 to 120 μg of DNA. DNA yields from the CTAB mini-preparation ranged from 20 μg (*Russula* and Chestnut) to 34 μg (Mallow). This represents enough DNA to do 100-400 typical RAPD reactions. DNA yields per gram of plant tissue from the control isolation procedure were 150 to 360 μg/g and the CTAB mini-prep yields were 80 to 140 μg/g plant tissue. While the DNA yield from the control method was 66% higher, the PCR fragment patterns were not different between isolation techniques for each tissue sample (Fig. 3.2). RAPD analysis employing other 10 base primers gave equivalent results for the two methods of DNA preparation (data not shown).

When doing population studies using RAPD, often a primary time consuming step is isolating DNA from numerous samples. Three days were required to process 6 samples by the control CTAB procedure (Doyle and Doyle 1987) including a CsCl gradient. Twenty to thirty samples can be processed per day using the Doyle and Doyle procedure without the CsCl gradients. In contrast, greater than a hundred tissue samples can be processed in a day using the CTAB mini-prep procedure. For researchers using amplification techniques on hundreds of plant DNA samples, large yields are likely to be less important than speed and cost of sample preparation. Additionally, polysaccharides, which are abundant in peanut and mallow leaves are known to be bound by PVP (Doyle and Doyle 1987), thus eliminating the need for additional removal methods for these compounds (Do and Adams 1991, Fang et al. 1992). Since the method uses disposable homogenizers and is done in microcentrifuge tubes there is diminished possibility for cross contamination between samples. Care should be taken to homogenize

samples individually in a chemical hood with adequate air flow to protect both the researcher and the DNA preparation. However, this procedure, with minor modifications in equipment, may useful for DNA extractions in the field. The CTAB miniprep procedure can be directly applied to many different plant species from polysaccharide-rich mallow and peanut to leathery-leaved cranberries.

Figure 3.1. The homogenizer used for DNA isolations: a deburring tool is pushed onto a racked, plastic 1000 ml Eppendorf pipette tip (any brand should suffice). This forces the end of the pipette tip to be bent at a ~45° angle. The deburring tool's conical head had the following dimensions: 9 mm maximum outside diameter and 20 mm in length.

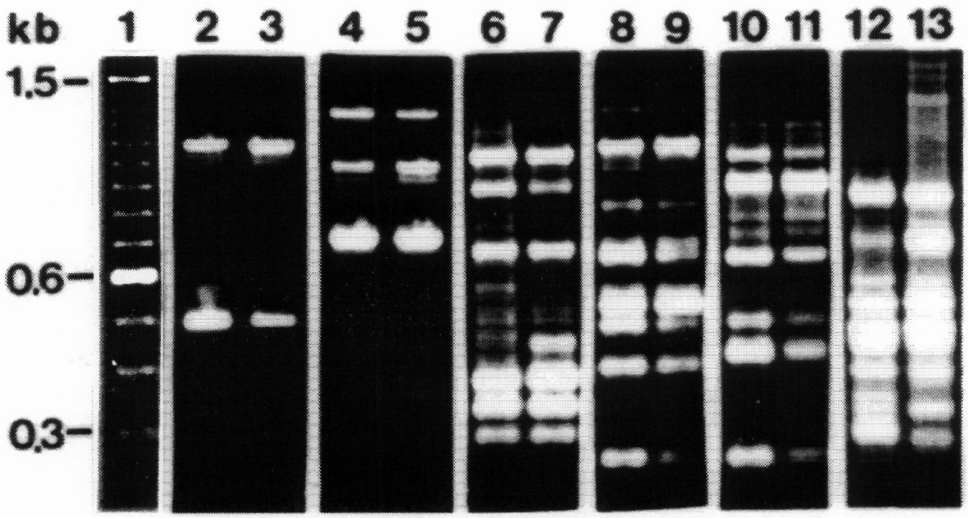

Figure 3.2. Comparison of RAPD patterns using 2 DNA isolation methods. Lane 1 contains the 100 bp marker (Gibco-BRL, Gaithersburg, MD). In each of the following pairs of lanes the CsCl-gradient purified DNA is presented first followed by the CTAB mini-prep DNA. The samples presented are American cranberry (lanes 2 and 3), Peters Mountain mallow (lanes 4 and 5), American chestnut (lanes 6 and 7), geranium (lanes 8 and 9), peanut (lanes 10 and 11) and *Russula* (lanes 12 and 13) amplified with primer OPA-04 5'AATCGGGCTG (Operon Tech. Inc., Alameda, CA).

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Chapter Four

Assessing Population Genetic Structure and Variability with RAPDs Data: Application to *Vaccinium macrocarpon* (American Cranberry) [9]

[9]This chapter was the product of a collaborative effort with Laurent Excoffier, (Department of Anthropology, University of Geneva, Switzerland), and Peter Smouse, (Center for Theoretical and Applied Genetics, Cook College, Rutgers University), and will be revised for submission to Molecular Ecology. Their contributions were in some of the biometrical aspects of the work (LE and PS) and alterations in the WINAMOVA program (LE) to fit the needs of this project. A computer program to carry out the analysis of molecular variance and the nonparametric test of heteroscedasticity is available via using the anonymous file transfer protocol (FTP) on the internet computer site acasun1.unige.ch in the directory pub/amova as winamova.zip.

Introduction.

Random amplified polymorphic DNA (RAPD) profiling, a molecular genetic method that utilizes the polymerase chain reaction (PCR), is gaining wide usage in genetic, taxonomic, ecological, and behavioral research: in bacteria, plants, and animals (Hadrys et al. 1992). RAPD profiling uses single short (5-15 base) oligonucleotide primers and Taq^R DNA polymerase (or other thermostable polymerases) to amplify DNA segments between priming sites. Amplified DNA fragments may then be visualized on horizontal or vertical gels, and bands scored as presence/absence character states. A composite genetic profile is therefore the product of all DNA bands amplified using primers that reveal useful polymorphisms. These bands have been shown to be inherited in Mendelian fashion, and therefore useful as molecular markers for qualitative and quantitative traits (Williams et al. 1990, Hadrys et al. 1992).

Molecular methods such as RAPD profiling are being increasingly used in population surveys because of the simple methodology, relatively low cost, and the numerous distinguishable polymorphisms. However, since most RAPD markers are dominant (heterozygotes are indistinguishable from homozygotes) (Tinker et al. 1993), estimation of individual genotypes and allele frequencies is difficult from the raw data (Lynch and Milligan in press). One can use traditional population analyses based upon allele frequencies if Hardy-Weinberg assumptions are made. This is often not appropriate, especially because of small sample sizes and non-random mating. Therefore, researchers employing DNA typing methods such as RAPD profiling often address populational questions such as genetic

heterogeneity or population structure in a qualitative and nonstatistical manner (e.g. Gilbert et al. 1990, Brauner et al. 1992, Lu et al. 1992).

Attempts have recently been made to use RAPD profiling in a more rigorous population genetics framework (Lynch and Milligan in press). A general methodology based on an analysis of molecular variance (AMOVA) for the estimation and the testing of population genetic structure using both haplotype frequencies and molecular information has recently been introduced (Excoffier et al. 1992). Its application to RAPD data is not straightforward because of the dominance pattern of these markers and the lack of information on the exact genotypes of diploid individuals. However, if we assume that organisms reproduce 100% clonally, then RAPD profile data may be analyzed using a slightly modified AMOVA. One modification includes a permutational heteroscedasticity test.

In several research areas (e.g. resource management, conservation, ecology), the comparison of the amount of genetic variability among populations is often as important as the determination of their genetic structures. I therefore introduce a nonparametric test for the homogeneity of variance (HOMOVA) based on Bartlett's statistic (Bartlett 1937). *Vaccinium macrocarpon* (American cranberry) RAPD population data with small sample sizes are utilized. The underlying biological question of interest is whether distributionally marginal cranberry populations are less heterogeneous than central populations (i.e., molecular variation is greater in central than in marginal populations).

Vaccinium macrocarpon and most other bog ericads are self-fertile (Reader 1977) and flower morphology and pollination biology suggest that autogamy is the reproductive norm

(Roberts and Struckmeyer 1942, Reader 1977). In homogeneous populations, greater amounts of geitonogamy (self-fertilization within spreading clones) would increase already high selfing frequencies to approach 100% (Handel 1983). Artificial cross-pollination has been reported in cranberry (Bain 1933) and fruit yield is reported to be greater in heterogeneous populations (Marucci and Filmer 1964). No cause and effect relationship has been elucidated concerning cross-pollination and yield (Sarracino and Vorsa 1991). To the contrary, there have been no data to date demonstrating that cross-pollination occurs in nature, or that heterosis or inbreeding depression exists in *V. macrocarpon* (Reader 1977, Dana et al. 1989). It is therefore a valid assumption that when *V. macrocarpon* does reproduce sexually that it is by autogamy.

Materials and Methods.

Samples. I sampled three marginal populations (Dare County, North Carolina (NC), Johnson County, Tennessee (TN), and Pocahontas County, West Virginia (WV)) and three central populations (Schenectady County, New York (NYF), Oswego County, New York (nye), and Houghton County, Michigan (MI)) (Fig. 4.1). Cranberry clonal spatial arrangements within sites are patchy and individual genets cannot be casually observed. The populations were small (< 1 ha) and discrete. The marginal populations (especially TN and NC) were geographically isolated. DNA was extracted from four leaf samples taken 20 m apart along a transect using a modified Doyle and Doyle (1987) procedure (Stewart and Via 1993). RAPD reactions were performed according to methods outlined in Stewart and Via (1993) with 5 ng of template per sample (Fig. 4.2). Reproducible band states (105) were scored and data coded +/- for the eight primers utilized (OPA4: AATCGGGCTG, A7: GAAACGGGTG, A9: GGGTAACGCC, A11: CAATCGCCGT, A13: CAGCACCCAC, A18: AGGTCACCGT, B4: GGACTGGAGT, B18: CCACAGCAGT; Operon Technologies, Alameda, CA).

Estimating population genetic structure with RAPD data. The estimation of population genetic structure in an analysis of variance framework (AMOVA) has been fully described for haplotypic markers such as mitochondrial DNA RFLPs (Excoffier et al. 1992). The principle of AMOVA is to extract variance components and analogs of F-statistics (called Φ-statistics) from a matrix of squared euclidian molecular distances between genets collected into populations, themselves arranged into groups of populations. The main difference between

conventional codominant molecular markers, such as RFLPs or DNA sequences, and dominant RAPD markers lies in the unavailability of genotypic information for the assayed individuals: one has to deal with phenotypic information only. Therefore, as the nature of the chromosomes present in one particular individual cannot be assessed, inter-chromosomal distances are not directly available. However, in completely asexual organisms or 100% selfers, RAPD profiles may be treated as haplotypes. For RAPDs I will estimate unknown distances by making several assumptions. These additional assumptions allow the use of highly polymorphic RAPD markers, but at the cost of a decreased precision of the analysis compared to that of codominant markers, as already pointed out by Lynch and Milligan (in press). Throughout this study, I will assume the following properties of RAPD markers in populations of diploid individuals:

1) The banding pattern on gels is interpreted without ambiguity.
2) A band on a gel identifies a strictly two-allele locus. A (1) will denote the presence of a band and a (0) its absence and (1) is completely dominant over (0).
3) Different band positions can be considered as different loci.
4) Chromosomes are inherited modularly.

I use the same hierarchical model of population genetic structure as described in Excoffier et al. (1992), with individuals collected into populations, and populations nested into groups, leading to the analysis of variance format shown in Table 4.1. The model assumes that the j-th haplotype frequency vector from the i-th population in the g-th group is a linear equation of the form

$$x_{jig} = x + a_g + w_{ig} + c_{jig} . \quad (1)$$

The vector x is the unknown expectation of x_{jig}, averaged over the whole study. The effects a, for group, w, for population within a group, and c, for individuals within a population, are assumed to be additive, random, independent, and to have the associated variance components (expected squared deviations) σ^2_a, σ^2_w, and σ^2_c, respectively. The total molecular variance is the sum of the variance components. Not considered here is the variance due to differences between chromosomes within individuals, as there is no way to estimate how haplotypes are associated within individuals using dominant markers. The vector x_{jig} takes the form of a boolean vector of dimension m, equal to the total number of RAPD loci surveyed, where the presence of a site is coded as a (1) and its absence as a (0). A sum of squared deviations may be expressed as a function of inter-haplotypic distances (Excoffier et al. 1992) as (for the total sum of square deviations)

$$SSD(T) = \frac{1}{2N}\sum_{j=1}^{N}\sum_{k=1}^{N}\delta^2_{jk}, \qquad (2)$$

where N is the total number of diploid individuals surveyed in all populations. As the RAPD loci are assumed independent and providing the same amount of information, the genetic distance between individuals j and k(δ^2_{jk}) is of the form

$$\delta^2_{jk} = (x_j - x_k)'(x_j - x_k)\ . \qquad (3a)$$

This euclidian distance is equivalent to the number of different RAPD loci between two haploid genomes and may be rewritten as

$$\delta^2_{jk}=\sum_{s=1}^{M}(s_{sj}-x_{sk})^2. \qquad (3b)$$

where the subscript 's' indexes the m RAPD loci. As RAPD data consist of dominant markers, we can only compare individual phenotypes. An AMOVA can be performed if we can translate inter-haplotypic distances in terms phenotypic distances between the RAPD profiles of two individuals as is done with haplotypic distances described previously (Excoffier et al. 1992).

Testing variance components significance. Under the null hypothesis of no population structure, all the individuals can be assumed to be drawn from a single panmictic population. This is tested by using a permutational analysis as described in Excoffier et al. (1992). Essentially, the haplotypic variance is partitioned within populations, among populations within regions, and among regions. The resulting distributions are compared to null distributions resulting from the AMOVA that randomly samples individuals and places them in arbitrary populations and regions, and places populations in arbitrary regions. We can thus obtain the null distribution of the statistics of interest by randomly permuting individuals across populations and regions, and compare these to the actual data (Excoffier et al. 1992).

Testing molecular variance homogeneity. It is conventional in analyses of variance to assume homogeneous variances among populations. With normality of the variates, this assumption is crucial for the proper parametric testing of variance ratios, but they are not absolutely required when using nonparametric methods for testing variance components significance (Excoffier et

al. 1992). In many ecological or biodiversity studies, testing for variance homogeneity is important in itself (Antonovics 1984, Ford-Lloyd and Jackson 1986, Altukhov 1990, Millar and Libby 1991). Conventional testing procedures, such as Bartlett's test of homogeneity of variances (Bartlett 1937), are parametric and assume normality of the variates for proper testing procedure. However, Bartlett's statistic (B) is meaningful as it expresses a deviation of population variances from the mean total variance. It can be formulated in terms of sum of squared deviations as

$$B=\frac{(N-P)\ln\left(\frac{SSD(T)}{N-P}\right)-\sum_{i=1}^{P}(N_i-1)\ln\left(\frac{SSD(WP)_i}{(N_i-1)}\right)}{1+\frac{1}{3(P-1)}\left(\sum_{i=1}^{P}\frac{1}{N_i-1}-\frac{1}{N-P}\right)}, \qquad (4)$$

where P is the number of populations and $SSD(WP)_i$ is the sum of squared deviations within the i-th population given by equation (2) with N replaced by N_i. B should follow a Chi-squared distribution with P-1 degrees of freedom if haplotype fequencies were to be normally distributed. As this assumption does not hold, we propose to test if the observed B value is significant by computing the nulll distribution of Bartlett's statistic using a permutational approach (Excoffier et al. 1992): a random collection of samples is generated by allocating individuals to random populations, keeping population sizes constant; population statistics (sums of squared deviations, molecular variances and Bartlett's statistic) are then computed for these new samples; the null distribution is obtained by repeating this procedure many times. If the above test reveals significant differences then we use a Tukey-like multiple comparisons (Zar 1984) to evaluate patterns of population variance. Here, pairwise population variance differences are taken and

compared to analogous differences from the resampled populations (null distribution). Absolute values are compared and observations made as to which variances differ.

Results and Discussion.

Population genetic structure and variance components. All clones in central populations were discernable from one another. In marginal populations NC and TN, RAPD profiles were nearly monomorphic (Fig. 4.2), where, on average, 97% of RAPD bands shared identity. The nested AMOVA procedure estimated the variance among groups (marginal and central) to be 15.5%, among populations within groups to be 48.2% and within populations to be 36.3%. When discounting groups as a factor, 61.8% of the variation was among and 38.7% was within populations. Significant differences were found ($p < 0.05$) among populations within groups, and within populations, but not among groups. Sample sizes of groups is low (d.f. = 1), and there are only 45 ways to permute populations, therefore it is statistically impossible to even test at the 0.05 level. However, the population variances are significantly different (Fig. 4.3), and the populations extremely well differentiated. Given the mode of reproduction, and life history strategy, this is not unexpected (Hamrick and Godt 1989). In fact, the amount of among population variation is high, but within the ranges of woody, selfing plants (Hamrick et al. 1992).

Molecular variance homogeneity. The HOMOVA procedure showed populations had significantly different variances ($p < 0.05$). Therefore, we used multiple comparisons to locate the differences. Based on $n=4$ marginal and central genetic heterogeneity patterns were observed and were statistically significantly different (Fig 4.3). Marginal populations (NC, TN)

were nearly monomorphic and had significantly lower ($p=.027$) molecular variances than WV and central populations (MI, NYF, nye). Another study showed that in TN, for example, 19 out of 24 samples were part of the same clone (Chapter 6). The WV population, which was *a priori* grouped with marginal populations, seems to have intermediate genetic heterogeneity between marginal and central populations. However, one outlier genet (WV4) which is substantially different from the other WV genets, is the basis of this result (Figs. 4.2 and 4.3). Whereas TN and NC populations are isolated, WV is proximate to several other cranberry populations. These results suggest that gene flow is higher among central populations than marginal populations. Alteratively, the former are, on average, larger and contain more genets because of their ecology.

Vaccinium macrocarpon is a vegetatively spreading clonal plant that reproduces sexually primarily by selfing. Another study has shown that clonal spread is positively associated with marginality (Chapter 6). That is, in marginal sites there are few widespread clones compared to many smaller clones in central sites. Genetic diversity has not been successfully measured by allozyme analysis in cranberry, presumably because of high homozygosity (Hill and Vander Kloet 1983, Breuderle et al. 1991, Hugan et al. 1993). However, RAPD profiling reveals many polymorphisms within and among populations. HOMOVA estimates differences in population genetic heterogeneity that are obvious in cranberry. However, larger sample sizes may be needed to resolve obscure differences. It is commonly thought that genetically homogeneous populations are less stable and flexible than genetically heterogenous populations (Millar and Libby 1991). Quantifying population genetic homogeneity is therefore important in conservation

biology and ecological settings and the HOMOVA provides a direct estimation of this parameter. This generalized method may be used with other molecular markers in clonal organisms, with few required assumptions. Thus, dominant marker DNA typing methods such as RAPD that do not directly provide allele frequencies, but reveal many polymorphic markers may be used in a comprehensive population analysis using the AMOVA and HOMOVA in tandem.

Table 4.1. General design for the analysis of molecular variance (AMOVA) of *Vaccinium macrocarpon*. MSD = mean squared deviations.

Source of variation	d.f.	MSD	Expected MSD
Among regions	1	MSD(a)	$\sigma^2_c + 8\sigma^2_w + 24\ \sigma^2_a$
Among populations within regions	4	MSD(w)	$\sigma^2_c + 8\ \sigma^2_w$
Among individuals within populations	42	MSD(c)	σ^2_c

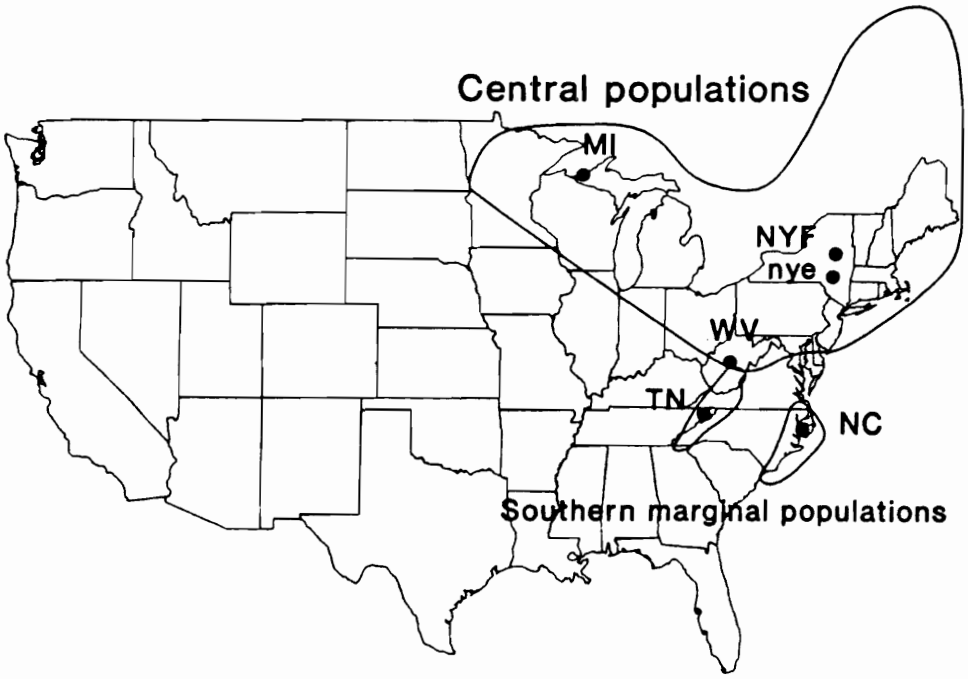

Figure 4.1. Geographic locations of cranberry populations sampled for RAPD profile analysis. See text for site locations.

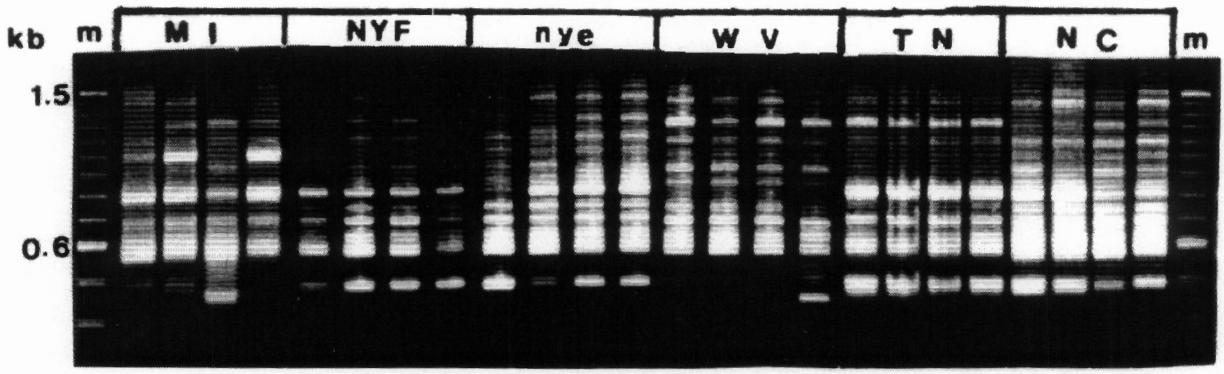

Figure 4.2. RAPD profiles of central and marginal cranberry clones. Profiles are representative of clones in these populations. Lane m contains 100-bp marker (GIBCO BRL/Life Technologies, Gaithersburg, MD). Samples were amplified with OPA-13 (Operon Technologies, Alameda, CA).

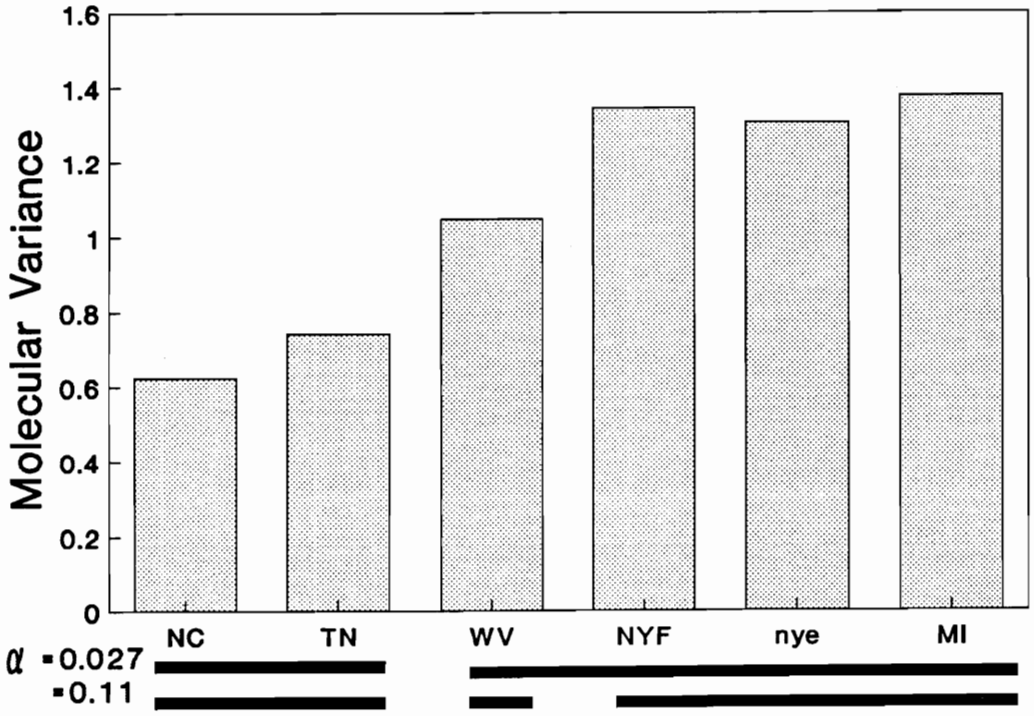

Figure 4.3. Molecular variance among cranberry populations. Homogeneity of variance (euclidean distances) significance of 6 cranberry populations. Bars indicate significant differences at two significance levels.

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Chapter Five

Phenotypic Plasticity and Genetic Heterogeneity of *Vaccinium macrocarpon*, the American cranberry. I. Reaction Norms of Central and Marginal Clones in a Common Garden.

Introduction.

Genetic differentiation among ecologically diverse populations has been widely documented (e.g. Turesson 1922 a,b, Clausen et al. 1940, Quinn 1978, Silander 1985b). Phenotypic plasticity, the morphological and/or physiological responses of a genotype to spatial or temporal environmental heterogeneity, has been hypothesized to be an important aspect of genetic differentiation within populations (Bradshaw 1965, Sultan 1987). Gause (1947) proposed that phenotypic plasticity could be an alternative mode of adaptation compared to "genoadaptation" or intrapopulational genetic differentiation. This inverse relationship between phenotypic plasticity and genetic variation has been rejected on empirical grounds (Moran et al. 1981, Hume and Cavers 1982, Scheiner and Goodnight 1984, Wood and Degabriele 1985, Schlichting and Levin 1986, Taylor and Aarssen 1988), and on the findings that phenotypic plasticity has a genetic basis (Bradshaw 1965, Schlichting 1986, Scheiner and Lyman 1991). However, there have been findings that support the tradeoff hypothesis (Pedersen, 1968, Cook and Johnson 1968, Jain 1978, Wu and Jain 1978, Zangerl and Bazzaz 1983, Silander 1984, 1985a, MacDonald and Chinnappa 1989, Thompson et al. 1991).

Although substantial research has recently involved the nature of phenotypic plasticity and its role in plant fitness, very little work has been done using clonal plants (Silander 1985b, deKroon and Schieving 1992). As a subset of plasticity studies, findings about clonal plant plasticity are mixed (Cook and Johnson 1968, Hume and Cavers 1982, Scheiner and Goodnight 1984, Silander 1984, 1985a, Taylor and Aarssen 1988, Thompson et al. 1991). However, the

clonal species studied are almost exclusively foragers or consolidators *sensu* deKroon and Schieving (1990) not conservative growers, which would include most woody clonal species and all dwarf shrubs. Life history strategies of long-lived clonally-spreading plants suggest that phenotypic plasticity could be adaptively significant (Silander 1985b, Sultan 1987). deKroon and Schieving (1990) have provided a framework for characterizing clonal plant life histories. Most vegetatively spreading facultative clonal shrubs are classified as conservative growth plants. These are homologous to the category of 'stress tolerators' (Grime 1979, Chapin 1980). This category includes arctic, boreal, and temperate bog dwarf shrubs, such as *Vaccinium macrocarpon*. These conservative growers respond plastically to increased nutrient availability resulting potentially in rapid site filling by one or a few genets, although Chapin (1980, 1987) argues that phenotypic plasticity would not be an important mode of adaptation for these plants. Guerilla growth (Lovett Doust 1981) would allow a single clone to spatially exclude possible competitors from a site, thereby rendering selective advantage to plastic genets. This scenario suggests that in long-lived perennial plants, plasticity could indeed be an alternative to genetic differentiation in a location with heterogeneous microsites. Restated, individuals may be adapted to microsites by producing variable phenotypes, or alternatively, by selection for specialized nonplastic genotypes. Opportunity for site filling by few clones would be contingent upon long periods between colonization events (i.e., low immigration), competitive exclusion, and small site area.

For this study I used *Vaccinium macrocarpon*, the commercially important cranberry that occurs naturally in peatlands in the northeastern U.S., the Great Lakes region and southeastern

Canada. Among centrally distributed populations there is higher gene flow and larger suitable habitats than in marginal populations (Ogle 1984). Distributionally marginal populations of *V. macrocarpon* are found in the central and southern Appalachian mountains and North Carolina coastal plain (Fig. 1.2). Marginal populations are smaller, have lower sexual reproduction and are thought to be relicts of the Pleistocene ice age (Wieder et al. 1981, Ogle 1984, Stewart 1993). Moreover, marginal cranberry populations have been shown to have lower genetic heterogeneity than central populations (Chapter 4). Specifically, there were fewer discernable clones and less interclonal molecular variation in marginal populations compared to central populations. Southern clones were larger and presumably older, suggesting decreased recruitment within marginal sites. The objective of this study is to determine the importance of phenotypic plasticity to clonal differentiation in central and marginal *V. macrocarpon* and answer the question: Are clones from marginal populations more plastic than clones from central populations?

Materials and Methods.

Sites sampled. Clones from three disjunct southern populations (NC, TN, WV), and from three northern, centrally distributed populations (NY, WI, MA) were used in a common garden experiment. The clone from Dare County, North Carolina (NC) was collected from a 0.5 ha coastal pocosin. At the site, small patches of *V. macrocarpon* grow among larger ericaceous (typical pocosin) shrubs. The Tennessee (TN) clone was from a small bog on a Johnson County farm. This 0.1 ha site had been fenced for 12 years to exclude cattle. The WV clone was sampled from a small bog on Droop Mountain, West Virginia. TN and WV sites have been studied with respect to vegetation (Stewart and Nilsen 1993, Chapter 2), phenotypic plasticity, and clonal variation (Chapter 6). The NY clone originated from Featherston Haugh Lake in Schenectedy County, New York. This clone was collected from the floating mat on the north side of the lake. The cranberry area was small (< 1ha). The Wisconsin (WI) and Massachusetts (MA) clones are cultivars originally isolated from natural populations and vegetatively propagated for commercial production. The WI clone ('Searles') was originally grown in Wisconsin Rapids in 1893 and was selected from that vicinity (Dana 1983). Likewise, a Cape Cod, MA clone ('Early Black') was first commercialized in 1857 in Harwich, MA (Dana 1983). The Searles and Early Black accessions used in these experiments came courtesy of Ocean Spray Cranberries, Inc.

RAPD profiling. Clonal identities and genetic distances were assessed by RAPD profiling (Williams et al. 1990). DNA was isolated either by the Doyle and Doyle (1987) method or

using the Stewart and Via (1993) protocol, which are of equivalent quality for RAPDs. RAPD cycling parameters have been described elsewhere (Stewart and Via 1993, Chapter 3).

Experimental design. Mature cranberry clones each representing a population were randomly assigned to 1600 cm^2 plastic pans containing Canadian peat, so that each pan had ramets of all six clones. The experiment was established September 1991, fertilizer added March 1992, and above-ground tissue was harvested September 1992. Nutrient treatments were also randomly assigned. The pans were in two raised beds (blocks). Thus, the experimental design was: 6 clones x 4 nutrient treatments x 2 blocks x 4 replicates = N = 192 plants. Although this was a balanced complete factorial design, because of plant over-wintering mortality (probably transplant shock) the data set was not balanced.

The nutrient treatments were:

i. Nitrogen addition 1.1 g/m^2 supplied as slow release urea.

ii. Phosphorus addition 1.5 g/m^2 supplied as triple superphosphate.

iii. Nitrogen and phosphorus added together at the same rates as above.

iv. No nutrient additions.

The nutrient application rates were derived from other studies (Eck 1964, 1990, Eaton 1971a,b). The fertilization rate represented minimal amounts to elicit growth responses. Nitrogen and phosphorus were chosen because they are the two most limiting bog soil nutrients. The fertilizer was applied in forms recommended for commercial cranberry growers (Eck 1990).

The original experimental design was to include the fertilization regimes as classification

variables in the profile analysis of variance. However, unexpected complications demanded another statistical approach. The pans had holes drilled in the bottom to allow for water drainage. This was done to avoid soluble salts buildup, since municipal water was the irrigation source for the experiment. The result was an unexpected alteration of pH and macronutrients, presumably caused by capillarity from the mineral soil underlying the pans. These nutrients swamped the effect of the experimental nutrient additions. This result was confirmed by the analysis of variance of proposed classification variables and the measured soil nutrient levels, which showed insignificance (data not shown). Therefore, data were primarily analyzed by analysis of covariance (ANCOVA), using the measurements of soil nutrient concentrations for root medium in each pan at the end of the experiment and clones as independent variables.

pH and soil macronutrient (P, K, Ca, Mg) concentrations were measured at the beginning and end of the experiment by the VPI & SU soil laboratory using a double acid extraction. Ionic measurements were done using a inductively-coupled plasma spectrophotometer (Donahue and Gettier 1988). Ammonium pools were analyzed by the indophenol blue method (Keeney and Nelson 1982) on a second set of soil samples collected on the same date.

Growth and Phenology. The functional plant modules in *V. macrocarpon* are upright stems, which are important vegetatively and in sexual reproduction; and runners, which are important for vegetative spread only (Eck 1990). Above ground biomass was harvested at the end of the experiment (September 1992). Leaves were counted and biomass partitioned according to organ and module type. Table 5.1 lists vegetative traits measured. These traits were chosen because

they are important indicators of growth habit and rate. Flower timing was measured in early summer 1992, and midwinter upright leaf anthocyanin levels were taken February 1993, on incidental leaves that remained after harvest.

Statistical analyses. Flower phenology was analyzed using profile analysis (Morrison 1976, Simms and Burdick 1988). An analysis of covariance (ANCOVA) and multiple comparisons (LSD) was used to assess clonal differentiation, plasticity, and differences in reaction norms. Clones and soil nutrients were the main effects in the model. The clone main effect tested for significant means of traits; i.e., tested if clonal reaction norms had different heights. Phenotypic plasticities were determined by simple linear regression by clone; i.e., tested $H_o\ \beta = 0$. To minimize variance, and as a normalization procedure, log log (base 10) transformations were done preceding regression analysis (by clone), which was performed to determine significant traits, nutritional factors, and to estimate plasticities (slopes). The slopes (reaction norms) were compared by using an ANCOVA homogeneity of slopes model, i.e. slopes were different if the nutrient*clone interaction term was significant (SAS Institute 1990). Total plasticity among clones was assessed by using an ANOVA. Reaction norm analysis is appropriate as a result of the clonal replicates utilized.

RAPD data were analyzed by scoring band presence or absence for each clone. Genetic distances were estimated using the euclidean distance (Excoffier et al 1992, Chapter 4). Pairwise matrix comparisons using the Mantel test (Mantel 1967, Rohlf 1988) were performed to pairwise compare the following distance matrices: geographic, genetic, trait, and plasticity

distances among clones. Relationships were also shown visually with UPGMA dendrograms (Rohlf 1988).

Results.

Fruit production in this study was negligible, although several clones produced flowers (Figs. 5.1, 5.2). Since sexual reproductive effort was low, most of the traits measured and reported were vegetative. In general, clones were phenotypically differentiated from one another (different interclonal trait means), but most clones were not phenotypically plastic.

Environmental conditions. The climatic conditions in Blacksburg, VA experimental site, (mean July temperature = 21.3°C, mean yearly precipitation = 108.7 cm) were similar to northeastern U.S. commercial cranberry centers. When comparing among clonal origins, Blacksburg's climate most resembled that found at the TN site (mean July temperature = 21.0°C, mean yearly precipitation = 116.4 cm), followed closely by MA (mean July temperature = 22.2°C, mean yearly precipitation = 120.7 cm) and WV (mean July temperature = 20.0°C, mean yearly precipitation = 111.8 cm). The NC site (mean July temperature = 23.8°C, mean yearly precipitation = 111.8 cm) was warmer, and NY (mean July temperature = 17.7°C, mean yearly precipitation = 96.5 cm) and WI (mean July temperature = 17.0°C, mean yearly precipitation = 78.7 cm) sites were colder (Ruffner 1985).

Soil nutrients in the experimental plots were significantly altered by the influx of ions from the mineral soil below the pans. As a result, most of the variation observed in soil nutrient measurements was not from the fertilizer treatments (data not shown). Nutrients and pH were significantly higher than those of most northern bogs and even marginal bogs (Stewart and Nilsen 1993). Means and standard deviations were as follows: pH: 5.37$\pm$0.69, ammonium: 81.9

$\pm$ 15.8 $\mu g/cm^3$, phosphorus: 0.85 $\pm$ 0.34 $\mu g/cm^3$, potassium: 5.42 $\pm$ 0.85 $\mu g/cm^3$. Experimental ammonium concentrations and pH levels were higher than those found in ombotrophic or minerotrophic bogs, and phosphorus and potassium concentrations were higher than those found in ombotrophic bogs, but comparable to minerotrophic bog (fen) soil (Heinselmann 1970). In contrast to the means, the standard deviations represented field-level variation. Therefore, the experimental conditions were climatically realistic, but not edaphically realistic for natural bogs. Furthermore, the edaphic variation was fine-scale. However, soil nutrient concentrations did fall well within the ranges of commercial cranberry bogs (Fisher 1951, Eck 1990).

Plant responses. **Clonal differentiation--** Clonal differentiation was much stronger than for phenotypic plasticity. Of the traits measured, 72% were significantly different among clones tested at $\alpha=0.05$ (Table 5.2). Clone MA had the greatest PTWT, L/UP, L/R, and was generally most vigorous vegetatively, whereas WV was the smallest clone (least ULWT, UPW, RLW). There did not seem to be any obvious central and marginal patterns regarding clonal differentiation. However the MA and NY clones had similar reaction norms, as did TN and WV (Table 5.2, Fig. 5.3). For some traits, marginal clones had generally lower runner trait means than central clones (Table 5.2).

The only clear pattern regarding marginality and centrality was that of flower phenology as central clones flowered earlier (Figs. 5.1, 5.2). Profile analysis showed only marginally significant differences among clones in the time contrasts ($p = 0.09$). The relatively small and

uneven sample sizes of flowering ramets (from four to 12) may explain the lack of significant differences (at $\alpha = 0.05$). The conspicuous differences in profiles may be biologically important nonetheless. This temperature and latitudinal related pattern of flowering is typical of other long day crops, in which northern clones flower earlier compared to southern clones when grown in a common garden (Salisbury and Ross 1978). Primarily, this phenomenon can be attributed to the increased sensitivity of temperature cues in northern clones (Billings 1974).

Plasticity in flower phenology could not be assessed because of the small sample of flowering ramets.

Phenotypic plasticity-- At the fine scale of soil environmental manipulation, very little phenotypic plasticity was noted in the measured traits. The log log type II or type III general linear models showed no significant differences with the following exceptions: TN clone (SW/R, RW; all independent variables and interactions significant at $\alpha=0.05$) WI clone (PTWT, TOTUPW, STOM, RLW, LW/U, SW/U; all independent variable and interactions significant at $\alpha=0.05$). Type I model significant differences are shown (Table 5.3) to show some possible trends and indicator environmental variables. Generally, soil ammonium concentration (N) was the predominant main environmental factor. pH was also a significant independent variable for some traits and clones (Table 5.3). Noteworthy is the tendency for a main effect (such as N) to be significant in central clones, whereas marginal clones display more significant interactions (Table 5.3). The ANCOVA homogeneity of slopes model (nutrient*clone interactions) revealed no significant differences at $p=0.05$.

Clones had significantly different levels of total phenotypic plasticity (Table 5.4). WI and NC had the greatest plasticities in response to N and pH. With log log transformations, these two clones exhibited the only positive slopes for N responses.

RAPD profiling. Of the 40 primers (A and B kits from Operon, Alameda, CA) screened, 25% revealed interclonal polymorphisms (over 10% average dissimilarity). The following primers were used to generate 105 polymorphic bands: A4, A7, A9, A11, A13, A18, B4, B18, which resulted in a composite genetic profile for each sample. The presence or absence of these markers were used as input to calculate a euclidean distance matrix. This matrix was used as the input for a UPGMA cluster analysis (Rohlf 1988) that showed coastal clones NC and MA formed a cluster and were joined successively with the WV and TN clones (Fig. 5.4). The two northern inland clones, WI and NY also clustered together, but were relatively dissimilar (euclidean distance of 20). Excoffier et al. (1992) have shown that for profile data ("haplotypes") that the euclidean distances indicate the number of bands that mismatch between clones. So, in this case, a euclidean distance of 20 indicates about 81% profile similarity.

Matrix comparisons. Distance matrices (geographic, RAPD profile, trait mean, plasticities) were standardized to a mean of 0 and a standard deviation of 1 (SAS Institute 1990). Standardized matrices, when performing pairwise comparisons with one another, showed no highly significant correlations, with the exception that the nitrogen-response plasticity matrix

negatively correlated with RAPD, means, and geographic matrices, but with low r ($r < 0.5$) (Table 5.5). Dendrograms generated from geographical, genetic trait and plastic matrices also showed no consistent patterns (Fig. 5.4).

Discussion.

The objective of this study was to address the importance of phenotypic plasticity to clonal differentiation regarding marginal and central populations. Two aspects of the marginal-central question will be addressed here. One is the historical context that the concepts of centrality and marginality may be applied to *V. macrocarpon*. The other is the degree of environmental stress and uncertainty present at marginal versus central sites.

I used a common garden experiment to assess differences in reaction norms (levels and slopes) in response to nutrient manipulations. Any variation in means or slopes would be due to genetic differences, since they were grown in a common environment. Generally, all clones responded similarly with certain exceptions. These will be put in the context of the two points above to determine whether or not we can assess possible long term ramifications of a changing climate for *V. macrocarpon* and other bog shrubs.

Genetic variation. *Vaccinium macrocarpon* is a temperate bog mat plant. The fruits are presumed to be bird-dispersed; potentially for long distances (Ogle 1984). Following Pleistocene glaciation *V. macrocarpon* migrated from now distributionally marginal sites to currently central sites. Genetic distances estimated from RAPD phenotypes show that the clones with the largest distances between them (WI, TN) genetically are about 75% similar. This hypothesized intraspecific phylogeny is not well modelled by the UPGMA cluster analysis (Fig. 5.4, panel B), which has a dissimilar relative topology from that of a tree constructed when a larger sampling of clones is used (Stewart 1993, unpublished data). However, for our purposes of

comparing different matrices, phenograms are sufficient visual aids.

It is recognized that the species is very homozygous, as allozyme variation within the species is very low (Hill and Vander Kloet 1983, Hugan et al. 1993). In contrast, blueberries, which are outcrossers, contain many allozyme polymorphisms (Breuderle et al, 1991). The genetic homogeneity of *V. macrocarpon* is probably, at least in part, due to the breeding system, the recent distributional history, and island-like distribution. The latter is not shared with the *Cyanococcus* section of *Vaccinium*. Unlike the more generalist blueberries, cranberries are specialized to bog habitats. It is likely that springtime migratory birds quickly dispersed *V. macrocarpon* seeds to available bare suitable sites as glacial ice retreated (Ridley 1930, Ogle 1984). This rapid colonization and gene flow would have served to homogenize a genome that had already been through a distributional bottleneck. Although *V. macrocarpon* is now found in somewhat isolated populations, especially at its southern limits, this distribution is only a recent (last 5000 years) occurrence (Ogle 1984). For a species with this distributional history, it is not unexpected that genetic distances between now geographically distant clones are relatively low. Therefore, it follows that phenotypic responses would also be similar among clones. That is, strong ecotypic differentiation would not be expected in *V. macrocarpon*. The results of this study agree with the historical development of *V. macrocarpon* discussed above. A consideration of these ideas follow.

Environmental stress and marginality. *Vaccinium macrocarpon* is adapted to low nutrients, and cold temperatures. In addition, peatlands have relatively low dissolved oxygen as a result

of high water tables (Small 1972a). It is believed that evergreen, sclerophyllous leaves are a means to provide a high nutrient use efficiency to cope with chronically low nutrient status (Monk 1966, Small 1972b, Chapin 1980). In many commercial cranberry growing regions, such as in Wisconisn, there is a year-long possibility of frost (Curtis 1959). Since *V. macrocarpon* is a dominant member of the vegetation where these conditions are prevalent, it is presumably adapted to these abiotic stresses. One could argue that at its southern limit, where it exists as a relict, *V. macrocarpon* faces stresses that it is not adapted to, such as heat and interspecific competition with more vigorous plants. This scenario could favor directional selection for heat tolerance and guerilla growth characteristics (i.e. phenotypic plasticity). Even though commercial *V. macrocarpon* clones are reported to be neither heat nor shade tolerant (Eck 1990), increased plasticity was not observed in the predefined marginal populations.

Several studies with clonal plants have found that clones from ecologically marginal (stressful) sites have greater phenotypic plasticity. Grant and Antonovics (1978) found that *Anthoxanthum odoratum* plants from adjacent marginal sites responded more quickly to environmental change compared to a central population. However ecotypic differences were greater than plasticity. Lotz and Blom (1986) found that in *Plantago major*, plants from stressful environments contained higher plasticity. Few studies have examined bog plant plasticity, and none have compared genetic variation and phenotypic plasticity. *Vaccinium vitis-idaea* was found to be the least plastic of bog dwarf shrubs in a high arctic nutrient study (Chapin and Shaver 1985). In a common garden study Riebesell (1981) found that more stressed alpine populations of *Ledum groenlandicum* had higher plasticity in photosynthesis rates than

central bog populations. Ohlson (1989) compared low nutrient (stressed) and higher nutrient populations of the mire plant, *Saxifraga hirculus*, and found higher plasticity in marginal clones. She found large amounts of ecotypic differentiation as well.

In *V. macrocarpon*, there seems to be neither strong ecotypic differentiation nor phenotypic plasticity. Although many clonal trait means are significantly different, there seems to be no pattern in the differences, and the differences in many instances are biologically slight (Table 5.2). There are also no obvious patterns when comparing geographic, genetic, trait mean or plasticity distance matrices (Table 5.5, Fig. 5.4). In other instances where these types of comparisons have been made, the matrices or trees also did not correlate (Schlichting and Levin 1988, Vasseur and Aarssen 1992). These authors concluded that phenotypic plasticity was independent of traits and a was trait itself (Schlichting and Levin 1988), or that plasticity was not necessarily adaptive (Vasseur and Aarssen 1992). In *V. macrocarpon*, the most parsimonious explanation is that the genetic and phenotypic similarities among clones are due to the recent clonal ancestry and low genetic divergence.

In the current study the only hint that phenotypic plasticity may be of any importance to growth of *V. macrocarpon* comes from the overall analysis of phenotypic plasticity (pooling all traits) using an ANOVA (Table 5.4). Clones WI and NC, had statistically significantly similar responses (plasticities) to N and pH when subjected to log log transformation. A non-parametric one-way ANOVA analog yielded similar results (Hollander and Wolfe 1974). This explanation is weak, given the insignificance of the individual trait plasticities, especially in NC. However,

as an exercise in speculation, let us assume that the "optimal" climate for *V. macrocarpon* is at its highest density and geographical center of contemporary distribution, Cape Cod, Massachusetts. NC and WI sites have the greatest temperature deviations from the MA site. Temperature difference from the cranberry optimal may be used as an index of environmental stress. Perhaps the wrong criterion of marginality was invoked (distributional versus environmental) in my initial classification of clones. Perhaps TN and WV should be included with central sites because of their near Massachusetts-like climate, and WI should be classified as marginal, because of its very cold temperatures that could be stressful. This could be formally tested by including additional *a priori* northern marginal clones from areas such as Minnesota and Canada and southern clones from the North Carolina coast, or the newly discovered clone south of Nashville, Tennessee (Hal DeSelm, pers. comm.). Common garden and reciprocal transplant studies of additional bog species (temperate, boreal and arctic) need to be performed to address the adaptive significance of phenotypic plasticity to stress-tolerating clonal plants.

Table 5.1. Key to trait abbreviations

Abbreviations	Traits
L/U	avg. leaf number per upright module
ULWT	avg. upright module weight per leaf (mg)
LW/UP	avg. weight of all leaves per upright module (mg)
SW/UP	avg. weight of stem per upright module (mg)
PTWT	weight of above-ground individual plant (mg)
TOTUPW	weight of upright modules per plant (mg)
UPW	avg. weight of upright modules (mg)
UP/P	number of upright modules per plant
STO	avg. stomatal density per 0.04 mm^2 of upright leaf
L/R	avg. leaf number per runner module
RLW	avg. runner module leaf weight (mg)
LW/R	avg. weight of all leaves per runner module (mg)
SW/R	avg. weight of stem per runner module (mg)
RW	avg. weight of runner modules (mg)
TOTRW	weight of runner modules per plant (mg)
R/P	number of runner modules per plant
UW/RW	ratio of upright module weight to runner module weight
U/R	ratio of upright module number to runner module number
ANTHO	midwinter leaf anthocyanin concentrations (optical densities at 510 nm, Lloyd et al. 1992)

Table 5.2. Multiple comparisons of trait means (untransformed) with clones. Different letters in rows denote significance at $\alpha = 0.05$. determined using Fisher's LSD (Zar 1984). (ns) = model reveals no significant differences at $p = 0.05$. See Table 5.1 for trait abbreviations and text for clone provenances.

	Clones					
TRAITS	NC	TN	WV	MA	NY	WI
L/UP	30.9 ab	28.1 bc	28.0 bc	32.1 a	25.7 c	29.0 abc
ULWT	1.72 bc	1.73 bc	1.41 d	1.70 c	1.89 b	1.96 a
LW/UP	52.8 a	48.9 a	39.6 b	54.7 a	48.6 a	55.3 a
SW/UP	27.4 a	23.1 c	21.1 c	23.6 ab	25.0 ab	27.3 a
PTWT	4005 a	3991 a	3915 a	5536 b	4369 a	3950 a
TOTUPW (ns)	1701	1926	1756	2216	1229	1897
UPW	80.0 ab	72.0 b	61.0 c	78.4 ab	73.6 ab	82.9 a
UP/PT (ns)	21.1	27.8	28.8	28.0	16.9	21.4
STO (ns)	28.9	27.8	29.4	29.0	27.3	28.9
L/R	40.3 b	36.8 bc	37.8 c	47.1 a	40.6 b	35.8 c
RLW	2.39 c	2.63 bc	2.07 d	2.76 b	3.10 a	2.58 bc
LW/R	95.7 b	97.1 b	78.5 c	129 a	128 a	93.9 b
SW/R	106 ab	81.5 c	81.8 c	121 a	129 a	88.7 bc
RW	202 b	178 bc	160 c	250 a	256 a	188 bc
TOTRW	2282 ab	1827 b	1781 b	2887 a	3151 a	1582 b
R/PT (ns)	11.0	10.2	10.8	11.6	11.4	7.79
UW/RW	1.17 c	2.84 a	1.32 bc	1.19 c	0.776 c	2.78 ab
U/R (ns)	2.81	6.24	3.26	3.32	2.26	5.30
ANTHO (ns)	0.713	0.758	0.744	0.596	0.743	0.586

Table 5.3. Significant factors (α = 0.05) of the complete factorial general linear model. Traits and factors were log transformed. See Table 5.1 for trait abbreviations and text for clone provenances. Abbreviations of the independent variables in the model are: N = Ammonium, P = Phosphorus, PH= pH.

Populations	NC	TN	WV	MA	NY	WI
TRAITS						
L/UP	N*P	PH		PH		
ULWT	N*PH	N*PH	N	N	N	PH
LW/UP	N*P	N*PH	N*P	PH		
SW/UP		N*PH		PH		
PTWT	N*P	N	N*P		N, P	N
TOTUPW			N*P			
UPW	N*P	N*PH		PH		
UP/PT	N		N, N*P			
STO		P*PH			N	
L/R		N		N	N	N
RLW	N	N	N	N	N	N,PH
LW/R	N	N, PH	N	N, N*PH	N	N,PH
SW/R	N	N, PH	N	N, PH	N	N
RW		PH				
TOTRW	N	N	N	N, PH	N	N
R/PT	N*P				N	N
UW/RW	N		N	N	N	N
U/R	N*P					N
ANTHO		N	N	PH,P		

Table 5.4. Clonal differentiation for slope versus soil nutrient concentrations and log log transformed traits and nutrients. Different letters in columns denote significant differences at the $\alpha = 0.05$ level.

Soil Nutrient Factors						
Log log transformed				Untransformed		
pH	P	N	Clone	pH	P	N
0.76 a	-0.20 b	0.50 a	WI	130.6 ab	-2.70 a	1.87 ab
-0.17 b	0.24 a	-0.28 b	NY	35.8 bc	3.59 ab	5.27 a
-0.40 b	0.11 ab	-0.20 b	MA	-145.5 a	2.54 ab	-4.02 c
-0.28 b	0.40 a	-0.13 b	WV	-76.3 ab	8.68 b	8.69 bc
-0.20 b	0.05 ab	-0.23 b	TN	-59.1 ab	0.32 ab	0.52 abc
0.43 a	0.06 ab	0.35 a	NC	68.4 bc	0.27 ab	5.27 a

Table 5.5. Pairwise matrix comparisons of RAPD profiles, geographic distances trait means and plasticities. P values of Mantel test (1000 iterations) are listed. For slopes, the variable on the x axis is listed. See text for details about test and factors.

FACTORS	RAPD	Geog. dist.	Trait means	Slopes X = pH	Slopes X = P
Geographic distances	0.18				
Trait means	0.50	0.42			
Slopes X = pH	0.27	0.45	0.44		
Slopes X = P	0.27	0.31	0.46	0.32	
Slopes X = N	0.11 r = -0.31	0.04 r = -0.44	0.04 r = -0.44	0.28	0.29

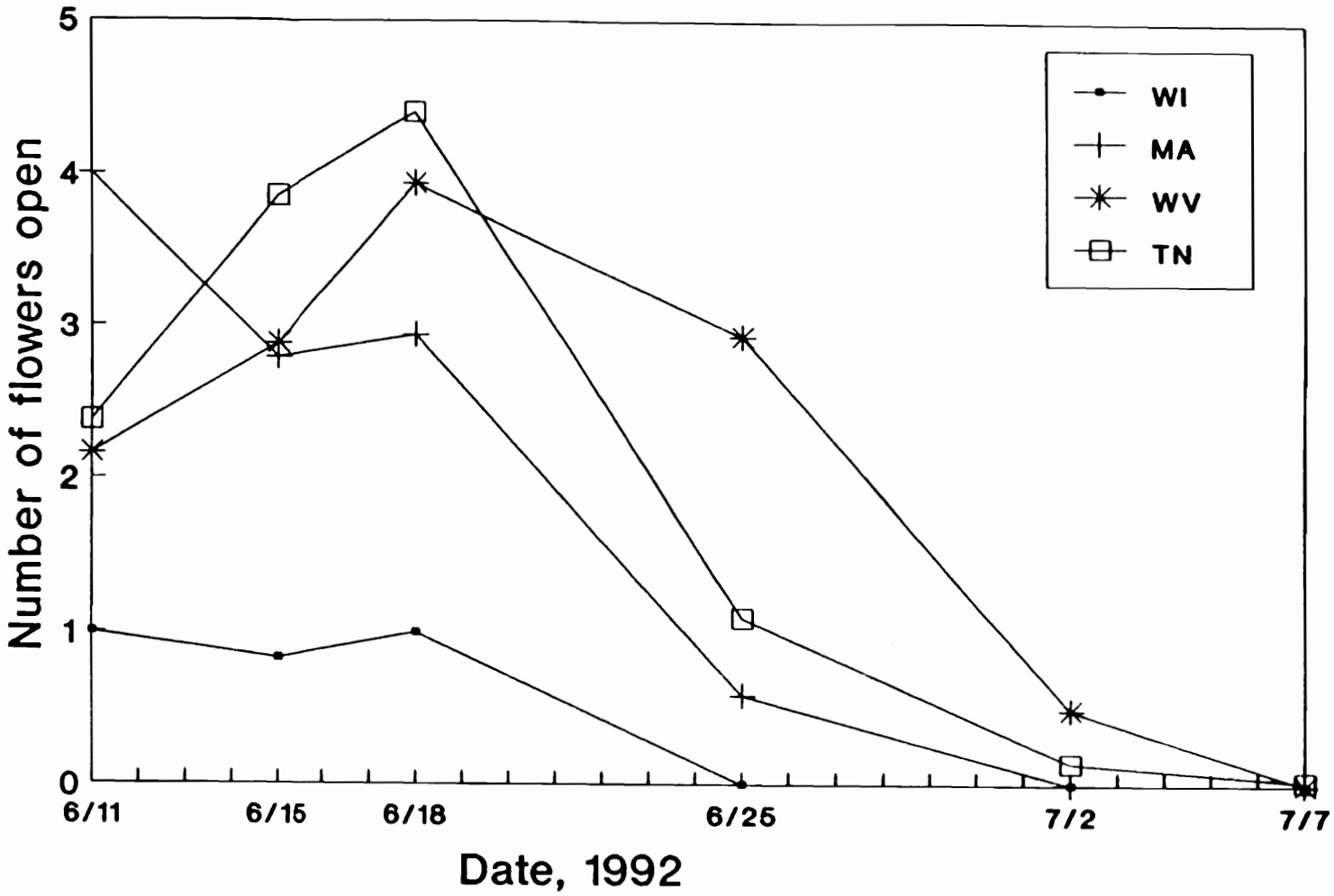


Figure 5.1. Flower phenology of *Vaccinium macrocarpon* in the common garden experiment.

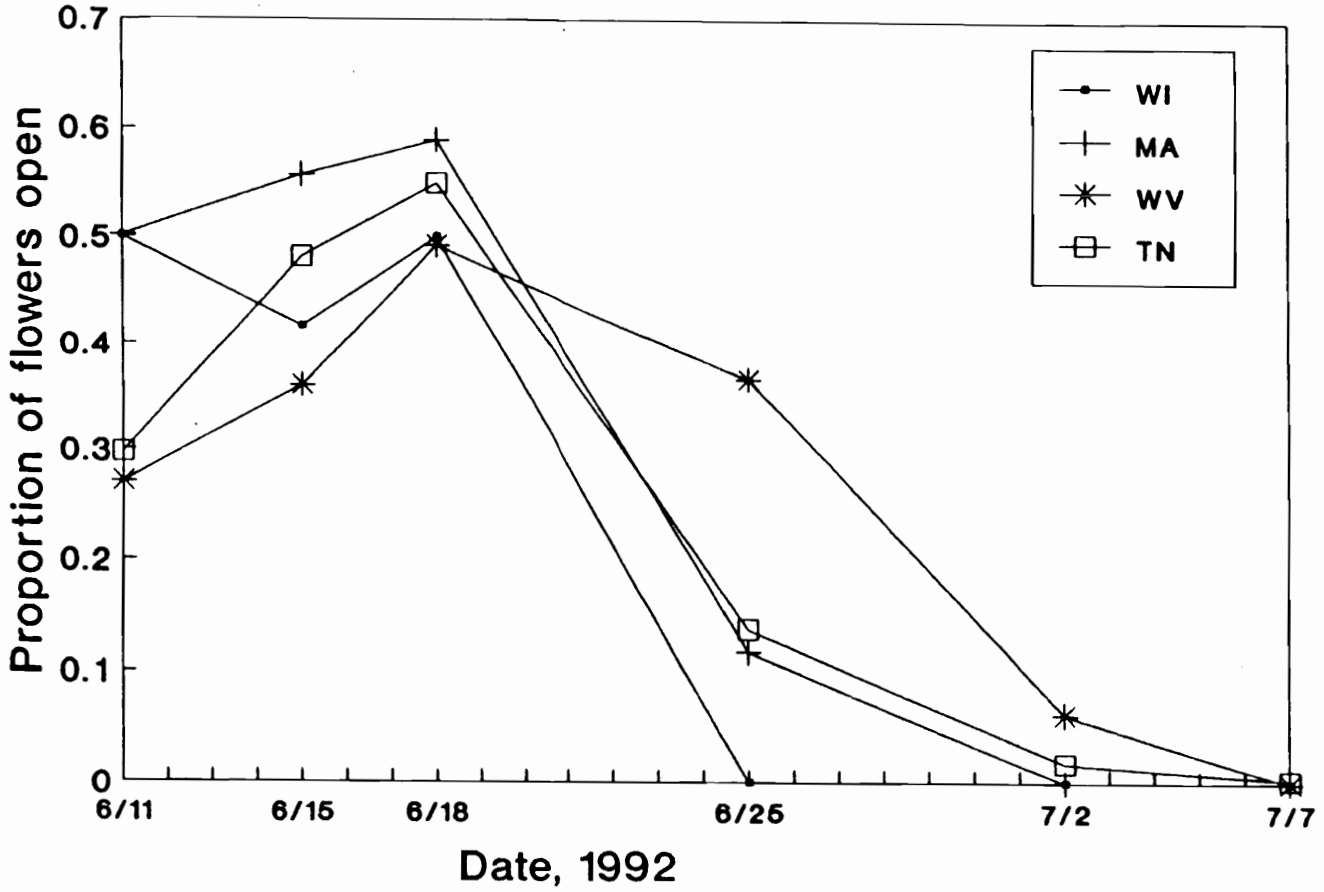
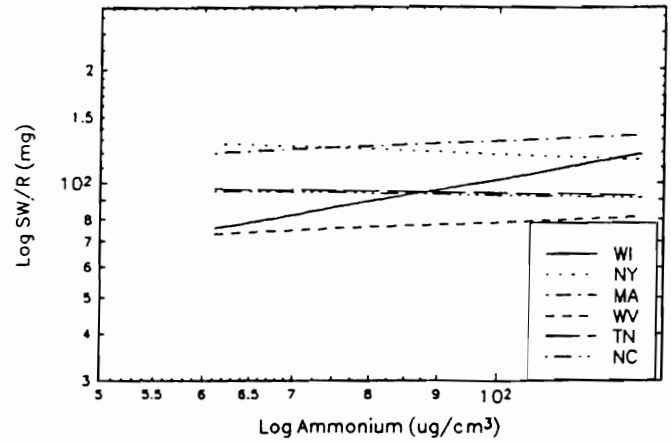
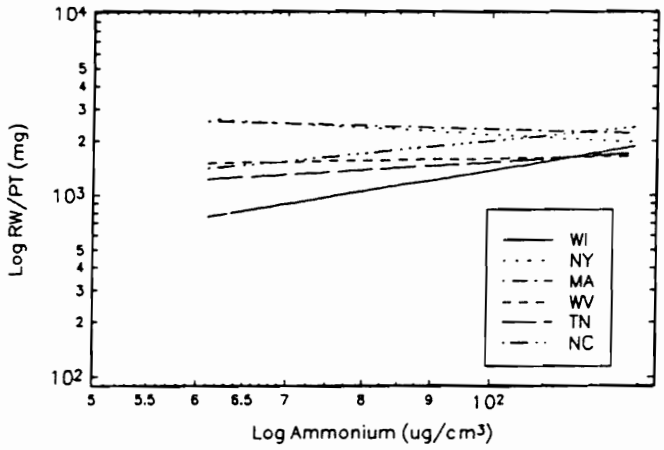


Figure 5.2. Flower phenology of *Vaccinium macrocarpon* in the common garden experiment. Proportions of flowers on clones are presented to better show timing.





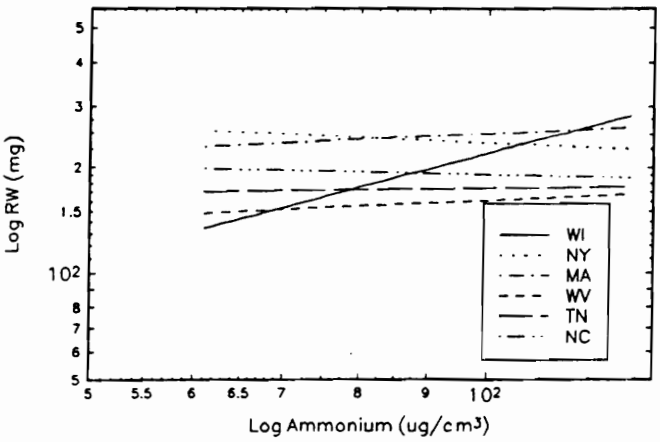


Figure 5.3. Norms of reactions of 3 traits: stem weight per runner (SW/R), runner weight per plant (RW/PT), and runner weight (RW) versus soil ammonium concentration. See Table 5.1 for trait nomenclature. Traits and ammonium were log log transformed to minimize variances.

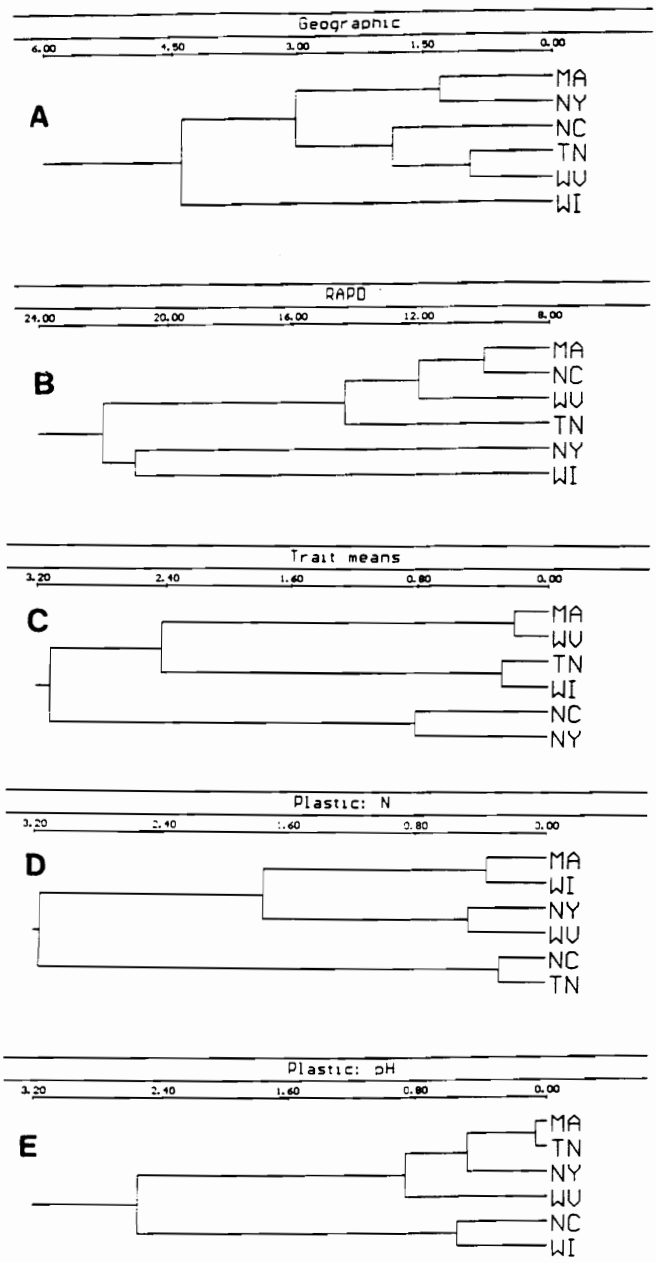


Figure 5.4. UPGMA phenograms showing clonal relationships using various distance matrices: geographic (A), RAPD (B), trait means (C) and trait plasticity in response to N (D) and pH (E). Euclidean distances (Excoffier et al. 1992) were used for RAPDs, and the Canberra metric was used for phenotypic data (Rohlf 1988).

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Chapter Six

Phenotypic Plasticity and Genetic Variation in *Vaccinium macrocarpon*, the American Cranberry. II. Reaction Norms and Clonal Identity in Two Marginal Populations.

Introduction.

Hypotheses abound about the possible evolutionary significance of phenotypic plasticity in plants (Sultan 1987). In much of the plant literature phenotypic plasticity is considered an adaptive strategy (e.g. Bradshaw 1965, Sultan 1987). One hypothesis suggests that there is an inverse relationship between intrasite genetic variation and phenotypic plasticity (e.g., Jain 1978). The rationale behind this hypothesis is that individuals may be adapted to microsites through variable (plastic) phenotypes, or by specialized genotypes in each microsite. However, since phenotypic plasticity is under genetic control, researchers have pointed out that genetic variation and plasticity need not be exclusive (Schlichting and Levin 1986, 1988, Scheiner and Lyman 1991).

Perennial vegetatively spreading plants such as *Vaccinium macrocarpon* are excellent systems for testing some basic hypotheses about fitness in clonal plants in general. There are two main components of fitness in clonal plants: asexual or clonal and sexual (Eriksson 1992). Clonal plant genets exist as reiterative modules (ramets) that may dominate a site rapidly, given the requisite environmental conditions. Therefore, it is conceivable that the 'fittest' clone within a population could monopolize a site, excluding possible conspecific competitors. Recruitment from seed, although generally considered the most important component of fitness, is only important among, rather than within populations for clonal plants. It has been documented that there is often low recruitment from seeds in populations that contain large standing clones, especially among boreal bog plants (Eriksson 1992). Therefore, the vegetative or clonal (non-sexual) fitness component may be the most important factor in regulating genotype success in

local populations. In fact, it has been shown that ecologically marginal populations of *V. macrocarpon* have low genetic heterogeneity, based upon RAPD profiling, compared with central populations (Chapter 4).

Vaccinium macrocarpon is the commercially important cranberry species that is prevalent in bogs in the northeastern and Great Lakes regions of the U.S. There are distributionally marginal populations in the mid-to southern Appalachians and the North Carolina coastal plain. These plants may exhibit phalanx (predominantly upright modules present) or guerilla (runners are predominant) morphology (Lovett Doust 1981). Plasticity in module type would, in this case, constitute a switch in life history strategy. In phalanx growth, uprights are tightly packed into an area, which serves to exclude competitors spatially. Uprights bear most of the flowers and fruit in *V. macrocarpon*, therefore, phalanx growth leads to greater sexual reproduction. Contrastingly, guerilla growth allows genets to explore new areas via runners. Guerilla growth could lead to attainment of greater area by clones or species, and is therefore more important for vegetative spread within a site. Within a population, one could expect clones that had high plasticity for growth morphology to be larger than nonplastic clones. There is evidence that *V. macrocarpon* may switch between these two morphologies depending upon nutrient status (low: phalanx; high: guerilla), which is consistent for stress tolerant clonal plants ([Addoms and Mounce 1931, Kender and Childers 1959, Torio and Eck 1969, Eck 1971, 1976] cited in Eck 1990, deKroon and Schieving 1990). However, in a recent common garden study, there was no switch in morphologies in *V. macrocarpon* response to nitrogen or phosphorus fertilization (Chapter 5).

Many recent studies have documented the spatial distribution of clonal plants within populations using molecular methods (e.g. Silander 1979, 1984, Huenneke 1985, Nybom and Schaal 1990, Rogstad et al. 1991, Aspinwall and Christian 1992, Smith et al. 1992, Parks and Werth 1993). In addition, there is a large growing body of data on sizes and patterning of clonal plants (reviewed in Ellstrand and Roose 1987). Although clonal spatial distributions are themselves interesting, the objectives of this study focused on relating clonal plant molecular variation and identity with ecological responses. The research objective presented in this chapter was to determine the spatial pattern of genets in two bogs and compare the genetic diversity and phenotypic plasticity between the dominant genet and incidental genets. First, I determined clonal identity and spatial distribution of clones within sites using RAPD profiling. Second, I determined whether larger clones (the dominant clone in each site) had higher fitness (higher means for characters and/or appropriate plasticities in response to nutrient variation). The approach was to select two marginal populations, one in Tennessee and one in West Virginia, collect *V. macrocarpon* samples throughout each bog, and use RAPD markers to distinguish genets. Reaction norms of the genets in the bogs (*in situ*) were determined in response to nutrient manipulations. I could then compare the reaction norms of the dominant genet to incidental genets in each bog. The general hypothesis tested is that the dominant genet will have greater phenotypic plasticity and /or trait means than the incidental genets. In addition, I tested the hypothesis that there will be no difference in genetic diversity between the genets from two marginal bogs.

Materials and Methods.

Study sites. The study targeted small *V. macrocarpon* populations that differed in marginality (defined by geographical location), resource availabilities (soil nutrient concentrations), and resource heterogeneity: Two study sites were examined:

1. The West Virginia site (WV) is in Pocahontas County on the Allegheny Plateau, 38° 5'N, 80° 17'W. The bog is 0.45 ha in area, has an elevation of 920 m, and is located on top of Droop Mountain. This bog lies within the corredor of such bogs in the Appalachian Mountains. It is one of two adjacent bogs (site 3 from Stewart and Nilsen (1993), Chapter 2). The surrounding forest consists mainly of *Tsuga canadenesis* and *Picea rubens*. Topography would suggest that this is an ombrotrophic bog, since it receives no mineral nutrients from runoff and has a deep peat layer (> 1 m). The nutrient status may be characterized as very poor (Table 6.1). The genetic heterogeneity of this site is intermediate between marginal sites (such as the Tennessee site) and central sites (Chapter 4).

2. The Tennessee site (TN) is located in the Ridge and Valley physiographic province, 36°30' N 81° 57' W. The treeless area (bog) is about 0.16 ha in area. The site has an elevation of 1010 m and lies at the headwaters of Beaverdam Creek. It is located on Cross Mountain, TN, and lies adjacent to Shady Valley, a onetime large (>4000 ha) boggy area (Killebrew and Safford 1874). The site is bordered by forest on one side and by pasture on the other sides, and is situated on a cattle farm. The bog itself has been fenced for about 9 years to exclude cattle. The surrounding forest is an oak-hickory association. This bog is disjunct from the main Appalachian corredor. The nutrient flux into the bog has probably increased because of

agriculture over the last 150 years. Because of this minerotrophic influence, the nutrient status is higher than that of the WV site (Table 6.1).

Experimental design. Sixteen 1 m^2 plots were established in each population in September 1990, where *V. macrocarpon* dominates or co-dominates in the ground layer with *Rubus hispidus*. Nutrients were added in March, 1991. Soil nutrient levels were heterogeneous among plots before the experiment started. Thus, fertilization increased resource gradients and heterogeneity.

The nutrient treatments were:

i. Nitrogen addition 1.1 g/m^2 supplied as slow release urea.

ii. Phosphorus addition 1.5 g/m^2 supplied as triple superphosphate.

iii. Nitrogen and phosphorus added together at the same rates as above.

iv. No nutrients added.

The nutrient application rates were derived from other cranberry studies (Eck 1964, 1990, Eaton 1971a,b). The fertilizers applied were minimal amounts known to elicit growth responses, and were forms recommended for commercial cranberry growers (Eck 1990).

Soil surface layer (10 cm) samples for macronutrient (P, K, Ca, Mg) concentrations were taken before the experiment started (September, 1992) and at harvest (September, 1993). pH and macronutrients were analyzed by the VPI & SU soil laboratory using a double acid extraction (Donahue and Gettier 1988). Ionic measurements were done using a inductively-coupled plasma spectrophotometer. Ammonium pools were analyzed by the indophenol blue

method (Keeney and Nelson 1982). Nitrogen mineralization rates were estimated using anaerobic incubations (Keeney and Nelson 1982).

Growth and Phenology. The functional plant modules in *V. macrocarpon* are upright shoots, which are important vegetatively and in sexual reproduction (Eck 1990), and runners, which are important for vegetative spread. Approximately fifteen shoots per plot were randomly sampled by a haphazardly thrown 10 cm^2 string at harvest in September. Shoots were dried to even weight for one week at 65°C. Leaves were counted and biomass was partitioned according to shoot type and age. Fruit on uprights was counted and weighed. Table 6.2 lists the traits measured.

RAPD profiling. Clonal identity was assessed by RAPD profiling (Williams et al. 1990). Fresh leaf samples were taken from each plot and other areas where *V. macrocarpon* was observed growing. DNA was isolated either by the Doyle and Doyle (1987) method or using the Stewart and Via (1993) protocol (chapter 3). Best quality DNA and highest yields were obtained from newly produced leaves and apical meristems. RAPD cycling parameters are described elsewhere (Stewart and Via 1993). Reproducible bands were scored for presence or absence.

Statistical analyses. Genetic distances among clones within populations were estimated using a squared euclidean distance of RAPD characters, resulting in a triangular distance matrix

(Excoffier et al. 1992, Chapter 4). This is represented graphically as a UPGMA cluster dendrogram (Rohlf 1988). Clonal relationships as revealed by RAPD fingerprinting are also shown by superimposing a minimum spanning tree (MST) over a principle components analysis (PCA) plot of the clones in two or three dimensions (using the first two or three principle components on the axes). Phenotypic plasticity was assessed using simple linear regression as part of an analysis of covariance (ANCOVA) using ammonium concentration, phosphorus concentration, nitrogen mineralization, and pH as independent variables. With clonal replicates the regression line is the best estimate of the clonal reaction norm. The regression slope is indicative of the magnitude and direction of the plastic responses. Trait means are equivalent to the average heights of the reaction norms. To determine whether the predominant clone in each population had different reaction norms than incidental clones, I used an ANCOVA heterogeneity of slopes model (SAS Institute 1990). A significant clone-independent variable interaction term indicated unequal slopes (differing reaction norms).

Results.

Soil nutrients. The TN site had higher soil ammonium concentrations (N), phosphorus (P) concentrations and pH than the WV site. Within each site, the addition of N and P fertilizers increased soil N and P concentrations, but pH remained unchanged (Table 6.1). Bartlett's heteroscedasticity test revealed significantly higher post-fertilization variances for N and P concentrations (Bartlett 1937). Therefore, both N and P means and variances significantly increased after fertilization. This served to make both sites more edaphically heterogeneous than before fertilizer was added.

Clonal patterns. The criterion I used to select primers was the ability to reproducibly reveal intrapopulational genetic polymorphisms. Of the 40 primers screened (Operon Technologies, Alameda, CA., A and B kits) six revealed polymorphisms within populations. Of these, four were used in the population clonal analysis (OPA-7, OPA13, OPA-17, OPB-4). Within TN, five of 44 (11.3%) markers were polymorphic, and within WV 18 of 52 (34.6%) markers were polymorphic. Typical profiles are shown in Figures 6.1 and 6.2. Sample sizes were 22 in TN and 39 in WV. Attempts were made to sample every *V. macrocarpon* genet. Relationships among clones revealed significantly more genetic homogeneity among TN samples than WV samples. More clones were detectable (21 versus 5) and higher genetic distances were evident in WV than TN (Figs. 6.3 and 6.4) (Chapter 4). Both the cluster analysis and MST showed more complex clonal relationships in WV than in TN. However, in both sites there were one

or two widespread clones that monopolized each bog (Figs. 6.5 and 6.6). There were two large clones in WV, which had similar RAPD phenotypes (euclidean distance of 1 separation, Fig. 6.6). In addition, there were many incidental clones in WV that are separated by a euclidean distance of 1 from each other. This could suggest that many but not all WV clones may share a common vegetative lineage, in which members may have diverged by somatic mutation.

Plant responses. Clonal differentiation-- In the ecological experimental plots, the predominant clone was in 13 sampling plots in TN and 10 in WV. Three plots contained the incidental clones in TN and six in WV. Three different ANCOVA models were implemented regarding clonal identity. In model X, incidental clones were disregarded, and clonal identity was not a factor. Thus, significant regressions were determined only for predominant clones (Table 6.3: columns X). Full clonal identity was a factor in the ANCOVA model Y (Table 6.3: columns Y). The predominant clone in each site had several replicates, but the individual incidental clones often did not. Thus this test was not very powerful in distinguishing among clonal reaction norms. In model Z clonal identities were grouped within populations as two classes: "predominant" and "incidental" (Table 6.3: columns Z). The purpose of the third model was to better test the hypothesis that clonal identity was an important factor in means and reaction norms, specifically, that predominant clones responded more adaptively (had greater slopes) and had higher trait means than non-predominant clones.

In TN, because the sample size of non-predominant clones was very low, there was low statistical power in model Y. However, there was no evidence that nonpredominant clones were

different than predominant clones for any traits (Table 6.3, Figs. 6.7-6.9). The ANCOVA models X, Y, and Z differed only in the ratio UP1/2 (Table 6.3). This shows that the reaction norms of the predominant clone in TN were generally the same as the profile of all clones in TN.

Contrastingly, in WV, there were significant differences for clonal differentiation using model Y of traits FRUWT and UWT2 (Table 6.3, column Y) and several additional traits when non-predominant clones were using model Z (Table 6.3, column Z). In addition, model X results did not correspond with models Y and Z except for UPWT (Table 6.3). In some traits with model X, (ULWT, L/UP2, LWT/UP2, RLWT) there was a linear relationship against nitrogen mineralization (NM), whereas when analyzed with models Y and Z, there was not. Reaction norms were linear for other traits such as FRUWT using models Y and Z but not using model X (Table 6.3). The WV clones were more genetically heterogenous than TN clones, so greater ecological clonal differentiation in WV than TN was expected. Although direct statistical comparisons between TN and WV clones were not possible because they were not grown in a common environment, reaction norm plots show that TN clones had higher trait means than WV clones, suggesting population level differentiation as well (Figs. 6.7-6.9.).

Phenotypic plasticity-- There were more plastic traits in response to N and pH gradients in TN than WV, especially runner traits (Table 6.3). In WV, there were a few traits responding to increased nutrients, but no patterns emerged (Table 6.3). Reaction norms were in different directions (TN, –; WV, +) for sexual reproductive traits (Fig. 6.7). Unequal

reaction norms (slopes) are indicative of genetic-environmental interactions. WV clones had flat reaction norms for flower and fruit number, and TN clones produced fewer flowers and fruits in response to N soil concentrations. There were slight decreases in fruit weight in response to N. For vegetative traits (Figs. 6.8-6.9), TN clones seemed to be slightly more plastic in response to increased ammonium availability. The exception is in the ratio of 1 year upright modules to older uprights (Fig 6.8: UP1/2). TN clones had a greater number of young uprights to old uprights as N levels increase. TN clones responded with a positive slope in most vegetative traits and WV clones had flat or slightly positive reaction norms. This contrasts with the near absence of plasticity shown in a common garden experiment (Fig 6.9, Chapter 5).

Discussion.

Clonal patterns-- RAPD markers have been used successfully in assessing plant and fungal clonal patterns (Brauner et al. 1992, Smith et al. 1992, Wilde et al. 1992, Eriksson and Bremer 1993, Stewart and Porter in review.). Although allozyme electrophoresis has been the method of choice for clonal detection for over 20 years, DNA methods have also been shown to be useful and sensitive. DNA fingerprinting (Jeffreys et al. 1985), although sensitive, is notoriously difficult in plants, and requires relatively large amounts of purified DNA (Waugh and Powell 1992). RAPD fingerprinting can detect intrapopulational polymorphisms, and therefore differentiate between clones, where allozymes may not, and does not require large quantities of purified DNA (Hadreys et al. 1992, Waugh and Powell 1992). An even more attractive feature is that RAPD presumably provides an unbiased sampling of the genome. A potential problem is that RAPD fingerprinting is so sensitive that it may detect somatic mutations within a vegetative lineage (J. Mitton pers. comm.). However, this could be considered an asset. Another potential problem is that RAPDs are dominant markers and could lead to confounding in population analyses (Chapter 4).

Theoretically, in very old clonal perennials, the likelihood of all living cells in the plant body having the same genomic sequence is low. Therefore, if one were to employ the most sensitive molecular assay for clonality, DNA sequencing, one might find dozens of "clones" in a single integrated individual. The practicality of sequencing all the DNA of a metapopulation

of cells in a single plant is very low; however, RAPD fingerprinting is moving in that direction. For example, if two individuals share 40 RAPD bands, then they also share 800 bases in the same order (40 x 10 x 2) and approximately the same number of bases between each band's priming sites.

Clonal identities-- Of the 23 different clones detected at WV, 10 were separated by a euclidean distance of 1. This means that they differed pairwise in sequences sampled by one each of 1040 bases sampled, assuming all RAPD positive markers represented heterozygous loci, which we know is not a good assumption (Hadrys et al. 1992). This means that although profiles differ by one band, there must have been two mutation events (at least in the case of the loss of a band). A better explanation perhaps is that there have been low levels of sexual reproduction, leading to the production of genetically similar, but not identical clones.

In TN however, all clones are connected to the single large clone by one mutation each. The two clones (12 and 27) that differ each from the large clone by a euclidean distance of 2 differ from a band shift in one primer. That is, because bands were coded as present or absent, the loss and subsequent gain of bands appears as if bands were gained and lost independently. Probably they each were caused by an insertion or deletion in a heterozygous RAPD marker. So it is likely that the present day TN clones are the product of a single vegetative lineage, but not so in WV.

Assuming that both TN and WV sites are relict bogs and are thousands of years old, then there has been ample time for clonal structure to reach equilibrium. There is, of course, no way

to age *V. macrocarpon* clones unless they are excavated. Even then, there would likely be discontinuities. In WV, clones A and B, although not contiguous, contain ramets 112 m (A); and 125 m (B) apart. So the clones must be at least 350 years old given an average runner length of ⅓ m (although yearly runner lengths may exceed 1 m, pers. obs.). However, they are probably much older than that given clonal meandering and the forest invasion of the bog[10]. Their minimum age would make these clones older than the surrounding spruce and hemlock trees.

Large ericaceous clones have been reported in the literature. Harberd (1961, 1967) cites studies in *Vaccinium*, *Erica*, and *Gaylussacia* in which clones spread over 800 m. In the case of *Gaylussacia*, the box huckleberry, Wherry (1972) reports a clone spread over 2000 m, and estimated to be 13,000 years old. Noble et al. (1979) report that ericaceous *Vaccinium myrtillus*, and *Calluna* had among the largest genet width to height ratio of the woody plants they surveyed. These estimates were based upon uniform morphological characters and the absence of seedling recruitment within clones. Clonal spread of *V. macrocarpon* is consistent with these reports, but it does not approach the mass of these other ericads. In other plants and fungi, there are reports of very large *Spartina* clones (hectares in breadth) on the U.S. gulf coast (C. Werth pers. comm.), expansive *Populus* clones (Kemperman and Barnes 1976), large grass (*Holcus*) clones (Harberd 1967), 1000 m long bracken clones (Parks and Werth 1993), and the 15 hectare clone of *Armillaria bulbosa* (Smith et al. 1992).

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[10]I excavated *V. macrocarpon* stems that were over 30 years old. They were found about 10 m into the surrounding forest, where actively growing cranberry shoots are presently absent.

Clonal responses-- When performing ecological experiments in natural populations, inequitable environments prevent statistical comparisons among sites. Common garden and reciprocal transplant experiments circumvent this difficulty, but often are not realistic. There are several advantages for field manipulations: one may better assess how plants react in their natural environments, there is no transplant shock, plants should seemingly be adapted to the environment of the experiment, and should therefore respond more sensitively to fine-grained environmental manipulation.

In most plant ecology experiments, no attempt is made to determine genetic profiles of the experimental plants, and the mosaic of genotypes is pooled in the analysis. When genotypes have been determined (e.g. Silander 1979, 1984), the experimental focus was ecotypic differentiation in contrasting environments. However, the goal of this study was to compare the responses of different genotypes in the same, relatively homogeneous, natural environments.

In general, the clonal responses to soil nutrients of the large and incidental clones were different in WV, but the large and incidental clones responded similarly in TN. This corroborates the molecular data, which suggest one vegetative lineage in TN but multiple vegetative lineages in WV (Table 6.3, Figs. 6.7-6.9). Although statistical comparisons were not possible because of different environments, TN clones had higher means for most traits, and several traits were more plastic compared to those for the WV clones. For example, TN had a much steeper reaction norm of UP1/2 in response to N (Fig. 6.8). As N levels increased there

was less longevity of persistent (> 1 yr) leaves, which is consistent with other studies (Shaver 1981, 1983). As N levels increased, there was greater plasticity for sexual reproductive output in TN compared to WV (Fig. 6.7). Thus, based on several diagnostic traits the more marginal site appears to have greater phenotypic plasticity.

Do incidental clones in WV appear to be less adaptive than the large clone? For some traits the answer is "yes" (Figure 6.10). For most traits for which clonal significant differences were found, the predominant clone had greater means than the collection of incidental clones. There are two possible explanations for these differences. First, if incidental clones are somatic mutants of the predominant clone, a mutational load may have hampered performance (Muller 1964). Muller proposed that over time an asexually reproducing organism accumulates deleterious mutations resulting in decreased relative fitness. In this view, one of the advantages of sex over clonal growth is the ability of a population to purge these mutations through recombination. However, in a 100% selfing homozygous population, sex would be equivalent to clonal reproduction (Maynard Smith 1989). Muller's ratchet, where stochastic events may reiteratively extirpate the most fit individual in a population, is probably not applicable in these *V. macrocarpon* populations. This is because, as noted above, ericaceous genets may be quite large and fragmented. Therefore, the most fit genet will persist barring environmental change that may favor another genet. In light of mutational load and natural selection, the most fit clone, in the absence of perturbation, environmental change, or heterogeneity, will increase in size (Cook 1985). Additionally, with an increase of environmental stress, this process could be quite rapid and clonal diversity would decrease (Cook 1985, Silander 1985, Cwynar and

MacDonald, 1987, Harrison and Durance 1992). However, with adjacently different ecological conditions, and outbreeding plants (*Agrostis stolonifera*), extreme stress did not decrease clonal diversity (Wu et al. 1975). Also, if genotypes are very similar, and/or microgeographic variation is present, clonal heterogeneity may persist (Sebens and Thorne 1985).

In summary, each *V. macrocarpon* population was dominated by one or two genets. Also present were several incidental genets that were genetically similar to the dominant genet. The more isolated bog had fewest incidental genets and the lowest genetic diversity. The data support the hypothesis that genetic diversity decreases in marginal populations, and that one genet gains dominance. There were few data in this study that support the second hypothesis. Measureable difference in the phenotypic plasticity of the dominant and incidental genets was infrequent. This study can only weakly support the assertion that one genet gains dominance in marginal populations because it has higher phenotypic plasticity.

Table 6.1. Soil macronutrient means and standard deviations (in parentheses) at Johnson Co., TN bog and Droop, WV bog. Nutrient concentrations at surface level before and after treatments are measured in $\mu g/cm^3$, and anaerobic nitrogen mineralization rates are expressed as $\mu g/cm^3/day$.

	September, 1992 (before treatment)	September, 1993 (after treatment)
TN		
pH	4.65 (0.20)	4.37 (0.43)
Ammonium	12.6 (5.37)	58.6 (34.8)
Phosphorus	1.50 (0.58)	13.4 (9.37)
Nitrogen mineralization	*	4.82 (4.38)
WV		
pH	3.65 (0.09)	3.59 (0.21)
Ammonium	2.75 (0.71)	20.3 (6.34)
Phosphorus	0.34 (0.15)	12.6 (7.33)
Nitrogen mineralization	*	1.16 (2.03)

Table 6.2. Key to trait abbreviations

ABBREVIATIONS	TRAITS
L/UP	avg. leaf number per current year upright module
ULWT	avg. current year upright module leaf weight (mg)
LW/UP	avg. weight of all leaves per current year upright module (mg)
SW/UP	avg. weight of stem per current year upright module (mg)
UPWT	avg. weight of current year upright modules (mg)
FRUWT	avg. weight of mature fruit (mg)
FRU	avg. number of fruit harvested
FL	avg. number of flowers on current year uprights harvested
L/UP2	avg. leaf number per old (>1 yr) upright module
ULWT2	avg. old (> 1 yr) upright module leaf weight (mg)
LW/UP2	avg. weight of all leaves per old (> 1 yr) upright module (mg)
SW/UP2	avg. weight of stem per old (> 1 yr) upright module (mg)
UWT2	avg. weight of old (> 1 yr) upright modules (mg)
TOTUPW	weight of upright modules per sample (mg)
UP1/2	ratio of the number of current year upright modules to old upright modules harvested
UPWT1/2	ratio of the of current year upright module weight to old upright module weight harvested
UP/P	number of upright modules per sample
L/R	avg. leaf number per runner module
RLWT	avg. runner module leaf weight (mg)
LWT/R	avg. weight of all leaves per runner module (mg)

SWT/R	avg. weight of stem per runner module (mg)
RWT	avg. weight of runner modules (mg)
UP/R	ratio of upright module number and runner module number
UWT/RWT	ratio of upright module weight and runner module weight
TUWT/R	ratio of the total upright module weight to the total runner module weight
TWT1/2	ratio of the total current year module weight to old module weight

Table 6.3. Summary of the analysis of covariance (ANCOVA). Type III model significance at α = 0.05. Trait abbreviations are in Table 6.1. Variables in the ANCOVA: C = clonal identity within each population; Soil nutrients: N = Ammonium; NM = Ammonium mineralization rates; PH = pH; P = Phosphorus. Model X (columns X) groups all clones together. Model Y (columns Y) tests with full clonal identity. Model Z (columns Z) group all incidental clones together within each population in an artificial class to better test H_o: predominant clone > incidental clones.

Site	**Droop Mt., WV**			**Johnson Co., TN**		
Model	**X**	**Y**	**Z**	**X**	**Y**	**Z**
L/UP						
ULWT	NM			N, PH	N, PH	N, PH
LW/UP			C			
SW/UP			C			
UPWT	NM	NM	NM			
FRUWT		C, P	C, N, NM, PH, P C*N, C*NM, C*PH			
FRU						
FL				P	P	P
L/UP2	NM					
UPLWT2						
LWT/UP2	NM		C*NM			
SWT/UP2						
UWT2		C	C			
UP1/2						N
UPWT1/2	NM	C				
L/R						
RLWT	NM			PH	PH	PH
LWT/R				N, PH	N, PH	N, PH
SWT/R			C	N, PH	N, PH	N, PH
RWT				N, PH	N, PH	N, PH
UP/R			C, PH, C*PH			
UPWT/RWT			C			

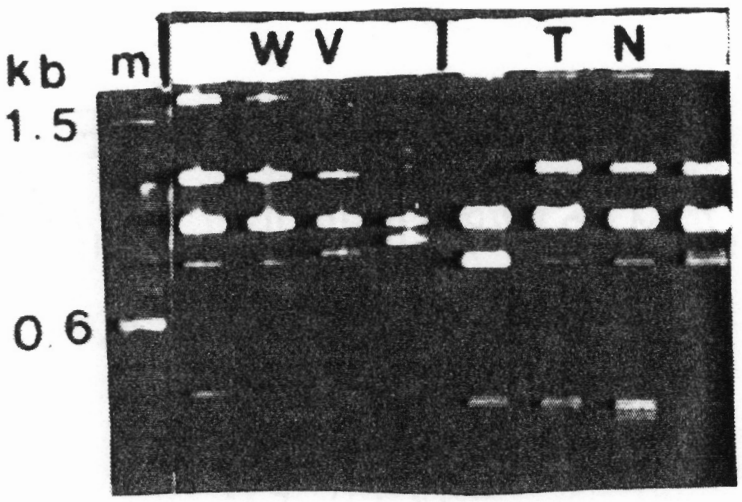


Figure 6.1. RAPD profiles of WV and TN cranberry clones. Profiles are representative of clones in these populations. Lane m contains 100-bp marker (GIBCO BRL/Life Technologies, Gaithersburg, MD). Samples were amplified with OPA-07 (Operon Technologies, Alameda, CA).

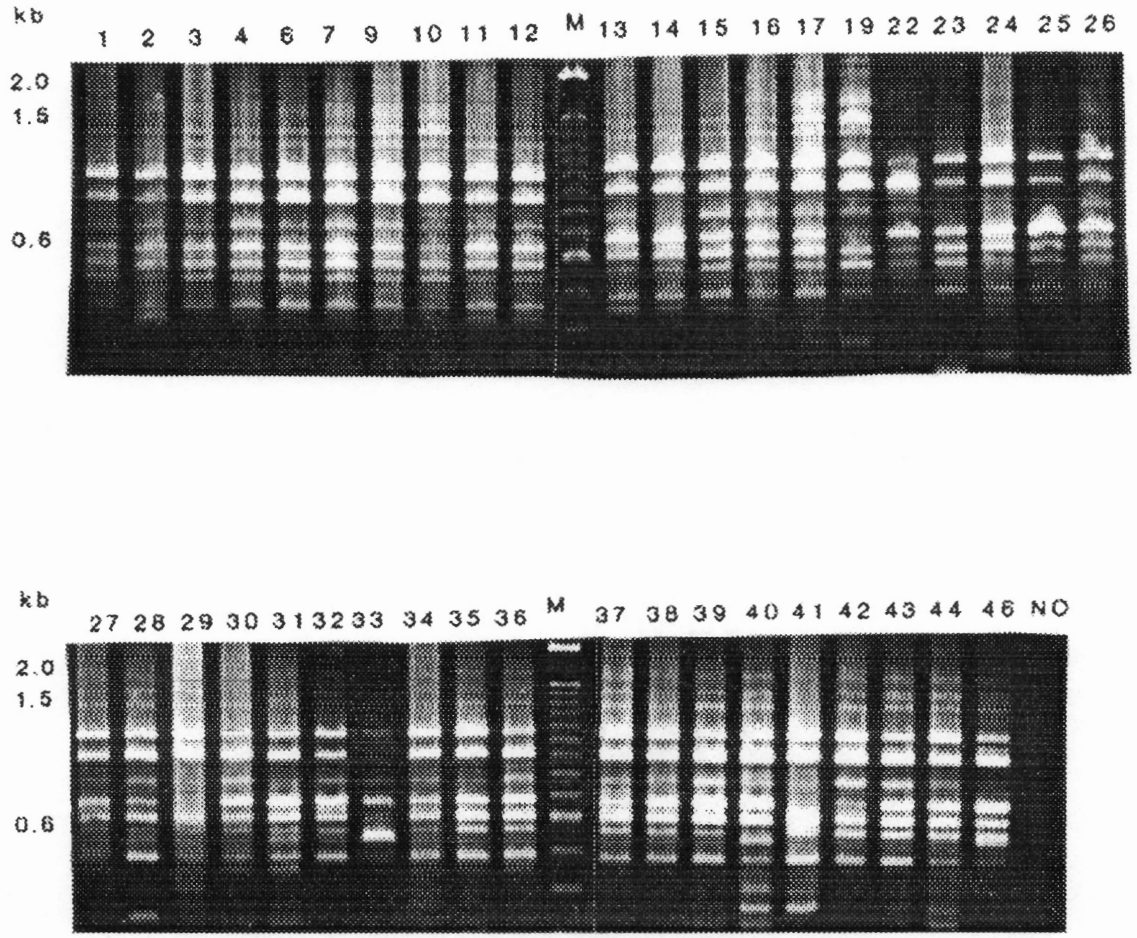


Figure 6.2. RAPD profiles of all WV samples. Lanes m contain 100-bp marker (GIBCO BRL/Life Technologies, Gaithersburg, MD). Samples were amplified with primer OPB-04 (Operon Technologies, Alameda, CA).

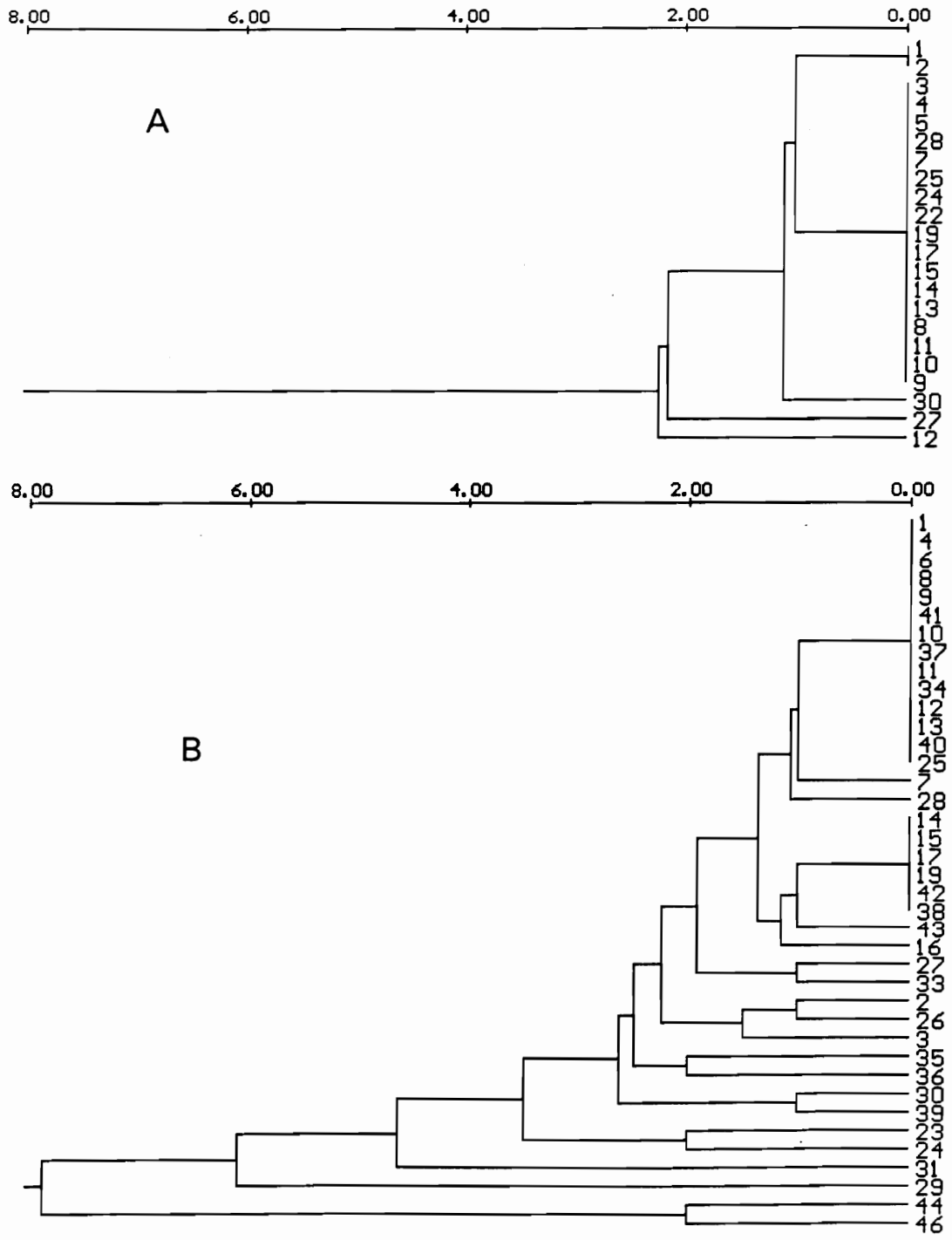
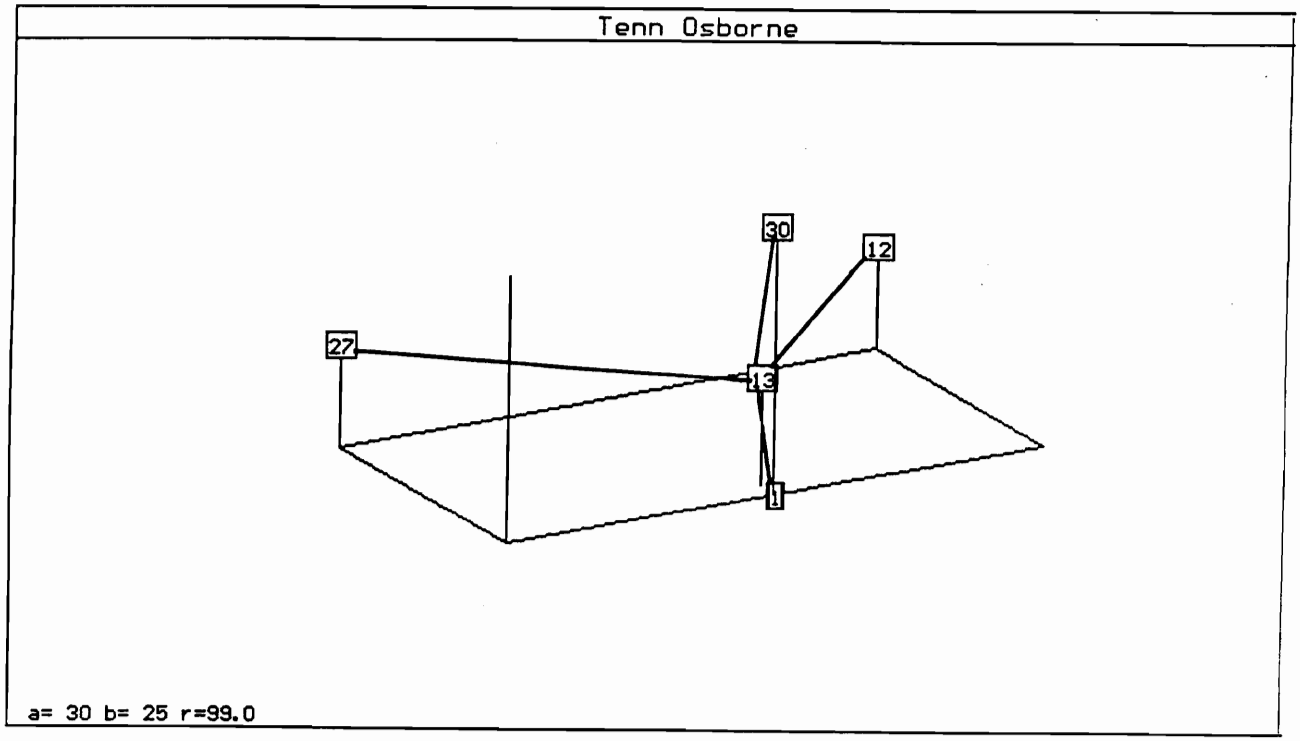


Figure 6.3. Cluster dendrogram by the unweighted paired group method using arithmetic averages (UPGMA) of A: TN, and B: WV populations. Scale is euclidean distance. At the tips are sampling points.



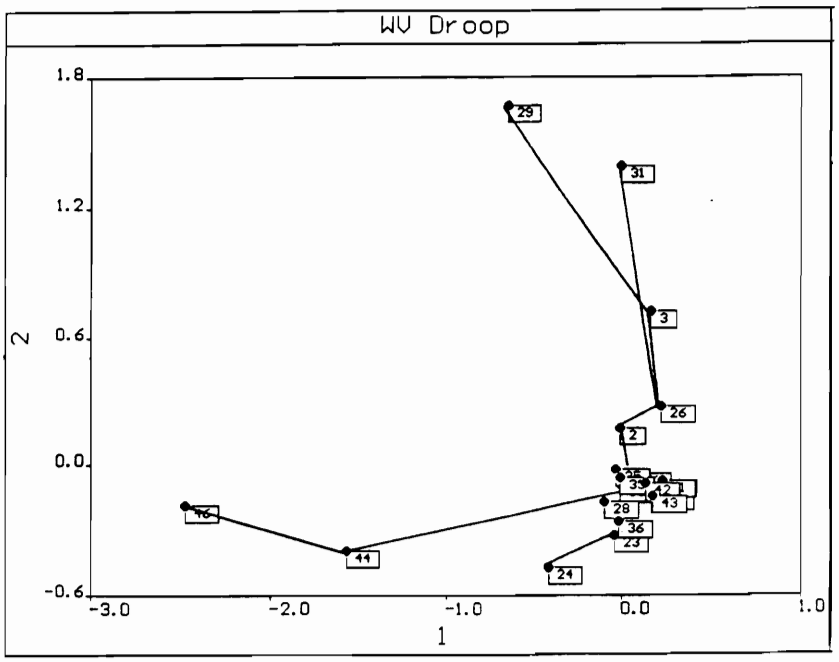


Figure 6.4 Minimum spanning tree (MST) of TN population (projected on the first 3 principle components) and WV population (projected on the first two principle components).

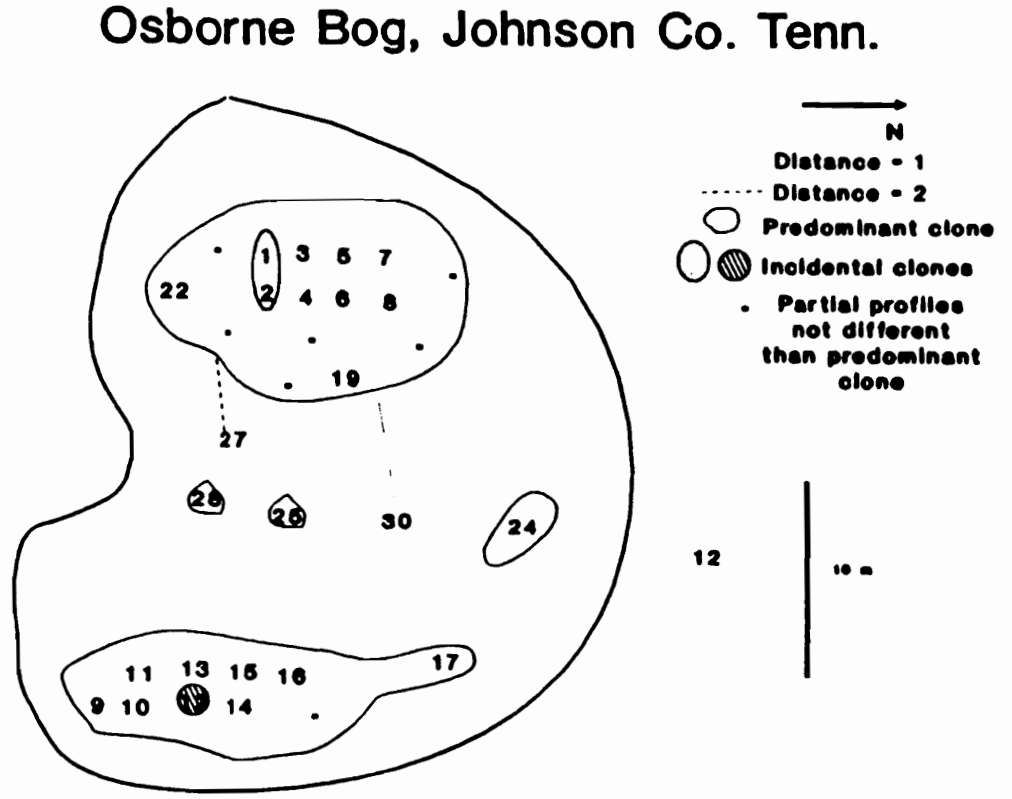


Figure 6.5. Spatial distribution of *Vaccinium macrocarpon* clones in TN.

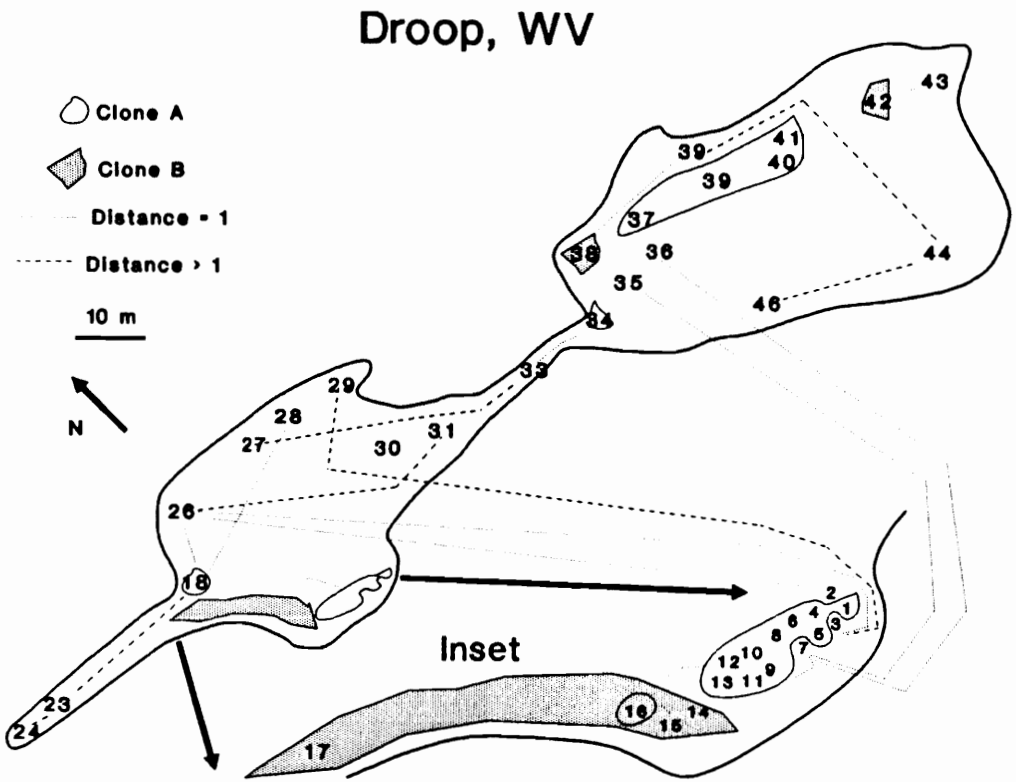


Figure 6.6 Spatial distribution of *Vaccinium macrocarpon* clones in WV.

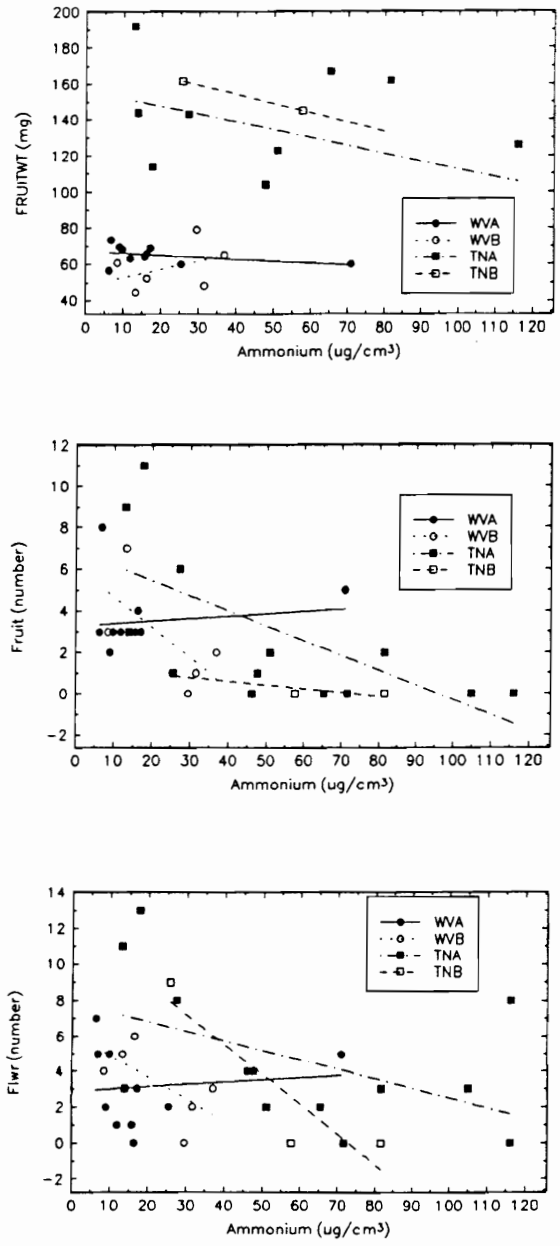
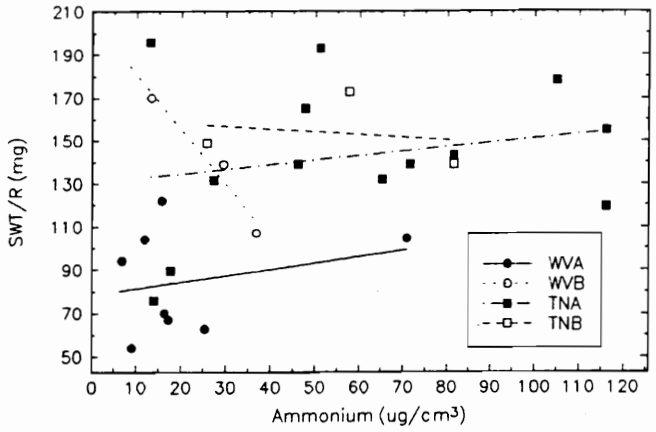


Figure 6.7 Norms of reaction for 3 sexual reproductive traits versus soil ammonium concentration: fruit weight (FRUTWT), number of fruit per plant (FRUIT), and number of flowers per plant (FLWR) for large clones (WVA, and TNA) and incidental clones (WVB, TNB) per site.



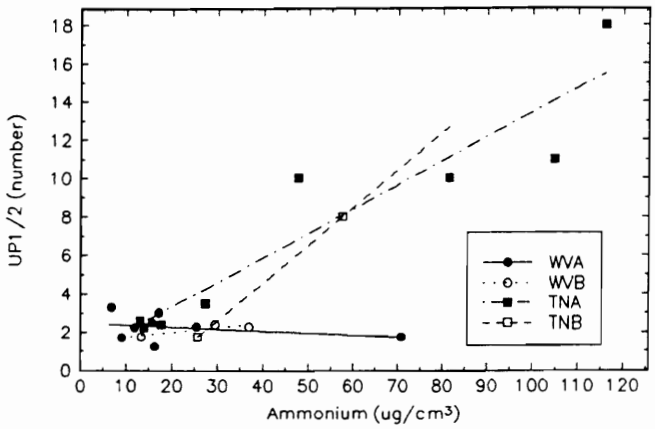


Figure 6.8 Norms of reaction for 2 vegetative traits versus soil ammonium concentration: stem weight per runner modules (SWT/R), and the ratio of upright module weight to runner module weight (UP1/2) for large clones (WVA, and TNA) and incidental clones (WVB, TNB) per site.

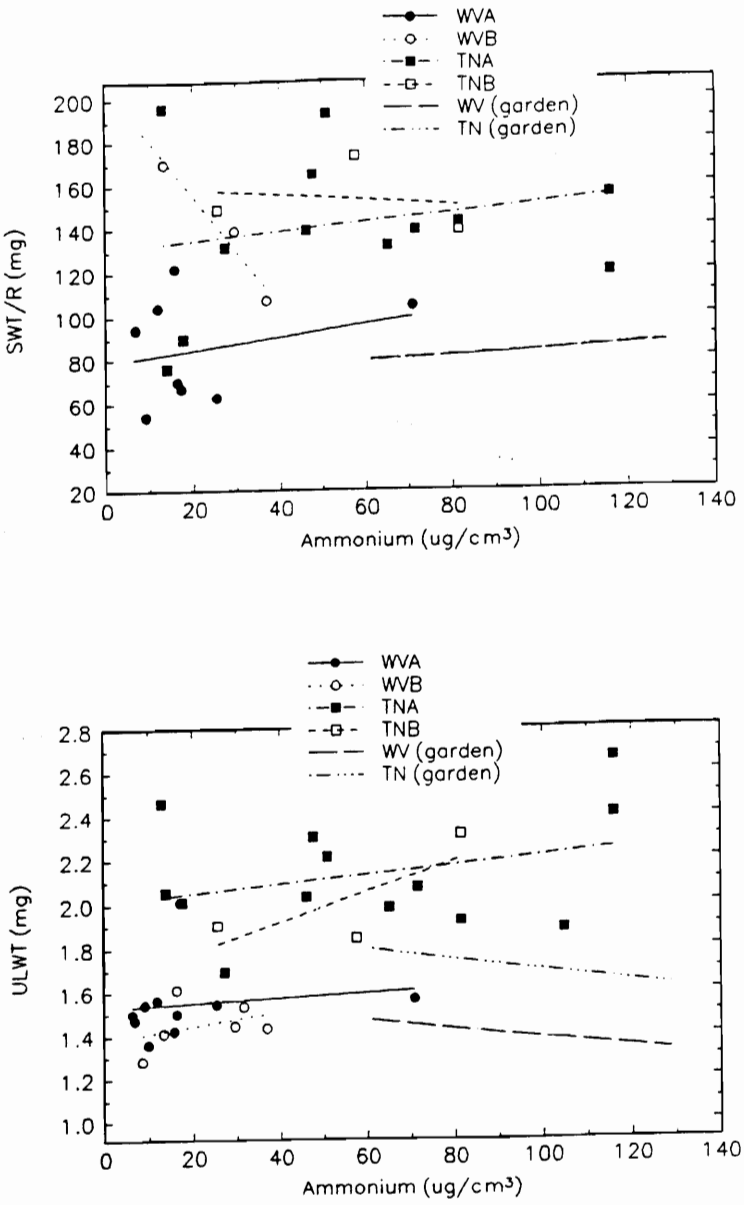


Figure 6.9 Norms of reaction for 2 vegetative traits versus soil ammonium concentration: stem weight per runner modules (SWT/R), and average leaf number per upright leaf (ULWT) for large clones (WVA, and TNA) and incidental clones (WVB, TNB) per site.
Shown for reference are reaction norms from a common garden study in Blacksburg, VA (Chapter 5) using clones TNA and WVA (WV and TN garden).

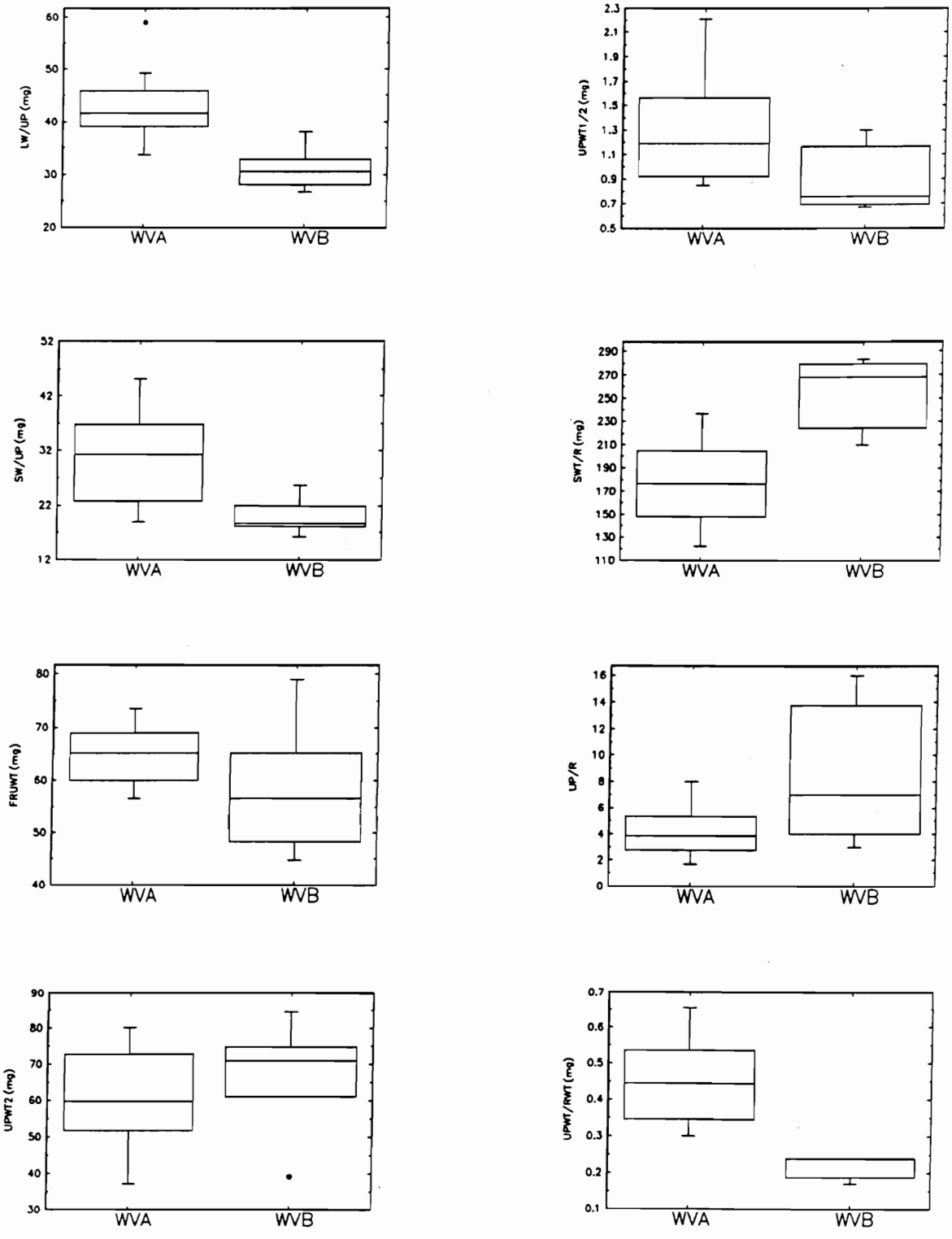


Figure 6.10 Box plots of traits (see Table 6.1 for trait nomenclature) that were significantly different for clones in WV: large clone (WVA) and incidental clones (WVB).

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Vita

C. Neal Stewart, Jr. was born October 24, 1960 in Winston-Salem, N.C., USA. He received his undergraduate education at North Carolina State University in horticulture and education. While teaching high school and middle school in Winston-Salem, he earned a Master of Arts degree in education from Appalachian State University. After resigning from public school teaching he studied with Erik T. Nilsen for the next five years at Virginia Polytechnic Institute and State University, of which the first two were spent on a Masters of Science project involving the insectivorous plant genus *Drosera*. At this time he became interested in genetics and the interface between genetics and ecology; the roles that phenotypic plasticity and genetic differentiation play in plant fitness. Research is planned for integrating transgenic plant technology, and other biotechnology to population and community ecology.