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Ecological Genetics of Local Adaptation: climatic races
in *Arabidopsis thaliana* and *Arabis fecunda*.

by

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B.S. Biology, State University of New York at Albany, 1995

Presented in partial fulfillment of the requirements

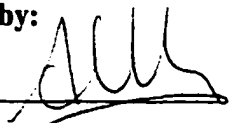
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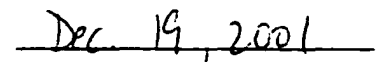
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Ecological Genetics of Local Adaptation: climatic races
in *Arabidopsis thaliana* and *Arabis fecunda*.

Chair: Anna Sala A.S.

Local adaptation, or the presence of genetically based trait differences among populations that result from natural selection, appears to be quite common in plant species. However, any two independent populations may diverge in phenotypes, even in the absence of differential selective pressures. The degree of population divergence that results from natural selection is therefore of fundamental interest to evolution as well as the applied sub-discipline of conservation genetics. In chapter 1 I review a body of theory which develops an expectation of both marker and trait divergence in the absence of natural selection. Based on this theory and data from 29 species I compare expected divergence at neutral markers and neutral quantitative traits. Results show that quantitative trait divergence among populations is greater than expected from neutral divergence, providing support for the ubiquity of local adaptation. Chapter 2 describes a case study of *Arabis fecunda* populations at high and low elevation sites that differ in water availability. Common garden screenings show that several specific quantitative traits likely to be important for drought adaptation have diverged among populations, despite a general lack variation or structure at neutral markers.

Ultimately, evolutionary constraints and the rate of adaptive evolution will depend on the genetic basis of adaptive traits. Drought (water availability) is thought to be one of the major climatic factors to which populations locally adapt, and a number of traits have been identified which may contribute to drought adaptation. Understanding the genetic basis of drought tolerance is an important goal in both ecology and agriculture, and provides an ideal framework to understand potential genetic constraints and correlated responses. In chapter 3 I examine natural variation in drought tolerance traits in *Arabidopsis thaliana*, a model system whose genome has been completely sequenced. A screening of natural accessions reveals a large positive genetic correlation between drought tolerance and time to flowering. I demonstrate that allelic variation at gene for flowering time (*Frigida*) is also responsible for variation in physiological and drought tolerance traits, suggesting that natural selection has caused the fixation of a pleiotropic gene.

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Introduction: The central theme of my dissertation research is adaptive evolution. Differences among species, populations within a species, and individuals within a population are a consequence of evolution. Since Darwin first produced *On the Origin of Species* (1859), speciation and adaptation have continued to fascinate students of evolution (Provine 1971). Despite the title of Darwin's influential work, the majority of his book was concerned with the origin of adaptation, the process by which natural selection generates differences between populations of a single species. Within a species populations may occupy different habitats and therefore face differences in local selective pressures. Populations of a single species can differ in morphological, physiological and life history traits. The term "ecotype" was coined by Turesson (1922) to describe heritable differences in traits between populations of a single plant species. I follow this theme, first generally considering the processes of population divergence, then more specifically adaptation to local climate and conservation issues in a rare plant, and finally, investigating the genetic basis of drought adaptation in a model system.

Adaptation, neutral evolution and population structure - Although Darwin is credited with being an adaptationist, he did make efforts to point out that some variation may serve "no particular use" to the organism. Gulick (1905) was the first to intensively study neutral variation, and concluded that reproductive isolation is likely to play a role in the evolution of non-adaptive traits. Fisher (1930) was the first to quantitatively model the effects of population size on the efficacy of selection and apply the diffusion approximation to models of genetic drift. Wright developed interest on the role of mutation, selection and drift and how these were influenced by population size, *Ne*

(Wright 1931). In molecular evolution, the now famous neutral allele theory proposes that for some loci, the fate of particular alleles is primarily determined by mutation and genetic drift (Kimura and Ohta 1971). A similar body of theory is available for neutral quantitative genetic traits (Lande 1992), providing a null hypothesis that variation within and among populations has evolved in the absence of natural selection.

In chapter 1 I review theory and data concerning the relationship between population structure at ecological traits, quantitative trait loci and neutral molecular markers. The relationship between markers and traits relates to the degree to which gene flow constrains adaptive evolution as well as the potential application of molecular methods to conservation. Direct comparison of population structure in markers with that of traits is made possible by the measure Q_{ST} , which partitions quantitative genetic variation in a manner analogous to F_{ST} for single gene markers. Comparing Q_{ST} with F_{ST} provides evidence for adaptive variation, since we expect Q_{ST} to equal F_{ST} in the absence of natural selection. Across 24 species mean Q_{ST} is typically larger than and poorly correlated with mean F_{ST} . Within species Q_{ST} varies widely among traits; traits experiencing the strongest local selection pressures are expected to be the most divergent from molecular F_{ST} . In addition to providing further support that important ecological genetic variation exists among populations in different habitats, these studies point out research needs concerning the relationship of markers and traits.

In appendix 1 I examine theory and data concerning the role of population structure on the probability of speciation. As a result of the modern synthesis we are now aware of the various microevolutionary processes that are responsible for genetic change over

time: selection, random genetic drift, recombination, mutation and migration. It is these population level processes that are responsible for generating differences within a species (inter and intrapopulation) and the process of speciation (cladogenesis). Adaptation can lead to speciation, but they can be distinguished. Adaptation is unique in its requirement for natural selection; it must have fitness consequences. Speciation, on the other hand, can occur in the absence of selection. It is an inevitable consequence of reproductive isolation (Turelli et al. 2001). Although genetic divergence of populations does not define a speciation event (Mayr 1963, Futuyma 1998), it is both a component and an influence on factors important in speciation (Turelli et al. 2001, Slatkin 1987). Accumulated genetic differences among populations can lead to phenotypic divergence and reproductive isolation (Tilley et al. 1990, Coyne and Orr 1989), both of which play critical roles in speciation. While traits important for reproductive isolation and speciation can evolve among connected populations (Barton and Bengtsson 1986), the relative genetic differentiation of populations provides an estimate of evolutionary independence and thus the relative probability of future speciation (Orr and Turelli 2001). Our results suggest that lower latitude populations are more likely to follow independent evolutionary trajectories, where mutation, recombination, selection and/or drift may lead to further divergence and speciation. Thus, for a given time period, we expect a greater number of species to originate at lower latitudes, although this does not necessarily imply a difference in the rate at which individual lineages diverge.

Conservation and Ecological Genetics - Impacts of human activity and future climate change make estimates of evolutionary potential of rare species crucial to accurately

assess the ability of species and populations to persist in both the short and long term. As species are typically not listed under the Endangered Species Act until population size has decreased dramatically (Wilcove et al. 1993), these species are likely to be at risk from both genetic and demographic causes of extinction (Lande and Shannon 1996, Lynch 1996). Therefore measuring genetic variation in rare or endangered species is a major focus in conservation. The availability of molecular techniques has provided biologists great insight into the patterns of genetic diversity in natural populations. Although preserving populations with the greatest difference in allele frequencies is advocated in conservation (Moritz 1994), there is no theoretical basis for assuming that the population with the highest level of genetic diversity will be the best genetic source for restoration. This discrepancy is reflected in the considerable debate whether variation at neutral markers (heterozygosity) can be used as an indicator of fitness or evolutionary potential (Cheverud et al. 1994, Savolainen and Hedrick 1995, Britten 1996, Lynch 1996, Storfer 1996, David 1998).

Patterns of genetic variation detected with random molecular markers are likely to reflect neutral genetic variation. Although convenient, studying the patterns of variation in molecular markers in rare species will not necessarily lead to an understanding of the species biology needed for conservation decisions. Genetic variation in quantitative characters is considered to be a better predictor of a species ability to persist (Lynch 1996, Storfer 1996), but is rarely measured in endangered species (Schemske et al. 1994, Knapp and Rice 1998). Maintaining variation in traits involved in local adaptation is a promising approach for conserving ecologically important variation, as it preserves the ability of the species to maintain fitness across a variety of habitats. Ignoring local

adaptation may lead to severe outbreeding depression when populations are mixed, a circumstance that can only be anticipated by studying the ecological genetics of local adaptation. Additionally, genetic variation in quantitative characters is acknowledged as a better predictor of a species' ability to survive genetic and non-genetic threats of extinction such as inbreeding depression and climate change. Management of rare species will benefit from studies of genetic variation in morphological and physiological traits that confer adaptation to habitat heterogeneity.

In chapter 2, I demonstrate local adaptation for traits conferring drought tolerance in a rare plant, *Arabis fecunda*. I compare that with a detailed survey of marker variation (allozymes and DNA sequences), revealing a nearly complete absence of genetic variation within the species, and little to no marker divergence between locally adapted populations. Increasingly, conservation studies quantify diversity at neutral markers, hoping this will reveal which populations of a rare species are most valuable or appropriate as sources for restoration or demographic rescue. These results, combined with the data from the other 23 species in chapter 1, suggest that a commonly used approach in conservation genetics may result in the loss of adaptive genetic variation.

Genetics of Drought Adaptation:

The study of plant adaptation to climate has a long history and remains of central concern in agriculture, ecology, evolution, functional genetics and physiology. Unlike motile animals, plants are exposed to all extremes of their environment at some stage of their life-history. Over 200 years of research on the genetics of ecological races within plant species provides overwhelming support for climate as an important selective pressure to

which populations must locally adapt. I focus on drought adaptation because variation in water availability is likely to be the major climatic selective pressure, as water is fundamental in almost all aspects of a plant's physiology. This is most apparent in agriculture, where drought is the major impact on agricultural yield, but is also supported by studies in natural populations demonstrating selection for drought tolerance traits. These experiments ask questions at an organismal level, e.g. how various traits and loci contribute to drought adaptation, as well as more general questions concerning the genetics of adaptation.

Drought Adaptation: Water is fundamental in almost all aspects of a plant's physiology (Bray 1997). In all plants CO² uptake and water loss to the air (transpiration) both occur through the stomata. Stomata are pores in the cuticle of the leaf, which allow gas exchange between the environment and the mesophyll. Although plants are able to regulate stomatal aperture in response to plant water status, the shared site of exchange results in a tradeoff of losing water for gaining CO². Across taxa, this trade-off between growing and losing water has resulted in a variety of strategies for reducing water loss, including the evolution of alternative photosynthetic pathways which can decouple CO² uptake and growth with loss of water to the air (Bray 1997, Ingram and Bartels 1996).

In habitats where plants encounter seasonal or chronic drought, selection has been shown to favor individuals that are able to minimize the tradeoff between growth and water loss (Dudley 1996), resulting in ecotypes adapted to drought. Water relations are likely to be involved in local adaptation, but little is known of their genetic basis in natural populations (Dudley 1996, Ingram and Bartels 1996, Geber and Dawson 1997). Water relations of a species or ecotype also determine habitat suitability and the outcome

of competitive interactions between species (Fowler 1986). Knowing a species' ability to tolerate or avoid drought, and the genetic basis of this ability is important in the conservation, management and restoration of plant species. The identification of genes involved in water use also has great potential for agriculture, both by marker assisted selection for traits in breeding, and the creation of transgenic crops (Martin et al. 1989, Ingram and Bartels 1996).

In chapter 3, I describe the completed portion of my ongoing research into the molecular and physiological basis of drought adaptation in *Arabidopsis thaliana*. *A. thaliana* has become the model angiosperm, where studies of functional genetics and gene expression have elucidated hundreds of loci that may play a role in climatic adaptation. However, nearly all studies of gene function employ a reverse genetics approach, identifying the phenotypic effect of "knocking out" the gene. With the exception of flowering time, almost no data exist on which of these loci are polymorphic in natural populations. Theory suggests that the alleles identified in mutant screenings will have deleterious pleiotropic effects on fitness, and thus are unlikely to be involved in adaptation. To understand adaptation, we need to determine the subset of loci, out of all of the approximately 25,000 loci that comprise the (completely sequenced) genome of *A. thaliana*, at which allelic variants have actually been selected in natural populations. Chromosomal regions identified as QTL are now being compared with the available data on candidate genes which map to the QTL regions. Once QTL are identified, these data will be used to test hypotheses concerning the genetic architecture of adaptation. Ultimately, sequence data from individual QTL and flanking regions can provide

additional information on the role of population genetic processes and the type of loci involved in the evolution of adaptation. This is particularly feasible in model systems such as *A. thaliana* where large amounts of genomic data are now being collected on a population scale. These data can ultimately answer the controversy over the extent to which "micromutationism" (many genes of small effect), or "macromutationism" (few genes of large effect) contribute to adaptation. This subject has received much theoretical attention, but ultimately requires experimental work. Particularly lacking are data on the number, mode of action and magnitude of effect of genes involved in adaptation in natural plant populations.

I examined patterns of genetic variance and covariance in two traits 1) time to flowering (drought escape) and 2) stable carbon isotope ratio, $\delta^{13}\text{C}$ (dehydration avoidance), and found a highly significant positive genetic correlation ($r_G = 0.98$). I then used mutants and near isogenic lines to test for pleiotropy in the form of positive mutational covariance between $\delta^{13}\text{C}$ and flowering time. Screening of 8 physiological mutants showed variation in $\delta^{13}\text{C}$ but not flowering time. However I found strong evidence for pleiotropic effects of an allele of *FRIGIDA*, a locus known to be responsible for natural variation in flowering time. An examination of the climate to which these genotypes are native provides evidence that natural variation in $\delta^{13}\text{C}$ may be an adaptation to differences in precipitation. Although genetic studies of flowering time in *A. thaliana* often assume adaptation, we suggest that genetic variation in flowering time may be explained in part by a life history tradeoff driven by the genetic correlation (pleiotropy) between $\delta^{13}\text{C}$ and time to flowering.

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Adaptive population divergence : markers, QTL and traits

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Keywords— adaptive potential, conservation, ecological genetics, evolutionarily significant unit, F_{ST} , gene flow, local adaptation, quantitative genetics, Q_{ST} , quantitative trait locus

Molecular markers appear to be poor indicators of adaptive genetic variation. Direct comparison of population structure in markers with that of traits is made possible by the measure Q_{ST} , which partitions quantitative genetic variation in a manner analogous to F_{ST} for single gene markers. Across 24 species mean Q_{ST} is typically larger than and poorly correlated with mean F_{ST} . Within species Q_{ST} varies widely among traits; traits experiencing the strongest local selection pressures are expected to be the most divergent from molecular F_{ST} . Thus Q_{ST} will be particularly relevant to short term management efforts where extant adaptation is important. Theoretical and simulation studies suggest however that in random mating populations F_{ST} is a better predictor of the pattern of allelic differentiation at QTLs than Q_{ST} , in which case allelic variation at QTLs (longer term evolutionary potential) may be better assessed by molecular markers than will extant variation in the traits themselves.

“A major unresolved issue is the relationship between molecular measures of genetic diversity and quantitative genetic variation.”

(Frankham¹)

Molecular genetic markers have played a major role in evolutionary biology. As molecular methods have become cheaper, faster and involve less invasive sampling, they have become increasingly popular in conservation^{2,3}, where there is often a clear need for rapid decision-making. For example since genetic differences among populations are often considered worthy of conserving⁴⁻⁶, many studies apply a criterion, assigning

conservation priority to populations (or clades of populations) which are reciprocally monophyletic, because these likely represent independently evolving clades or Evolutionary Significant Units (ESU's)⁶. There are several definitions of ESU in the literature, which vary in the degree of emphasis placed on molecular vs ecological criteria^{2,4-6}. Such definitions have critical importance in the light of conservation legislation, where boundaries of ESU's must be drawn before legal protection status can be assigned². However, locally adaptive genetic diversity within units, is likely of greater importance in choosing populations that are most suitable as translocation or restoration sources. In this case, adaptive genetic differences among populations can lead to outbreeding depression if divergent populations are mixed⁷.

Several reviews have identified testing the assumption of an association between marker diversity and adaptive diversity as a pressing research concern^{1,8-10}, and warn against the gradual replacement of ecological data with molecular criteria, when diagnosing units of conservation^{2,3,10}. Reed and Frankham¹⁰ recently conducted a formal meta-analysis of the relationship between molecular and quantitative variation, and found there to be little association for a variety of measures. We here focus on measures of divergence or population structure, since these are most relevant to the definition of "units" in conservation. Much recent theoretical work is relevant to the identification of ecologically adaptive divergence among populations. We review the comparison of population genetic structure in markers, traits and quantitative trait loci (QTLs), drawing upon both the empirical studies available to date as well as theoretical arguments. We suggest that genes, markers and traits will each behave differently in the adaptive divergence of populations, and therefore arguments from one type of variation to another

must be made with caution. However, these considerations also suggest promising approaches to outstanding questions and to determining the appropriate role for molecular markers in defining units for the conservation of local adaptation within taxa.

Comparing Divergence at marker loci and quantitative traits

Tests for adaptive variation can proceed most easily by comparison against the null hypothesis that variation is selectively neutral¹¹⁻¹³. Neutral theory is well developed for molecular markers¹³, and a similar body of theory has recently been developed for neutral quantitative genetic traits (Box 1). Population genetic structure is often quantified using F_{ST} or related statistics, for a variety of marker types and mutation models¹⁴. A precisely analogous measure for quantitative genetic traits is Q_{ST} ¹⁵. Provided that quantitative genetic techniques are applied to measure and partition genetic variation (rather than phenotypic variation) within and between populations, Q_{ST} is straightforward to calculate, and can be directly compared to F_{ST} .

For neutral additive traits, the expectation of F_{ST} and Q_{ST} will be equal (Box 1). The magnitude of the difference between Q_{ST} and F_{ST} can be used to infer the degree of local adaptation^{11,12,15,16}. Under divergent selection pressure, Q_{ST} is expected to be greater than F_{ST} of molecular markers^{11,12,16}. For example across a latitudinal cline in Scots pine (*Pinus sylvestris*), a common garden screening revealed that among population divergence in the timing of budburst was much greater ($Q_{ST} = 0.80$) than population divergence in allozyme, microsatellite, RAPD and RFLP markers ($F_{ST} < 0.02$)¹⁷. While this is a particularly striking example, it appears to be the rule rather than the exception.

Figure 1 summarizes data for 24 species in which it is possible to statistically compare F_{ST} and Q_{ST} directly (Table 1). Three patterns emerge. First, for 19 of 24 species Q_{ST} , averaged across traits, is higher than F_{ST} (or G_{ST}) averaged across marker loci (paired t-test). This represents a significant (sign test, $P < 0.005$) departure from the 50:50 one would expect if Q_{ST} and F_{ST} were equal on average. There is no a priori reason for this to be so, and although $Q_{ST} > F_{ST}$ for most traits, a minority exhibit Q_{ST} significantly lower than F_{ST} ¹⁵, suggesting selection acts on those traits towards the same optimal phenotype in each population. Second, these empirical studies reveal little evidence that population differentiation at quantitative traits is well predicted by the differentiation of neutral molecular markers (Figure 1). What little correlation there is between Q_{ST} and F_{ST} seems to derive primarily from the tendency for Q_{ST} to be greater than F_{ST} . In the lower panel of Figure 1, we re-plot the data against Nm , the number of migrants inferred from F_{ST} , to illustrate that high levels of neutral gene exchange do not seem to prevent adaptive differentiation (see below). Finally, there is considerable scatter (range of Q_{ST}) among the different quantitative traits assayed within each species, indicating that selection is specific to individual traits. The overall picture from empirical studies is of adaptive divergence of specific traits taking place in the face of gene flow, with little relationship to patterns exhibited by molecular markers.

Theoretical considerations of adaptive and neutral divergence

Selection and migration constrain but do not eliminate one another.

Rather than gene flow "overcoming" selection (or vice versa) there exists a balance between them which determines both the equilibrium level of differentiation, and the rate of approach to that equilibrium¹⁸. Adaptive differences between populations develop in spite of considerable gene flow¹⁹⁻²¹, and strong selection can rapidly remove the genetic load imposed by immigrants, maintaining differences among populations^{22,23}. At the same time, studies comparing populations experiencing different levels of isolation show that gene flow constrains the adaptive differentiation, such that populations connected by high levels of gene flow are less differentiated than might be expected based upon the locally optimal phenotypes^{20,24-26}.

An example will illustrate this point. King and Lawson²⁰ document (i) adaptive differentiation between mainland and island populations in the banding patterns of water snakes as well as (ii) high levels of gene flow. Selection "overcomes" gene flow in that the populations are more differentiated for banding pattern than they might be under migration drift balance. At the same time the high levels of gene flow constrain local adaptation in that the populations are less differentiated than expected by selection alone. (ie the populations do not exhibit fixed differences). Moreover, the selection against the immigrant type is not strong enough to eliminate gene flow at allozyme markers, which exhibit F_{ST} values in the range of 0.019 to 0.093 between island and mainland populations. The conservation implications of such studies is clear. If the island populations were in some way endangered and in need of restoration, a large scale translocation of snakes from the mainland would greatly reduce the mean fitness of the island population. But neutral markers do not reveal this differentiation at the adaptive

trait. Indeed, had the island and mainland snakes been differentiated for a more cryptic (say, physiological) trait, the adaptive differences may well have gone undetected.

There are two important reasons for the disconnection between geographic patterns at neutral and selected traits. First, the **number** of migrants may be high enough ($Nm > 1$) to prevent neutral differentiation, while the **proportion** is low enough ($m < s$) to permit adaptive differentiation. Moreover, selection against immigrant alleles at a locally adaptive locus will present little barrier to the effective migration of neutral loci, unless such loci are tightly linked to the locus under selection²⁷. Second, the rate of approach to equilibrium is likely to be higher for loci experiencing selection than for those which are drifting unless population size is extremely small. Adaptive differentiation occurs at a rate proportional to the product of trait heritability and the selection intensity (the familiar $R = h^2 S$ of basic quantitative genetics). Typical rates of short-term evolutionary change are about 0.1 to 0.5 phenotypic standard deviations per generation²⁸ and cases of very rapid evolution are famous²⁹⁻³². For local adaptation occurring over fairly short periods ($\approx 50-100$ generations), substantial differentiation of quantitative traits can be achieved under sustained directional selection of moderate to strong intensity. By contrast, for many organisms, differentiation due to drift will be much slower, on the order of N_e generations³³.

Selective differentiation of polygenic traits will cause little differentiation of the underlying loci

For adaptive traits controlled by single Mendelian loci, we expect among population divergence of the allele frequencies at these loci. However, many adaptive

differences among populations involve polygenic traits, controlled by two or more unlinked QTL (we use the term polygenic rather than quantitative traits to distinguish from continuously varying traits controlled by a single QTL) With multiple loci affecting a polygenic trait, selection on the trait is diluted over many loci, such that each locus can itself behave as if nearly neutral³⁴. Simulation studies³⁵ show that under random mating within populations, QTLs differentiate little in the face of pronounced diversifying selection on trait values. In the simulations, neutral marker loci conformed to the expectations of migration/drift equilibrium regardless of the selective regime imposed on the polygenic trait. More importantly, F_{ST} calculated from the QTLs themselves was almost identical to that seen at the neutral markers. Thus, if divergence in a polygenic trait is created by local adaptation, not only will Q_{ST} be greater than the F_{ST} value seen at neutral markers - it will also be greater than F_{ST} of the QTLs!

Because the trait value is the sum of each allelic effect, the variance of the trait includes a contribution of the covariance of allelic effects. With neutral differentiation of the trait, unlinked loci differentiate independently among populations, giving covariances that are zero on average (Box 2). Adaptive differentiation of a polygenic trait among populations creates a parallel differentiation (i.e. a covariance) of allele frequencies at the underlying QTLs, because each QTL is responding to the same selection pressure. These covariances increase trait differentiation beyond what would be expected from the sum of each allele frequency difference. Thus, counter-intuitively, substantial trait differentiation is possible with only minor differentiation of allele frequencies at the underlying loci (or vice versa). Moreover, as the number of loci affecting the trait increases, the relative contribution of covariances increases exponentially. In the

extreme, very large Q_{ST} is possible with only trivial allele frequency differences acting in parallel over very many loci. If the QTL's (which are the targets of selection at the genetic level) themselves differentiate only slightly, there can be no reason to expect neutral molecular markers to reflect the adaptive differentiation of populations.

Conserving Present and Future Evolutionary Potential

This theoretical result suggests a critical distinction between the differentiation of traits and of their underlying loci. To illustrate, consider a hypothetical riparian species occurring along parallel streams that traverse steep elevational gradients. If populations at the same elevation experience the same environment, then trait means will be most similar among populations at the same elevation in different drainages (Q_{ST} highest among different elevations). But if gene flow occurs mostly along the riparian zone within drainages, allele frequencies (at Both QTL's and molecular markers) will be most similar within drainages (F_{ST} highest among different drainages). In the short term, evolutionary potential to respond to environmental changes will be determined by the standing pool of phenotypic variation (Q_{ST}). However, the allelic variation (F_{ST}) represents the underlying potential for longer term evolutionary change.

Perhaps the major motivation for conservation genetic studies is the identification of ESUs - populations that are sufficiently distinct to merit conservation status under existing legislation^{4,6}. Such ESUs are thought to preserve evolutionary potential which can re-create lost biodiversity, provided that evolutionary processes are able to operate⁶. While we tend to favour ESU criteria which include as much ecological information as possible^{2,5}, molecular genetic markers appear to hold out considerable opportunity to

make inferences about allelic variation underlying fitness traits³⁵⁻³⁸, and thus potentially the longer term evolutionary potential of a population.

However, genetic criteria are also frequently invoked to guide transfers of individuals between existing populations as well as restoration efforts where populations have been extirpated^{7,39,40}. With rapid translocations, it seems unlikely that evolutionary processes can operate rapidly enough to prevent significant loss of fitness in endangered populations⁷. From the recipient population's perspective, translocations can represent a very high proportion of immigrants, enough to substantially erode existing local adaptation. Alternatively from the perspective of the translocated individuals, the change in the environment, will potentially occur faster than adaptive change can take place without threat of extinction⁴¹. Thus long term evolutionary potential may not be enough to preserve populations through short term stresses imposed by movement between sufficiently different environments. While such translocations are extremely beneficial in many cases, both theoretical (Box 2) and empirical (Fig 1) results suggest that molecular genetic markers may provide a poor guide to adaptively similar units. Such short term efforts should in all cases emphasize the ecological criteria over and above molecular genetic markers. In some cases, adaptive variation may be predicted most reliably (and conveniently) by available data on ecological and climatic gradients⁴².

Research Needs

Ecological genetic experiments can directly estimate genetic variation in traits that affect fitness and therefore the demography of rare and endangered species, crucial

information that cannot be inferred directly from molecular data. However, while molecular genetic markers are applicable to almost any taxon, common garden studies are not feasible for many endangered or intractable species. Recent methods have been developed combining molecular markers to infer relatedness with field measures of ecologically important variation⁴³. These methods allow the estimation of both heritability and Q_{ST} from field studies of natural populations⁴³, and so hold out promise for the analysis of adaptive variation in any species. To help interpret such studies, however, it will be useful to apply detailed common garden methods to well studied organisms, so that the evolutionary processes that shape quantitative trait and QTL variation are thoroughly understood.

Does F_{ST} reflect QTL distribution?

While techniques for the identification of Quantitative Trait *Loci* become ever more sophisticated^{44,45}, assaying the *allele* frequencies at QTL's in most natural populations remains out of reach. Significant promise for estimating allele frequencies at QTL comes from well studied species for which QTLs have been mapped, and in some cases cloned. We have conjectured that while Q_{ST} and related approaches may be most relevant to the distribution of trait variance (short term conservation), F_{ST} may better reflect the distribution of allelic evolutionary potential, more relevant to longer term conservation. However, we cannot overstate that this conjecture is based entirely upon theoretical arguments assuming random mating, and thus may not be relevant to many species. *It remains to be empirically demonstrated that molecular markers do indeed reflect allelic variation at QTLs or other genes underlying fitness.* Uncritical inference

from molecular markers to QTLs may be just as damaging as the uncritical inference from markers to adaptive variation.

What evolutionary forces influence Quantitative traits ?

It will be useful to compare Q_{ST} v. F_{ST} across species representing a variety of life-histories, breeding systems and metapopulation demographics. Several authors (Fig 1) have used Q_{ST} to infer selection acting on individual quantitative traits by their departure from patterns seen at neutral molecular genetic markers, but other comparisons are possible. For example, comparisons across breeding systems, will help us to identify the nature of quantitative trait evolution in selfing systems. Similarly, in well studied organisms for which QTLs have been mapped, it would be instructive to compare Q_{ST} across traits with different numbers of QTLs or with non-additive inheritance (dominance and/or epistasis). If molecular surveys of candidate loci can be included in such studies, a complete understanding of the relationship between marker and trait variance should be possible.

Summary

We emphasize that none of the foregoing is intended to argue against the use of molecular markers or translocations, both of which can be extremely beneficial in ecological, evolutionary or conservation studies. However, we caution against an oversimplified interpretation of the results in which it is assumed that low marker differentiation inevitably precludes adaptive differentiation. We have argued on both theoretical and empirical grounds, that the interpretation of genetic variation must

distinguish among (1) molecular genetic markers (putatively neutral), (2) quantitative genetic (polygenic) traits, and (3) the genes (QTLs) underlying quantitative traits. Each is likely to have its own pattern of geographic distribution, which are likely to be poorly correlated across types of variation. Moreover, the relevance of these three classes of variation to the definition of ecologically and evolutionarily relevant groupings will vary depending upon the short and long term purpose of defining those groups.

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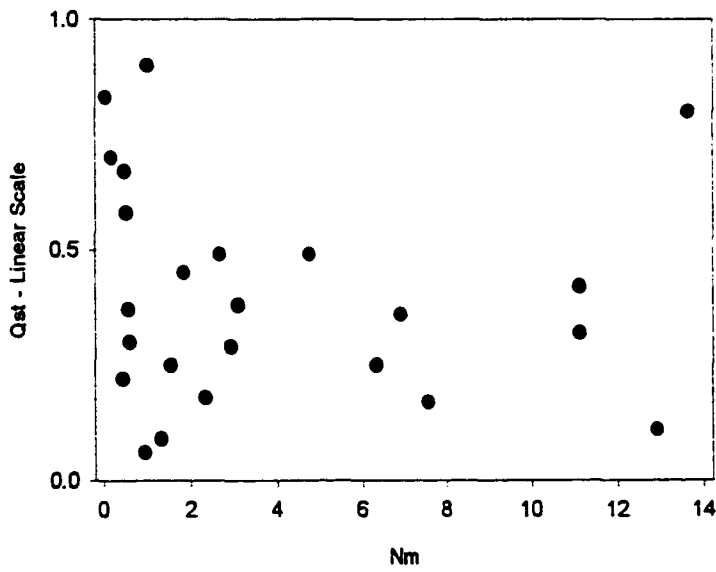
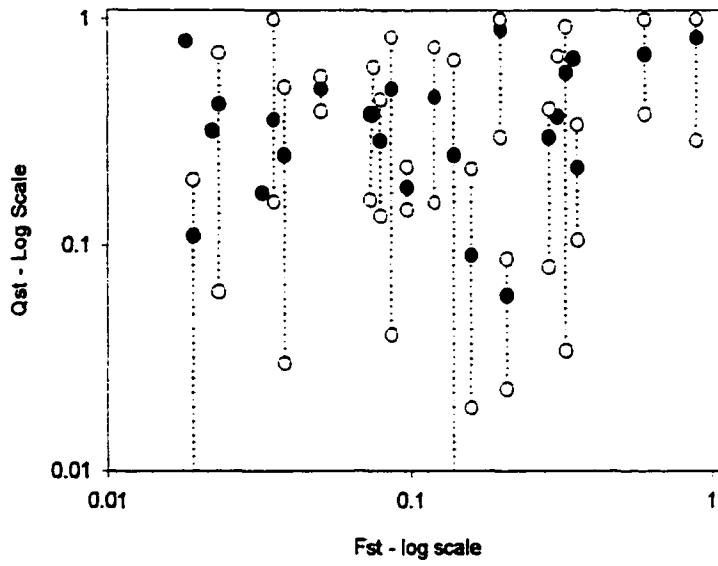
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Table 1 - Q_{ST} and F_{ST} from published data for 24 species.

Species	Refs	Qst	Qst Range	Markers ¹	Refs	Fst
<i>Arabidopsis thaliana</i>	16	.83	0.29,1	A, M	51	.890
<i>Arabis fecunda</i>	42	.98	0.3,1	A	42	.200
<i>Brassica insularis</i>	46	.06	0.023,0.087	A	46	.210
<i>Centaurea corymbosa</i>	46	.22	0.105,0.341	A	46	.364
<i>Clarkia dudleyana</i>	16	.38	0.158,0.611	A	16	.075
<i>Daphnia obtusa</i>	15	.30	0.08,0.4	A	15	.300
<i>Daphnia pulex</i>	16	.31	0,0.685	A, M	16	.310
<i>Drosophila buzzatii</i>	16	.17	N/A	A	16	.032
<i>Drosophila melanogaster</i>	16	.70	0.38,1	A	16	.600
<i>Larix laricina</i>	47	.49	0.39,0.553	A	48	.050
<i>Larix occidentalis</i>	49	.49	0.04,0.833	A	50	.086
<i>Medicago truncatula</i>	16	.58	0.034,0.928	RAPDs	16	.330
<i>Phlox drummondii</i>	51	.25	0.03,0.5	A	51	.038
<i>Picea glauca</i>	52	.36	0.155,1	A	53	.035
<i>Picea sitchensis</i>	54	.29	0.134,0.44	A	55	.079
<i>Pinus brutia</i>	56	.25	0.004,0.659	A	57	.140
<i>Pinus contota ssp. latifolia</i>	16	.11	0.006,0.195	A	16	.019
<i>Pinus sylvestris</i>	17	.80	N/A	A,M,R	20	.018
<i>Pseudotsuga menziesii</i>	58	.42	0.062,0.706	A	59	.022
<i>Quercus petraea</i>	16	.32	N/A	A	16	.022
<i>Scabiosa canescens</i>	16	.09	0.019,0.217	A	16	.160
<i>Scabiosa columbaria</i>	16	.45	0.154,0.75	A	16	.120
<i>Sequoiadendron</i>	60	.18	0.143,0.222	A	60	.097
<i>Wyomea smithii</i>	16	.67	N/A	A	16	.346

1- A=allozymes, M=microsatellites, R=Restriction Fragment Length Polymorphisms, RAPD=Random Amplified Polymorphic DNA

Figure 1 - Each filled circle represents the mean value of F_{ST} and Q_{ST} for a given species. A paired t-test shows that across species mean Q_{ST} is greater than mean F_{ST} ($t=-4.382$, $df=23$, $P<0.0005$). The vertical lines and open circles show the range of Q_{ST} across the traits where data were available for a species. The upper panel shows that there is non-significant positive correlation between log transformed variables Q_{ST} and F_{ST} ($r = 0.185$, $P = 0.323$), and Q_{ST} does not approach zero as F_{ST} approaches zero. This is also apparent in the lower panel where F_{ST} has been "transformed" into an estimate of Nm , and again there is no significant linear relationship. See table 1 for a list of species included.



BOX 1 - Population Divergence in Molecular Markers and Ecological Traits.

Wright's^a F_{ST} and related statistics^b provide a useful measure of the level of population genetic structure at single genes by quantifying the proportion of the total allelic variation that occurs between populations. Imagine that several populations derive from a common ancestor with genetic variance $\sigma_{g(0)}^2$ and diverge through drift. Wright^c showed that the partitioning of genetic variance at a quantitative polygenic trait into within $\sigma_{g(w)}^2$ and between $\sigma_{g(b)}^2$ population components is related to the partitioning of allelic variation as :

$$\begin{aligned}\sigma_{g(b)}^2 &= 2F_{st}\sigma_{g(0)}^2 \\ \sigma_{g(w)}^2 &= (1 - F_{st})\sigma_{g(0)}^2 \\ \sigma_{g(t)}^2 &= (1 + F_{st})\sigma_{g(0)}^2\end{aligned}$$

Such that

$$\frac{\sigma_{g(b)}^2}{\sigma_{g(t)}^2} = \frac{2F_{st}}{(1 + F_{st})}$$

We can therefore define a quantitative trait analog of F_{ST} labeled Q_{ST} by Spitze^d as

$$Q_{ST} = \frac{\sigma_{g(b)}^2}{\sigma_{g(b)}^2 + 2\sigma_{g(w)}^2}$$

and therefore $Q_{ST} = F_{ST}$ for neutral traits.

We have retained the subscript g throughout the above to emphasize that Q_{ST} must be calculated from components of *genetic*, not phenotypic variance. In practice, Q_{ST} is measured by quantifying the genetic components of variance within and among

populations, in randomized common garden experiments. In such experiments, the phenotypic differences between populations can be ascribed to genetic differentiation among populations. Phenotypic variation within populations includes both a genetic and environmental component that can be partitioned by assaying multiple individuals within (say) half sib families^e. Thus the most common design is a nested ANOVA with individuals nested within families within populations.

The evolutionary forces influencing Q_{ST} for neutral traits are well worked out by Lande^f. Briefly, variation among n demes is determined by the migration (m) and mutation σ_m^2 rates

$$\sigma_{g(\text{between})}^2 = \frac{(n-1)\sigma_m^2}{m}$$

while the variance within populations is determined by N (the effective population size, which determines how much variation is fixed through drift) and the mutation rate

$$\sigma_{g(\text{within})}^2 = 2nN\sigma_m^2$$

Thus by substituting,

$$Q_{st} = \frac{\frac{(n-1)\sigma_m^2}{m}}{\frac{(n-1)\sigma_m^2}{m} + 2 * 2nN\sigma_m^2}$$

which simplifies to

$$Q_{st} = \frac{1}{1 + 4Nm \left(\frac{n}{n-1} \right)}$$

(Eq 10a of Ref f) which is equivalent to F_{ST} for large numbers of demes. The relationship between F_{ST} and Nm applies strictly to large numbers of demes at equilibrium, in an island model, a situation which is rarely observed in nature. However, using a coalescent approach it can be shown^g that departures of F_{ST} from it's equilibrium (e.g., due to historical contingency) influence Q_{ST} in a parallel manner, since

$$Q_{st} = F_{st} = \frac{t_{total} - t_{within}}{t_{total}}$$

for coalescence times t (eqs 10, 11 of Ref g).

Thus for a species with any population structure, it is possible to test the null hypothesis that a given trait evolved by genetic drift, in which case F_{ST} (from neutral markers) will be equal to Q_{ST}^{fh} . If instead we find a trait with an estimate of Q_{ST} significantly different from F_{ST} , we reject the hypothesis that this trait is neutral as the difference will reflect the relative influence of natural selection. When two populations have adapted to different habitats, we expect Q_{ST} values for traits involved in adaptation to be greater than F_{ST} . If both populations are experiencing stabilizing selection for the same phenotype we expect Q_{ST} will be lower than F_{ST} .

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Box 2 - Adaptive divergence at polygenic traits and quantitative trait loci.

Under the simplest model of polygenic trait variation, the trait value is simply the sum of the allelic effects at each of two underlying loci (QTL). The variance of a sum is determined both by the variance of each of the parts (here the genetic variation at each locus) and the pattern of covariance among the parts (which can be interpreted as linkage disequilibrium among loci).

$$\sigma_g^2 = \sigma_{loc1}^2 + \sigma_{loc2}^2 + 2Cov(loc1, loc2) \quad a,b$$

As σ_g^2 is partitioned within and among populations (Box 1), so are each of the terms on the right. Thus the variance among populations (*i.e.* the numerator in the calculation of Q_{ST}) will be determined both by the differentiation of QTL allele frequencies (*ie* F_{ST} of the QTLs), but also by the correlation of allelic frequencies across populations.

This among population linkage disequilibrium of QTLs can be intuitively seen in the figures below. If the correlation is negative (top), opposite changes in allele frequencies at locus 1 and locus 2 cancel each other out, such that there is strong allele frequency differentiation, but no trait differentiation (the mean trait value in this example is exactly the same for each population). The more likely situation is depicted in the lower panel, where parallel clines in allele frequencies produce a stronger differentiation of trait values than would be predicted from either locus considered individually^c. To put it another way, pronounced adaptive differentiation of traits can be achieved with little differentiation of allele frequencies at the underlying QTLs. Moreover, the contribution of the covariances increases as the square of the number of loci (because there are $n(n-$

1)/2 locus pairs among n loci). Thus, the more loci influence a trait, the greater the impact of parallel differentiation of allele frequencies on trait differences, and the less differentiation is expected of the allele frequencies themselves.

While this model assumes additive effects of genes on the trait, epistatic effects on fitness are implicit in the assumption of stabilizing selection^d (Fenster et al, 1997) because it is likely that several combinations of alleles will create intermediate trait values. Epistatic interactions in the determination of the trait itself have not been directly modeled. However, the suggestion that differentiation for combinations of alleles effectively de-couples F_{st} and Q_{st} suggests that the presence of epistatic interactions will further blur the association between population structure of QTLs vs traits.

a Falconer, D.S. and MacKay, T.F.C. (1996) *Introduction to Quantitative Genetics* 4th ed.

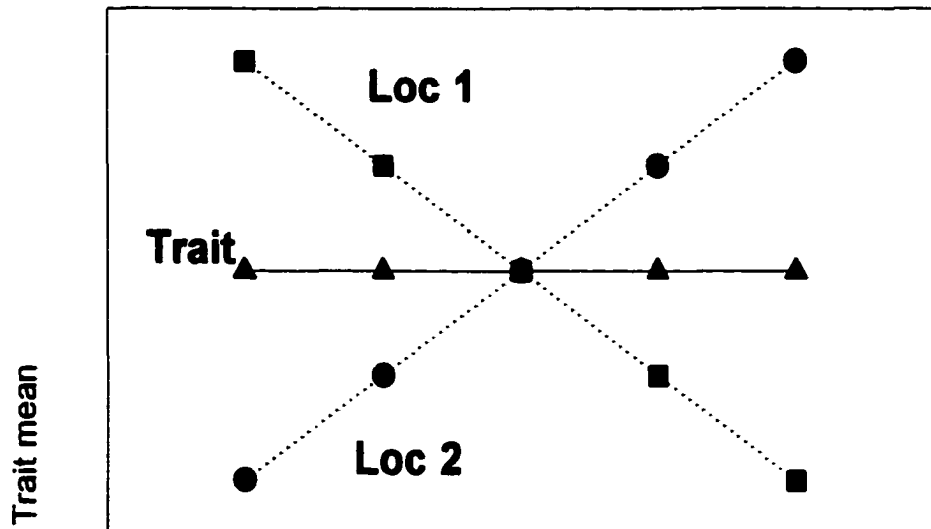
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b Barton, N.H. and Gale, K.S. (1993) Genetic analysis of hybrid zones. In *Hybrid Zones and the Evolutionary Process* (Harrison, R.G. ed.) pp. 13-45. Oxford Univ. Press, New York.

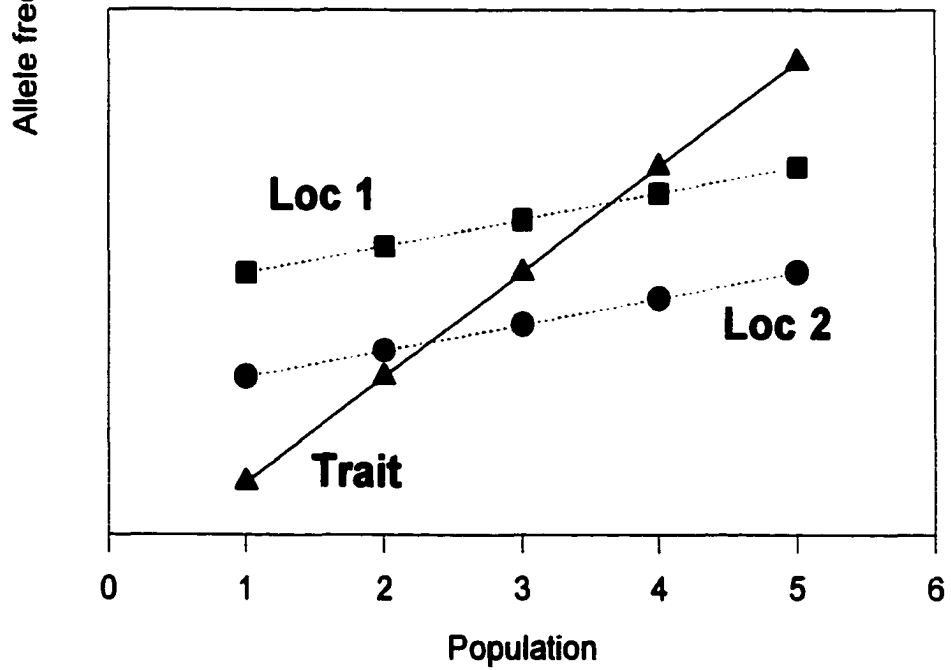
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a) Negative Covariance among Loci



b) Positive Covariance among Loci



Local adaptation across a climatic gradient despite small effective population size in the rare Sapphire Rockcress.

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SUMMARY When assigning conservation priority in endangered species, two common management strategies seek to protect remnant populations that 1) are the most genetically divergent, or 2) possess the highest diversity, at neutral genetic markers. These two approaches assume that variation in molecular markers reflects variation in ecologically important traits and ignore the possibility of local adaptation among populations that show little divergence or variation at marker loci. Using common garden experiments, we demonstrate that populations of the rare endemic plant *Arabis fecunda* are physiologically adapted to local microclimate. Local adaptation occurs despite 1) the absence of divergence at almost all marker loci and 2) very small effective population sizes, as evidenced by extremely low levels of allozyme and DNA sequence polymorphism. Our results provide empirical evidence that setting conservation priorities based exclusively on molecular marker diversity may lead to the loss of locally adapted populations.

1. INTRODUCTION

Measurement of genetic variation has become a major focus of conservation research, since many threatened and endangered species are likely to be at risk from genetic as well as demographic determinants of extinction (Lande & Shannon 1996). Typically, within and among population surveys of polymorphism using one or more marker type (allozyme, RFLP, microsatellites, and nuclear or organelle DNA sequence) are used to estimate phylogenetic and population genetic parameters (Vane-Wright et al. 1991; Moritz et al. 1995; Vrijenhoek 1994; Hamrick & Godt 1996). Based on the hope

that maximizing genetic marker variation will provide remnant populations with the greatest evolutionary potential and reduce negative consequences of inbreeding, conservation geneticists have advocated preserving the most divergent populations (Moritz et al. 1995) or those possessing the greatest level of genetic variation or heterozygosity (Vrijenhoek 1994). These data are also used to decide which populations are most suitable as restoration sources to and to designate evolutionarily significant units, which may be eligible for protection under the US Endangered Species Act (Haig 1998). Although convenient, the current emphasis on neutral marker diversity at the expense of ecological genetic information may lead to poor management decisions for rare species because molecular markers may not reflect variation in ecologically important traits or adaptation to local environmental conditions (Templeton 1986, Cheverud et al. 1994; Milligan et al. 1994; Hamrick & Godt 1996; Lynch 1996; Storer 1996; Parker et al. 1999; Crandall et al. 2000).

Marker diversity is frequently used to decide which populations are most suitable as translocation or restoration sources (reviewed in Templeton 1986; Haig 1998; Knapp & Rice 1998). For example, based on "low to moderate" levels of genetic differentiation at 4 microsatellite loci among the three remaining populations of the kokako (*Callaeas cinerea wilsoni*), an endangered passerine bird, Hudson et al. (2000) concluded that "there is no genetic barrier to translocations between the study populations". Similarly, based on low levels of genetic variation at RAPD markers among the four remaining populations of the woody shrub *Grevillea scapigera*, Rossetto et al. (1995) concluded that populations were not locally adapted, and recommended translocation. However, such management decisions are not without risk, especially if genetic differences among

populations lead to outbreeding depression when local populations are mixed (Fenster & Galloway 2000). In this case, genetic variation in quantitative characters may be more relevant (Geber & Dawson 1993; Schemske et al. 1994; Storfer 1996; Waldmann & Andersson 1998; Frankham 1999). Missing from this debate are data relating marker diversity to local adaptation. Here, we provide a clear demonstration that habitat heterogeneity maintains ecologically important genetic variation among populations of a rare plant, even when molecular variation and divergence are largely absent.

MATERIAL AND METHODS

(a) Study organism

We examined genetic diversity and local adaptation to habitat in populations of the plant *Arabis fecunda* (Brassicaceae), a rare perennial herb restricted to calcareous soil outcrops in western Montana, USA (Rollins 1993). *A. fecunda* is a partially self fertilizing species (Hamilton & Mitchell-Olds 1994), with 19 existing populations isolated into two elevational groups separated by 100km in distance (Lesica 1993). Like many rare plants (Schemske et al. 1994), major threats to this species are human development, competition from invasive exotic plants and cattle grazing (Lesica & Shelly 1996). Of particular concern is a group of four low-elevation (1500m) populations in the rapidly urbanizing Bitterroot Valley, where active management and reintroduction may be necessary. Differences in phenology, self-compatibility and geographic isolation make present day gene flow between the high and low elevation groups of populations extremely unlikely, hence divergence by genetic drift is expected.

(b) Habitat differences

We quantified climatic differences between sites in 1997 and 1998 in air temperature, soil temperature, relative humidity, precipitation, wind speed, global radiation, and vapor pressure deficit. Weather data were collected simultaneously during the growing season with automated weather stations (Campbell Scientific, Logan, UT, USA). In 1998, we also measured soil water potential using psychrometry (Rundel & Jarrell 1989). These data indicate that plants in the low elevation habitat were exposed to significantly greater drought stress than those at high elevation in both years (Table 1). Because water availability is a fundamental determinant of plant growth and survival (Stebbins 1952; Ingram & Bartels 1996) and dry environments are known to select for higher water use efficiency (WUE) (Dudley 1996), we predicted that differences in water availability would select for local adaptation in drought tolerance, as measured by ecophysiological traits (Comstock & Ehleringer 1992; Geber & Dawson 1993). We tested this prediction and compared the results to among and within population differentiation of isozymes and DNA sequences.

(c) Molecular markers

Populations were sampled for marker surveys by collecting seed from reproductive plants at least 10 meters apart. All plant material used for marker analysis was collected from plants grown in a growth chamber from field collected seeds. To assay genetic variation at allozyme loci, we sampled an average of 18 plants per population, from 8 populations of *A. fecunda*: 5 of 15 high elevation populations and 3 of 4 low elevation populations. Protein extracts were obtained by grinding 50-100 mg of

young leaves in liquid nitrogen and incubating on ice for 10 minutes with 60 μ l of dithiothreitol extraction buffer (1 mg/ml dithiothreitol in 0.05 M Na_2HPO_4 , pH 7.0). Isozymes were assayed on 11% starch gel in morpholine citrate buffer system. Among and within population genetic parameters were calculated using GDA (Lewis & Zaykin 2000).

To estimate effective population size we measured nucleotide variation at two unlinked nuclear loci. PCR primers for basic chitinase 1 (*Chi1*), and basic chitinase 2 (*Chi2*) were designed using *Arabidopsis* sequences, and optimized for *A. fecunda* as described in Bishop et al. (2000). All PCR amplifications included water-blank controls. The data for the two loci consists of 67 *Chi1* direct sequences (134 alleles), 837 bp each, from 10 populations; 52 *Chi2* direct sequences (104 alleles), 558 bp each, from 10 populations. Species and population level θ were calculated using DNAsp (Rozas & Rozas 1999). Comparisons to *Arabidopsis* are for coding and non-coding polymorphisms, whereas calculation of N_e from θ is based only on non-coding regions and synonymous substitutions. We estimated effective population size from $\theta = 4 N_e \mu$, where N_e is the effective population size and μ the mutation rate (Kimura & Ohta 1971), by using Koch et al.'s (2000) estimate of $\mu = 1.5 \times 10^{-8} \text{ yr}^{-1}$ as the neutral mutation rate for relatives of *Arabis*, a generation time of 3 years for *Arabis fecunda*.

(d) Among population differences in candidate traits

Based on climatic differences among *A. fecunda* populations, we examined traits that may confer adaptation to local climate. For example, WUE, carbon isotope ratio, and root investment (RMR) are traits that may be involved in adaptation to habitat differences in drought (Geber & Dawson 1993; Comstock & Ehleringer 1992). For each experiment all comparisons among genotypes are based on common garden screening in growth chambers or greenhouses. All parameter estimates are based on whole plant measures. In order to estimate within population variation, full-sib maternal families were produced from 2 generations of single seed descent in a common environment to reduce maternal effects.

In the first common garden experiment, instantaneous WUE was measured on 42 plants, representing 3 populations from each elevational group. Each plant came from 2 generations of single seed descent of field collected seed. Seeds were sown directly into 300 ml pots filled with sterile peat potting soils and vermiculite, and then cold treated (4 °C) in the dark for 30 days. Seeds were germinated and plants were grown at 25/15 °C, 50/65% relative humidity (day/night) at 15 hour days in an environmental growth chamber with fluorescent lighting (PAR = 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). We measured gas exchange with a LiCor 6400 (LiCor, Lincoln, NE, USA), equipped with a cuvette modified to permit whole plant measures of gas exchange, hence integrating leaf performance over the whole plant. Individual values were based on the average of 10 repeated measures and instantaneous water use efficiency (WUE) was calculated from the ratio of photosynthetic assimilation, A to transpiration, E, where

$$\text{WUE} = \frac{\text{A (CO}_2 \mu\text{mol m}^{-2}\text{s}^{-1})}{\text{E (H}_2\text{O mmol m}^{-2}\text{s}^{-1})}$$

(e) Quantitative genetic analysis

In this common garden experiment we determined the degree of heritable variation for WUE, rosette morphology and other ecologically important traits within and between both populations (high and low elevation) for which we collected climatic data. Seeds from eight full-sib families, from each of the 2 populations, were placed in petri dishes with filter paper and 2 ml of tap water. After germination, seedlings were transplanted into peat soil in a randomized complete block design. Plants were grown at 25/20 °C, 60/75 % relative humidity (day/night) at 17 hour days in an environmental growth chamber with fluorescent lighting ($\text{PAR} = 150 \mu\text{mol photons m}^{-2}\text{s}^{-1}$). Gas exchange was measured as described above in (d). Three months after germination we measured WUE, rosette height, rosette diameter, shoot biomass and total leaf area. Mortality due to transplanting led to inadequate replication in some families, therefore to avoid statistical artifacts of unbalanced design analysis was limited to 5 families per population ($n = 209$ plants). Root biomass was measured in a subset of plants ($n = 70$) to determine root mass ratio ($\text{RMR} = \text{root biomass} / \text{total biomass}$). WUE, leaf packing (total leaf area/ rosette volume), and leaf area were \log_e transformed to meet the assumptions of parametric analysis. The effect of family (FAM $\text{df} = 8$) was considered random and nested within population (POP $\text{df} = 1$), which was considered fixed. For the trait WUE, additional random effects of block and the interaction between family and block were significant and thus added to improve model fit. Significance of all effects was tested with ANOVA

using SAS 6.12 procedure GLM with type III SS (SAS Institute, Cary, NC), followed by a sequential Bonferroni procedure to control for multiple tests (Rice 1989).

(f) Comparative physiology

To test for convergent evolution of WUE in a sympatric *Arabis* species, we compared instantaneous and long-term WUE (using carbon isotope ratios) in *A. fecunda* and *A. holboellii* populations from the high and low elevation sites. Analysis of among population differences in carbon isotope ratios was based on six families per population, grown under well-watered conditions in a greenhouse at the University of Montana during the natural growing season. Discrimination against ^{13}C is greater with more open stomates and thus stable carbon isotope ratios can be used to compare WUE in plants with similar morphology growing in a common environment (Farquhar et al. 1989). Analysis of among population differences in instantaneous WUE was based on growth chamber screening of at least four individuals per population and growth conditions were as described above in (d).

4. RESULTS

(a) Molecular Markers. Levels of neutral genetic differentiation, assayed as variation at 14 allozyme loci and the two chitinase loci, were extremely low within and among *A. fecunda* populations. Of 14 enzyme loci (*HEX*, *DIA*, *IDH*, *G-6-PDH*, *6-PGD*, *ADH*, *SKDH*, *PGM*, *PGI*, *GOT*, *TPI*, *ME*, *ACP*, *EST*), only *PGI* was polymorphic. *PGI* was polymorphic in only 4 of 8 populations sampled (two high and two low), and may be

subject to balancing selection (Filatova & Charlesworth 1999). The remaining allozyme loci were monomorphic in all 8 populations ($n = 142$ plants) resulting in a total species diversity (H_T) of 0.0076. This level of variation in allozymes is very low, even when compared to mean levels for both endemic ($H_T = 0.096$) and selfing ($H_T = 0.124$) plant species (Hamrick & Godt 1996). We partitioned *PGI* variation into among-population-within-elevation group, and between elevational group components, by means of a hierarchical ANOVA of allele frequencies of the *PGI* locus (Weir 1996). The percent of genetic variation between high and low elevation groups (20%) was lower than the percent of variation contained among populations within the elevational groups (44%). At the two sites used for quantitative genetic analysis, within population allozyme diversity (H_S) was 0.0195 ($n=23$) and 0.0060 ($n=36$) for the low and high elevation populations, respectively.

To estimate the effective size of *A. fecunda* populations, we sequenced portions of two nuclear genes, *Chi1* and *Chi2*. Although we sampled over 170 KB of DNA sequence, only 6 of 2330 nucleotide sites were polymorphic and only 4 of 10 populations contained any nucleotide diversity. Based on all sequence data we estimated a total species θ of 0.00032. Hence, nucleotide polymorphism in *A. fecunda* is more than thirty-fold lower than that found for chitinase in the closely related and highly-selfing annual *Arabidopsis thaliana* (Kawabe & Miyashita 1999), where the total species $\theta = 0.011$. We also calculated the effective population size based on a published estimate of the neutral mutation rate for relatives of *Arabis* (see methods). For *A. fecunda* the average $N_e = 145$ (95% C.I.: 0 - 530) is more than an order of magnitude lower than the mean census population size of 5,000 (Lesica 1993).

(b) Local adaptation

Because *Arabis fecunda* populations show little divergence at most molecular markers, and polymorphism is absent in most populations, one might conclude that any population could serve as a transplant source for recovery at any other site. This conclusion is incorrect. We found that climatic differences (Table 1) have led to local adaptation between low (1,525m) and high (2,195m) elevation populations. First, we found substantial genetically based differences in WUE between *A. fecunda* from the two elevation groups. A Mann-Whitney U test showed that instantaneous WUE for the three populations from the drier, low elevation habitat, where mean WUE = 4.38 ($\mu\text{mol CO}_2/\text{mmol H}_2\text{O}$), was significantly greater ($p < 0.0001$) than three populations from the wetter, high elevation sites where mean WUE = 3.07 ($\mu\text{mol CO}_2/\text{mmol H}_2\text{O}$). These genetic differences in WUE among populations may result from physiological differences in gas exchange, as well as morphological differences such as rosette morphology. Second, we found significant genetically-based differences between populations in other traits affecting growth and physiological performance, further suggesting that *A. fecunda* populations are adapted to their local environments. Table 2 shows that plants from the drier, low elevation population have greater WUE and larger, more open rosettes, as revealed by a quantitative genetic analysis of our common garden study. We did not find significant genetic variation in root mass ratio in the experiment described here, perhaps because statistical power was reduced by small sample size for this trait. However, an independent experiment showed the low elevation populations have significantly greater root investment (data not shown).

The functional correspondence between genotype, WUE, rosette morphology, biomass allocation and environmental conditions suggests, but does not prove, adaptive divergence of this trait. Although reciprocal transplants provide a straightforward way to determine if the genetic differences among populations are adaptive, we felt this approach was not feasible for *A. fecunda*. This is based on concerns of erosion due to the steep, rocky habitat, and the danger that reciprocal transplants may cause inadvertent gene flow between populations, resulting in the loss of locally adapted or co-adapted genotypes (Fenster & Galloway 2000). Instead we conducted a comparative test for adaptation, predicting that if genetic differences in WUE were due to adaptation, then sympatric populations of a congener, *Arabis holboellii*, would show the same pattern of genetic divergence for physiology in response to the gradient in water availability. We tested this prediction in two additional common garden experiments. Figure 1 shows greater water use efficiency (as estimated by both instantaneous WUE and stable carbon isotope ratios) in the populations from the drier, low elevation site for both species. Among-site divergence of WUE for both species, combined with convergent evolution within each site, provides further evidence that among-site differences in WUE are due to local adaptation.

4. DISCUSSION

Preserving well-adapted populations is an important goal for species conservation plans. However, within- and among-population studies of marker diversity are routinely substituted for analyses of adaptation to local environments based on the assumption that marker diversity reflects evolutionary potential. In *A. fecunda*, we find that levels of

genetic variation at markers do not predict levels of variation in quantitative traits, either within or among populations. We found very low levels of genetic variation within *A. fecunda* populations, for both traits and molecular markers. The lack of quantitative genetic variation within populations is consistent with both the small effective population sizes in this partially-selfing species and the expectation of selection on traits involved in local adaptation. Consistent with results from other taxa (Waldmann & Andersson 1998; Lynch et al. 1999), we found no evidence for a correlation between heterozygosity and quantitative genetic variation within populations (data not shown).

Several features of *A. fecunda* may explain extremely low levels of molecular genetic variation within populations (H_S and θ_S). First, interspecific analyses show that chitinase experiences strong positive selection (Bishop et al. 2000), which may reduce sequence variation. However, tests of neutrality show no evidence for selective reduction of variation within species in *A. fecunda*, *A. holboellii*, (Bishop unpublished) or *Arabidopsis thaliana* (Kawabe & Miyashita 1999). Moreover, preliminary comparisons between the two groups of *A. fecunda* populations reveal no sequence polymorphism at *Adh*, and only 1 polymorphic microsatellite of 22. Thus it appears that a pattern of low molecular variation is consistent across the *A. fecunda* genome and among molecular marker types. Second, a combination of inbreeding and background selection may drastically reduce levels of neutral genetic variation in selfing plant populations, where linkage extends across large genomic regions (Charlesworth et al. 1997). However, the selfing rate in *A. fecunda* was estimated to be 0.37 (Hamilton & Mitchell-Olds 1994) and therefore does not explain why total species genetic diversity, θ and H_T , are much lower than that of *Arabidopsis thaliana* (Kawabe & Miyashita 1999; Bergelson et al. 1998) and

other highly selfing species (Hamrick & Godt 1996). The near absence of genetic variation within *A. fecunda* may reflect past population bottlenecks resulting from extinction and recolonization events (Pannell & Charlesworth 1999).

Under divergent selection pressure, the degree of population differentiation in adaptive traits, Q_{ST} , is expected to be greater than differentiation in neutral markers, F_{ST} (Rogers 1986; Lande 1992; Spitze 1993; Prout & Barker 1993). In fact the magnitude of the difference between Q_{ST} and F_{ST} can be used to infer the degree of local adaptation (Waldmann & Andersson 1998; Spitze 1993; Prout & Barker 1993). In *A. fecunda* comparison of Q_{ST} and F_{ST} also supports our hypothesis of local adaptation: mean trait divergence of high and low elevation populations ($Q_{ST} = 0.94$, 95% C.I. = 0.87- 1.0) is significantly greater than divergence at *PGI* ($F_{ST} = 0.20$). Our findings, along with a number of studies finding mean $Q_{ST} >$ mean F_{ST} (reviewed in Lynch et al. 1999), are consistent with predictions that population divergence for ecological traits is influenced primarily by natural selection, whereas population divergence for molecular markers is primarily the result of genetic drift. In addition, recent empirical and theoretical results suggest that a small number of loci may explain the majority variation in quantitative traits (Lynch & Walsh 1998; Orr 1998) and thus contradict Fisher's (1958) infinitesimal model. If this is generally true, local adaptation will have little influence on measures of overall divergence at neutral markers (Barton & Bengtsson 1986).

In *Arabis fecunda* a management strategy that used molecular markers to choose source populations for transplantation would be misled, because adaptation to local environments is not predicted by marker variation. Management of rare species will be

more successful if variation in neutral markers is compared to genetic variation in morphological and physiological traits expected to confer adaptation to habitat heterogeneity. Applying methods of ecological genetics to conservation can provide direct and inexpensive estimates of the non-neutral genetic variation imperiled species need to endure current and future abiotic stress. In addition to methods outlined in this study, Ritland (2000) reviews how inferences of relatedness, derived from molecular data, can be combined with field measures of ecological traits, to estimate levels of quantitative genetic variation, both within (heritability) and among populations (Q_{ST}). Finally, the criticism that such ecological genetic measurements are laborious does not justify inappropriate management based on convenient, but misleading, information.

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Table 1. *Microclimatic differences between A. fecunda habitats.*

Analysis is based on all data collected from each growing season, data shown are means at 16:00 from July 1998. a - Significant difference between means tested using a two-tailed t-test (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$). † Soil water potential was not measured in 1997

Habitat Differences (units)	Low Elev.		High Elev.	1997 ^a	1998 ^a
Vapor Pressure Deficit (KPa)	2.8	>	2.3	*	**
Wind Speed (m s ⁻¹)	2.0	<	3.5	*	***
Air Temperature (° C)	29.5	>	24.6	***	***
Soil Temp. at 15 cm (° C)	27.2	>	24.7	***	***
Soil Water Potential (MPa)	-0.60	<	-0.46	†	***

Table 2. *Quantitative genetic analysis of within and among population variation in putatively adaptive traits.*

Shown are means for high and low elevation populations and ANOVA *P*-values for independent variables Family and Population. Bold *P*-values indicate significant effects after using a sequential Bonferroni procedure.

Traits (units)	Low Elev.		High Elev.	FAM (<i>P</i>)	POP (<i>P</i>)
Leaf Packing (cm ² /cm ³)	0.031	<	0.047	0.352	0.0037
Leaf Area (cm ²)	9.72	>	4.84	0.279	0.0081
Root Mass Ratio (mg root dry mass/mg total plant dry mass)	0.28	>	0.24	0.020	0.0700
W U E instant. (CO ₂ μmol m ⁻² s ⁻¹ / H ₂ O mmol m ⁻² s ⁻¹)	2.53	>	2.01	0.200	0.0090
Rosette Height (mm)	16.38	>	8.89	0.070	0.0001
Rosette Diameter (mm)	37.46	>	19.59	0.001	0.0001

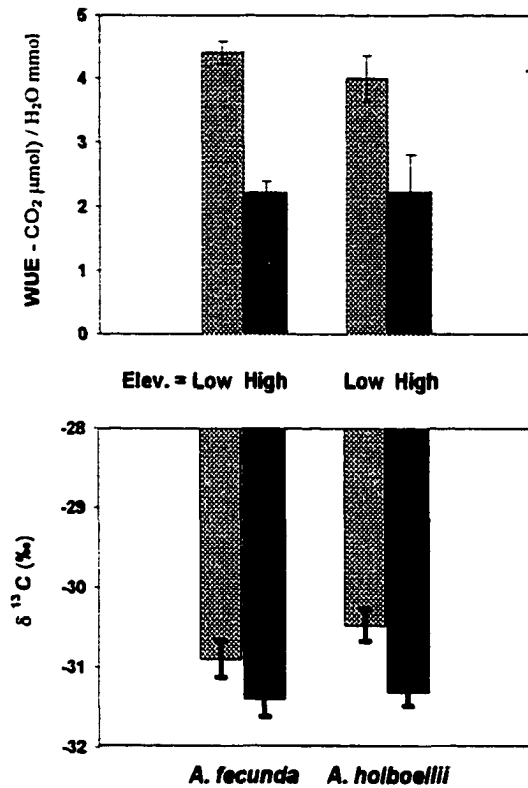


Figure 1. Comparative test of adaptation. Common garden comparison of two sympatric *Arabis* species from the high and low elevation habitats. Genetic differences in both instantaneous WUE (upper panel) and stable carbon isotope ratio (lower panel) were tested in independent experiments and indicate genetic differences in stomatal behaviour (Farquhar et al. 1989). Differences between low (lighter bars) and high elevation populations (darker bars) are heritable and significant for both species in both traits ($P < 0.005$), error bars = ± 1 SE.

Genetics of drought adaptation in *Arabidopsis thaliana*: I. Trait variation and co-variation in natural accessions, mutants and near-isogenic lines.

John K. McKay, James H. Richards, Anna Sala & Thomas Mitchell-Olds

Abstract - We examined patterns of genetic variance and covariance in two traits 1) time to flowering (drought escape) and 2) stable carbon isotope ratio, $\delta^{13}\text{C}$ (dehydration avoidance), putative adaptations to local water availability. A greenhouse screening of 42 genotypes of *A. thaliana* native to habitats spanning a wide range of climatic conditions, revealed a large, highly significant positive genetic correlation between $\delta^{13}\text{C}$ and flowering time ($r_G = 0.98$). This large correlation suggests the presence of a genetically based tradeoff between mechanisms of drought escape (early flowering) and dehydration avoidance ($\delta^{13}\text{C}$). We used mutants and near isogenic lines to test for pleiotropy in the form of positive mutational covariance between $\delta^{13}\text{C}$ and flowering time. Screening of 8 physiological mutants showed variation in $\delta^{13}\text{C}$ but not flowering time. However we found strong evidence for pleiotropic effects of an allele of *FRIGIDA*, a locus known to be responsible for natural variation in flowering time. An examination of the climate to which these genotypes are native provides evidence that natural variation in $\delta^{13}\text{C}$ may be an adaptation to differences in precipitation. Although genetic studies of flowering time in *A. thaliana* often assume adaptation, we suggest that genetic variation in flowering time may be explained in part by a life history tradeoff driven by the genetic correlation (pleiotropy) between $\delta^{13}\text{C}$ and time to flowering.

Introduction

The study of climatic adaptation began in plants (Matyas 1996), and has continued to be a major theoretical and applied interest in all major areas of plant biology including agriculture, ecology, evolution, functional genetics and physiology (Darwin 1859, Turesson 1920, Clausen et al. 1948, Comstock and Ehleringer 1992, Bohnert 1995, Dudley 1996, Ingram and Bartels 1996, Bray 1997). As sessile organisms, plants are exposed to all extremes of their environment at some stage of their life history, and therefore ecotypic differentiation (Turesson 1922, Ehleringer et al., Matyas 1996, Linhart and Grant 1996) among populations is not surprising. In addition, reciprocal transplant and phytometer experiments demonstrate that selection coefficients for local adaptation can be quite strong (Harlan and Martini 1938, Clausen and Heisey 1958, Schemske 1984, Jordan 1991, Nagy 1997). In short, over 200 years of research on the genetics of ecological races within plant species provides overwhelming support for climate as a selective pressure to which populations locally adapt. Clearly, plant populations experience spatially and temporally varying episodes of multi-variate selection (Clausen and Heisey 1958, Berg 1960, Nagy 1997). Despite this compelling evidence that local adaptation is common in plant populations, little is known about the combinations of traits (multi-dimensional phenotypes) involved in adaptation to climate (Arntz and Delph 2001). Even less is known regarding the evolutionary genetics of traits that contribute to the process of climatic adaptation, particularly genetically based correlations that exist among traits. Notably lacking are studies attempting to distinguish the degree to which selection, drift and pleiotropy contribute to the evolution of particular traits thought to be important in adaptation. Here we focus on drought, since drought stress is a major

component of climate, and a number of candidate traits may contribute to drought adaptation (Richards 1996).

Drought Adaptation - Water availability is likely a strong and recurring selective pressure, as it is fundamental in almost all aspects of plant physiology (Stebbins 1952, Bray 1997; Bohnert 1995). This is perhaps most apparent in agriculture, where drought has a major impact on agricultural yield, but is also reflected in plant distribution and abundance in natural systems. In crops, adaptation to drought involves both phenological and physiological traits (Passioura 1996). Ludlow (1989) describes three general strategies that plants have evolved to cope with drought stress: escape, avoidance and tolerance. These strategies are defined by the water status of both the plant and its environment. The drought escape strategy is attained through a short life cycle, allowing plants to reproduce before the environment becomes dry. This strategy is often employed in agriculture where breeders select plants that flower and fruit early enough to avoid drought (Passioura 1996, Richards 1996). The drought avoidance strategy refers to plants that maintain internal water status (avoid dehydration) in a dry environment by minimizing water loss and/or maximizing water uptake. The drought tolerance strategy refers to plants in a dry environment, which can survive internal water deficits, with an extreme example being resurrection plants that tolerate losing 95% of leaf water content (Scott 2000).

In all plants CO₂ uptake and water loss to the air (transpiration) both occur through the stomata, pores which allow gas exchange by diffusion between the

environment and the mesophyll. This shared site of exchange results in a trade-off between acquiring CO₂ for growth versus losing water. In habitats where plants encounter seasonal or chronic drought, selection has been shown to favor individuals who are able to minimize the tradeoff between growth and water loss (Dudley 1996), resulting in populations adapted to drought. This trade-off has been credited in the evolution of the variety of strategies for reducing water loss among taxa (Bray 1997; Ingram and Bartels 1996), including the evolution of alternative photosynthetic pathways (CAM and C4), which reduces the coupling of CO₂ uptake from loss of water to the air (Ehleringer and Monson 1993). Here, we focus on drought escape and avoidance in C3 plants. For short-lived annual C3 plants, dehydration avoidance by minimizing water loss via stomatal closure can be a rapid and effective drought avoidance strategy. Stomatal closure is not a sustainable strategy, as stomatal CO₂ uptake is also reduced and will limit photosynthetic assimilation and growth (Schulze et al. 1987).

Water use efficiency (*WUE*) represents this tradeoff between CO₂ uptake and H₂O loss, as the ratio of CO₂ assimilation (*A*) to transpirational H₂O loss (*E*).

$$WUE = \frac{A}{E} = \frac{Ca(1 - \frac{Ci}{Ca})}{1.6v}$$

where *Ca* and *Ci* represent the concentration of CO₂ in the ambient air and inside the leaf, respectively (Condon and Hall 1997). The factor 1.6 is the ratio of diffusivity of water vapor to CO₂, and *v* the vapor pressure difference between the inside of the leaf and the ambient air. Ambient air contains CO₂ mainly comprised of ¹²C but about 1.1% of atmospheric CO₂ includes the stable isotope ¹³C (Farquhar et al. 1989). Plants

discriminate against the heavier isotope, and thus the relative abundance of ^{13}C to ^{12}C is lower in C3 plant tissue than in source air. Because the two isotopes differ in their mass to charge ratio, the $^{13}\text{C}/^{12}\text{C}$ ratio of the tissue relative to a standard (henceforth carbon isotope ratio, or $\delta^{13}\text{C}$) can be quantified on a mass spectrometer, where a more negative $\delta^{13}\text{C}$ reflects greater discrimination. The degree to which plants discriminate against ^{13}C is a function of C_i , and therefore, all things being equal, discrimination against ^{13}C is greater with more open stomates (Virgona and Farquhar 1996). Because $\delta^{13}\text{C}$ is a negative function of C_i/C_a and WUE is a negative function of C_i/C_a (Condon and Hall 1997) under constant C_a and v , there is an expected positive correlation between WUE and $\delta^{13}\text{C}$, which has been confirmed in many empirical studies (Ehleringer and Monson 1993). Genetic variation in $\delta^{13}\text{C}$ is common and is often used as an indicator of variation in integrated WUE for plants grown in a common environment (Farquhar et al. 1989). Variation in $\delta^{13}\text{C}$ reflects variation in C_i/C_a , which may be due to differences in photosynthetic capacity (A_{max}) and/or stomatal conductance. Genetic differences in A_{max} may reflect differences in the capacity for carboxylation or the regeneration of ribulose biphosphate (Farquhar and Van Caemmerer 1981) and may be reflected by nitrogen concentration (Field et al. 1986, Virgona and Farquhar 1996, Lambers, Chapin and Pons 1998).

Flowering time is another important trait related to drought adaptation in both crops and natural systems, where a short life cycle can lead to drought escape (White 1993, Fox 1990). In many annual crops differences among genotypes in $\delta^{13}\text{C}$ are positively correlated with flowering time, suggesting later flowering genotypes may have higher WUE (Hall et al. 1990, Craufurd et al. 1991, Ehadie et al. 1991, Richards and

Condon 1993, Richards 1996). This positive, genetically-based correlation between $\delta^{13}\text{C}$ and flowering time suggests a tradeoff in drought adaptation strategies, with three possible causes: linkage disequilibrium generated by selection on both flowering time and $\delta^{13}\text{C}$, tight linkage between $\delta^{13}\text{C}$ and flowering time loci, or pleiotropy. Although it is plausible that selection on $\delta^{13}\text{C}$ and flowering time might be correlated, evidence for pleiotropy, tight linkage, or linkage disequilibrium is provided by a correlated response to selection (Menendez and Hall 1995). Population genetic models suggest that the ability of linkage disequilibrium to maintain large genetic correlations will increase with inbreeding (Lande 1984). We investigated variation and covariation in $\delta^{13}\text{C}$ and flowering time, in *Arabidopsis thaliana*, an almost entirely selfing annual. These two traits are likely to be important in climatic adaptation and are known to vary among accessions of *A. thaliana* (Neinhuis et al. 1994, Koornneef et al. 1998).

Functional Genetic Variation in *A. thaliana* - *Arabidopsis thaliana* has become the model angiosperm for functional genetics, generating a wealth of genomic information. Molecular population genetic studies in *A. thaliana*, reveal very low levels of within population variation, but large differences among populations at both molecular markers and functional loci (Bergelson et al. 1998, Stephan and Innan, Mitchell-Olds 2001 TREE). These data are consistent with observations of very high levels of selfing (Abbot and Gomes 1989). In comparison to molecular data, there is a dearth of data concerning genetic variation in ecological traits, although research in this area is increasing (reviewed in Pigliucci 1998, Alonso-Blanco and Koornneef 2000). For example, genetic differences among accessions have been found in a number of traits that may be

important in climatic adaptation, including: cold tolerance (Wanner, unpublished), relative growth rate (Li et al. 1998), response to elevated CO₂ (Norton et al. 1995), water use efficiency (Neinhuis et al. 1994, Masle et al. 1993) and flowering time (reviewed in Koornneef et al. 1998 and Juenger et al. 2000). Of these, flowering time has been the subject of the most intense genetic scrutiny. At the molecular level, expression of some of these traits (including drought tolerance and flowering time) is particularly well understood, permitting candidate gene approaches in population studies (Kliebenstein 2001, Thornsberry et al. 2001). Thus, as an experimental system to investigate adaptation to climate in natural populations, *Arabidopsis thaliana* has many outstanding qualities. In most systems, distinguishing pleiotropy from tight linkage is a major experimental challenge (Mather and Jinks 1982). In contrast, near-isogenic lines and mutant knockout lines at candidate loci in *A. thaliana* provide a unique opportunity to examine the genetic basis of trait covariance (see Methods).

The genetics of flowering time in *A. thaliana* has been studied intensively in the last 20 years (reviewed in Koornneef et al. 1998; and Levy and Dean 1998). Progress in dissecting the genetics of flowering time in *A. thaliana* has benefited from a combination of mutant screens and studies of natural variation using common garden studies and QTL mapping (Sanda et al. 1997, Johanson et al. 2000). Of at least 54 loci found to affect flowering time by the knockout approach (Levy and Dean 1998) only *FRIGIDA* (*FRI*) is known to be polymorphic in nature. *FRI* has been shown to have alleles of large effect on flowering time in a number of independent mapping populations and near isogenic lines (Napp-Zin 1957, Sanda et al. 1997, Johanson et al. 2000). *FRI* interacts epistatically with *FLOWERING LOCUS C* (*FLC*), to determine the time to flowering

(Koornneef et al. 1994). It is known that *FLC* encodes a transcription factor, which when up-regulated by *FRI*, acts as a flowering inhibitor but can be offset by cold treatment of the rosette (Michaels and Amasino 2001). Naturally occurring *FRI* null alleles have evolved multiple times (Johanson et al. 2000) and greatly reduce flowering time and the transcription of *FLC* (Michaels and Amasino 1999). In addition, null alleles of *FLC* have been found in lab strains (Ler and C24) and mutant screening for early flowering (Michaels and Amasino 2000).

We first examined variation in $\delta^{13}\text{C}$ and flowering time in 42 natural accessions of *A. thaliana* collected from a wide range of climates. We also use historical weather data from the source location of the accessions to test predicted correlations between traits and environment. We compared this natural variation to $\delta^{13}\text{C}$ and flowering time variation in 8 physiological mutants and a more detailed study examining variation in germination time, flowering time, leaf number at flowering, $\delta^{13}\text{C}$, and percent carbon and nitrogen of leaf tissue in 5 flowering time mutants, and near isogenic lines of a naturally occurring allele at a flowering time QTL. These experiments enable us to examine the spectrum of genetic variation and covariation in traits determining the acquisition and allocation of resources to growth and reproduction.

Methods

Our studies include natural variation, induced phenotypic mutants and near isogenic lines. We first describe these three experiments separately, including the rationale, genotypes used, traits measured, where plants were grown (greenhouse or growth chamber) and statistical analysis. In the final section we provide details of

growth conditions and trait measurement for the greenhouse and growth chamber screenings.

Natural Variation Our first experiment consisted of 42 natural accessions (Table 1) selected from the native range of *Arabidopsis thaliana* in Europe, Asia, and northern Africa, but not North America, where *A. thaliana* has only been recently introduced. Accessions were selected to represent a range of latitudes, elevations and climates (Table 1). In order to examine heritable variation in potentially adaptive traits, genotypes were compared in a common garden experiment in a greenhouse at UC Davis (growth conditions and phenotypic measures are described below).

The greenhouse screening of 42 accessions employed a randomized block design. The effects of block and genotype were considered random. The main effect of genotype was first tested by MANOVA and then tested by individual trait ANOVAs. Genetic correlations among traits were calculated using FreeStat and SAS. In both programs the genetic correlation is calculated as

$$r_g = \frac{\text{Cov}(i, j)}{\sigma_i \sigma_j}$$

where $\text{cov}(i, j)$ is the covariance among ecotype means for traits i and j , and σ_i and σ_j are the square roots of the among ecotype variance components for traits i and j (Robertson 1959, Falconer and MacKay 1996). Estimates of variance components were based on restricted maximum likelihood and carried out in SAS proc mixed.

We used historical climate data from the locations where each genotype was collected to test whether the correlation of $\delta^{13}\text{C}$ and flowering time versus climatic factors is attributable to putative selective pressures. Data on latitude and longitude are

to the nearest 0.5 degree, and were determined using detailed atlas data. For some accessions, the exact geographic origin is not clear and the coordinates are unknown. Data on elevation, and climate was taken from information provided for accessions by the Nottingham Arabidopsis Stock Center and supplemented with data from a long-term gridded climate data set (New et al. 1998). Correlations between traits and habitat variables are Spearman's rank. Genotypes are considered random replicates of adaptation (Felsenstein 1985), an assumption consistent with the well-supported star phylogeny of *A. thaliana* accessions (Innan & Stefan 2000, Sharbel et al. 2000).

Mutational Covariance: In this second experiment we used mutants selected for altered physiology or flowering time to examine patterns of among-trait covariance. We asked whether mutants selected for altered physiology also show altered flowering time in a direction consistent with the genetic correlation. Conversely, do mutants selected for late flowering also show less negative carbon isotope ratios? Physiological mutants were chosen which might alter photosynthetic capacity or stomatal conductance, which in turn affect carbon isotope discrimination (Table 2). Four mutations that affect flowering time, were known from the literature, and are described below.

Physiological mutants were selected based on phenotypes that were considered likely to alter $\delta^{13}\text{C}$. *Abal-4* is a mutant that does not synthesize abscisic acid (ABA), or at least very little (Koornneef et al. 1982). These mutants have much higher levels of transpiration than wild type in both light and dark, due to a lack of stomatal closure, resulting in wilting and death (Koornneef et al. 1982). These mutants require ABA treatment for germination and can be kept alive by bagging the rosette, or feeding the

plants 10 μ M ABA solution (Koornneef et al 1984); we chose the latter. Mutants *abi2-1* and *abi3-1* represent knockouts at two different loci affecting ABA sensitivity (Koornneef et al. 1984). These *abi* mutants both synthesize ABA and thus germinate readily, but show reduced sensitivity of germination and seedling growth to ABA. For *abi2-1* the phenotype extends into the vegetative stage, with increased transpiration and a "wilty" phenotype (similar, but to a lesser degree than *aba1-4*). For the other ABA insensitive mutant *abi3-1*, the ABA phenotype is mainly confined to the seedling stage. In the rosette stage, *abi3-1* has wild type stomatal behavior and ABA sensitivity in the vegetative stage (Koornneef 1984). We also examined a set of mutants at nuclear loci with altered chloroplast size and chloroplast number per mesophyll cell. *Arc1*, *arc2* and *arc3* are mutants with chloroplasts that are 1/2, 2 and 4 times the size of wild type, respectively. We also selected *hcs1* and *hcs2*, mutants at two loci that become chlorotic at high CO₂ (Artus 1990). All of these mutants were ordered from the Arabidopsis stock center in Ohio. These physiological mutants were grown in the greenhouse with the natural accessions.

Flowering time in *A. thaliana* is a highly plastic trait, which responds strongly to day length, seed stratification (cold treatment of imbibed seed) and vernalization (cold treatment of vegetative rosette) in many genotypes (Nordborg and Bergelson 1999). Vernalization is a well-studied environmental factor that decreases flowering time. Many mutations found to influence flowering time are classified by their response to vernalization, leading to the dichotomous classification of genotypes as winter or summer annuals. However, all known genotypes will flower in the absence of vernalization of the

rosette. Day length and light intensity are two environmental factors known to have a large effect on flowering time. We manipulated day length in order to create environmental variance in flowering time within genetic lines, and thus quantify environmentally induced covariance between $\delta^{13}\text{C}$ and flowering time. Four late flowering mutants were selected: *co-1* and *gi*, (both late flowering, vernalization insensitive) and *ld-1* and *fve* (both late flowering, vernalization responsive). All are in the Columbia background, were provided by R. Amasino and are described in detail by Redei (1962), Koornneef et al. (1985) and Michaels and Amasino (2001).

Late flowering mutants were compared in a growth chamber to examine covariance between flowering time and carbon isotope ratio (Table 2). Each genotype was replicated across two-day length treatments, treated statistically as experimental blocks: Block 1 (11 hour days, 3 replicates), (Block 2 - 13 hour days, 4 replicates).

Near Isogenic Lines

For the third experiment, we examined the pleiotropic effects of naturally occurring alleles at the *FRI* locus, a cloned QTL responsible for naturally occurring variation in flowering time. We used near isogenic lines (NILs) consisting of the dominant, functional *FRIGIDA* allele (**FRI**) of the naturally late flowering accession San Feliu-2 (*sf-2*) introgressed into the homozygous background of the Columbia strain (Col) and compared it with the early flowering wild type Col, which carries a null, recessive *FRIGIDA* allele (**fri**). In addition, we examine the effect of the *FLC* genotype, by using NILs where functional alleles **FRI** (*sf-2*) and **FLC** (Col) are introgressed into the

Landsberg erecta background (Ler), which carries a null allele (**flc**) at the *FLC* locus.

These NILs are further described in Lee and Amasino (1995) and Michaels and Amasino (2001) and were provided kindly by R. Amasino.

Statistical Analysis – All traits were analyzed with the following model, which was the best fitting five parameter genetic model for the flowering time data.

$$\text{Trait} = \text{FRI} + \text{FLC} + \text{FRI*FLC} + \text{BLOCK} + \text{GENOME} + \text{error}$$

For the *Frigida* experiments, blocks did not represent a day length manipulation and therefore block was considered a random effect in that analysis. Each effect was tested over the mean square error. In our model fitting we found no evidence of significant FRI*Block, FLC*Block or FRI*FLC*Block interactions. The residuals from this model were normally distributed for all traits but germination (Shapiro-Wilk statistic).

Growth Conditions

Greenhouse - At least 3 seeds of each genotype were sewn into four 250 ml pots filled with a peat soil-vermiculite mixture and 3-5 pellets of osmocote slow release fertilizer. Pots were then randomized into four complete blocks. Blocks were watered, covered with plastic and then placed into a walk-in cooler at 4° C for seed vernalization. After 5 days of cold treatment blocks were transferred to a warm, lighted greenhouse at UC Davis. The average day length (natural sunlight) during the experiment was a little over 10 hours, and supplemental lighting in the greenhouse was used to extend day length to 16 hours throughout the experiment. Supplemental light provided PAR levels of 350 ($\mu\text{mol photons m}^{-2}\text{s}^{-1}$) and maximum levels during full sunlight reached 1800 ($\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Relative humidity throughout the experiment ranged from 29 to 68%,

with a mean of 44%. Air temperature in the greenhouse ranged from 16.8 to 24.2 °C, with a mean of 23.1 °C.

Once transferred to the greenhouse each pot was checked daily for germination. 10 ml of 5 μ molar ABA solution was added weekly to the pots of the ABA synthetic mutant, *aba4* (Koorneef et al. 1984). All plants were kept well watered by bottom watering trays. Once a single germination date was recorded for each pot, subsequent germinants were removed. Replicates (pots) that did not germinate in the first 2 weeks were excluded from the experiment. Plants were checked daily for transition to flowering. When plants began flowering they were harvested at the developmental stage of 1-3 open flowers.

Growth Chamber - Plants were grown in 175 ml capacity pots (Super cell cone-tainers, Stuwe and Son). These pots were filled with UC Davis sunshine mix (peat potting soil and vermiculite)-with 0.625 ml of Osmocote slow release fertilizer. Soil was packed tightly into tubes and then tubes were placed in standing water until saturated. Five seeds of a given genotype were sown directly into the soil at the center of each pot. Once planted, tubes were randomized into the racks made to hold them (Stuwe), covered with black plastic and then cold treated (4 °C) in the dark for 8 days. Following cold treatment, blocks were transferred to an environmental growth chamber (Conviron) at 21/11 °C (day/night) with fluorescent and incandescent lighting, where PAR = 630 μ mol photons $m^{-2}s^{-1}$ at the level of the soil for both day length blocks. Relative humidity was not directly controlled, but ambient Rh in the chamber typically ranged 50/75 (day/night). The soil in each tube was kept moist by bottom watering throughout the experiment. In

addition, to insure adequate moisture for germination, tubes were hand watered with a syringe for the first 10 days in the growth chamber, where 3ml of water was added to the top of each tube per day. Germination was scored daily and only the first germinant per pot was retained for the experiment.

Trait Screening - All plants were harvested at the initiation of flowering. All aboveground biomass was transferred to glass test tubes and oven dried at 60°C (Farquhar et al. 1989). For natural accessions, some plants flowered in as little as 18 days and produced very little biomass. For the 14 plants with less than 1mg of aboveground biomass, all tissue was used for isotope analysis. For all other plants from the greenhouse (natural accessions and physiological mutants), between 0.8 to 1.2 mg of each sample was transferred to a tin combustion cup for isotope analysis. In addition in the growth chamber experiment, we measured percent mass carbon and nitrogen in the leaf tissue, which reflect differences in leaf biochemistry.

In the growth chamber, where maximum PAR(600) was two-thirds lower than in the greenhouse (1800), plants took longer to flower and produced much more biomass. Dried plant tissue was processed for carbon isotope analysis, by subsampling 100-200 mg of crushed leaf material from each individual into a 2ml microfuge tube containing 4 ball bearings, then shaking for one minute on a paint shaker. The result was a uniform powder, from which two subsamples (1mg and 3mg) from each plant were analyzed, to reduce measurement error. Samples were combusted in a Carlo Erba elemental analyzer. The gas produced from combustion was purified using cryogenic traps. Micrograms of Carbon and Nitrogen and are expressed as percentage of the sample mass. Purified CO₂

and N_2 were analyzed for isotope ratios on a dual inlet mass spectrometer at the Stable Isotope facility at UC Davis. Data are presented as carbon isotope ratios relative to the PDB standard (R_{PDB}), where $\delta^{13}C$ (‰) = $(R_s / R_{PDB} - 1) * 1000$ (Hubick et al. 1986). For plants where multiple subsamples were analyzed, the average is used for analysis.

It is worth mentioning why we express all values as carbon isotope ratio (lower case delta), since it is customary in plant ecophysiology to use carbon isotope discrimination, $\Delta^{13}C$ (uppercase Delta). While online measures of $\Delta^{13}C$ are straightforward (Evans 1986, Hubick et al. 1988), for tissue samples converting to $\Delta^{13}C$ requires the assumption a single value for the carbon isotope ratio for the air, which is particularly unlikely in greenhouse and growth chamber experiments. We did take periodic samples of the air inside the chamber and found a large fluctuation in the carbon isotope ratio in the source air, $\delta^{13}C_{air}$ inside the growth chamber. In this case converting to $\Delta^{13}C$ would require a temporal integration of the range of $\delta^{13}C_{air}$ weighted by maximum photosynthesis, A_{max} .

Results

Natural Variation - We found substantial variation in our sample of 42 accessions and significant genetic differences in both $\delta^{13}C$ and flowering time (Table 4). We found a strong positive genetic correlation ($r_G = 0.98$) between $\delta^{13}C$ and flowering time (Figure 1, upper panel).

Spearman's correlation shows the degree to which variation among accessions is related to environmental variables. The strongest relationship (Table 5) was a negative

correlation between spring precipitation and $\delta^{13}\text{C}$. We found no evidence that variation in flowering time or $\delta^{13}\text{C}$ are related to elevation or latitude.

Mutational Co-variance - The mutant lines with altered physiology showed variation in $\delta^{13}\text{C}$, but we found no evidence that these induced physiological mutations have pleiotropic effects on flowering time (figure 1, lower panel). We also used late flowering time mutants in an early flowering background. Figure 2 shows that mutations at loci that alter flowering time also affect $\delta^{13}\text{C}$ in the direction predicted - mutations that increase flowering time also increase $\delta^{13}\text{C}$. This is consistent with the genetic correlation that we found in natural accessions. We found no effect of genotype on germination time, % carbon, % nitrogen and C/N ratio suggesting that the pleiotropy is limited to flowering time, leaf number at flowering and $\delta^{13}\text{C}$ (table 7).

For the flowering time mutants we also manipulated day length. Longer days decreased flowering time and number of leaves at flowering for every genotype. Day length also influenced other measured traits, consistently across genotypes, with the exception of $\delta^{13}\text{C}$ (Table 6). In general, longer days increased germination time, $\delta^{15}\text{N}$, and %C and decreased time to flowering, leaf number at flowering and %N (see table 6 for significance tests). Within lines we found no evidence for a positive correlation of flowering time and $\delta^{13}\text{C}$ (four of five showed a non-significant negative correlation) suggesting environmental covariance does not contribute to the observed pattern.

Near Isogenic Lines - *FRIGIDA* and *FLC* NILs showed dramatic pleiotropic effects (figure 3, Table 7). The introgression of the late flowering **FRI** allele into a genotype

with a functional *FLC* increased flowering time and also caused an increase in $\delta^{13}\text{C}$. The genetic correlation in the NILs was 0.97, nearly identical to that found in the natural accessions. Our analysis (Table 10) shows that the *Frigida* genotype had significant effects on flowering time, leaf number at flowering, $\delta^{13}\text{C}$ and %C. *FLC* genotype had significant effects on flowering time and $\delta^{13}\text{C}$. The interaction, *FRIxFLC* only has a significant effect on flowering time. A significant effect of background genome also had a significant effect on flowering time and $\delta^{13}\text{C}$ (figure 3), suggesting the presence of (an) additional loci (locus, perhaps *erecta*) affecting these traits. The effect of block, which in this case represents unknown environmental effects, had a significant effect on all traits but flowering time. Within each NIL, there was substantial plastic variation in all traits but flowering time and $\delta^{13}\text{C}$ (Table 9). We found no evidence for a positive correlation of flowering time and $\delta^{13}\text{C}$.

Discussion

We found substantial genetic variation in ecologically important traits, and some of this variation is consistent with a hypothesis of adaptation. In particular, genetic variation in stable carbon isotope ratio was significantly correlated (table 5) with spring precipitation in the native habitat. This is consistent with the prediction that in dry climates plants have been selected for increased WUE, thus reducing *Ci/Ca* and decreasing discrimination against ^{13}C (increasing $\delta^{13}\text{C}$). Our data are consistent with the prediction that greater $\delta^{13}\text{C}$ is under selection in dry sites. Our most striking result was finding a large positive genetic correlation between flowering time and $\delta^{13}\text{C}$ among

naturally occurring ecotypes. This genetically based tradeoff in drought adaptation strategies suggests three possible causes: correlated selection, tight linkage or pleiotropy.

We tested for pleiotropy by examining mutational covariance introduced by alleles of known effect on one trait. Most mutants had some effect on $\delta^{13}\text{C}$, whether they were physiological or flowering time mutants. This was expected for physiological mutants, which showed no evidence for pleiotropy on flowering time (Figure 1, lower panel). These physiological mutations break up the tight positive genetic correlation observed among accessions, and nearly all reduce $\delta^{13}\text{C}$. The exception were the ABA insensitive mutants, in particular *abi3-1*, a transcription factor that mediates ABA regulated gene expression in seeds and response to cold/drought stress and is homologous to the seed germination locus, *vp-1* in maize (Girudat et al. 1992, Tamminen et al. 2001).

Our growth chamber screening of late flowering mutants and their earlier flowering wild type revealed evidence for pleiotropy, in the form of mutational covariance between $\delta^{13}\text{C}$ and flowering time (Figure 2). Three of four mutations described with a flowering time phenotype also show an effect on $\delta^{13}\text{C}$ in at least one photoperiod (Figure 2). It appears that the degree of pleiotropy depends on the environmental conditions (Fry 1996). We observed pleiotropy only in some flowering time mutants and not in the physiological mutants, showing that the mutational covariance is far from absolute, and depends on the particular genotypes and environments.

Stronger evidence for pleiotropy comes from the genetic covariance caused by *FLC* and *FRI* alleles, which are known to be responsible for natural variation in flowering time. Thus our data suggest that population differences in flowering time may involve

alleles that pleiotropically affect drought physiology. Although we did not find evidence for genetic variation in flowering time being correlated with environmental variables, it is reasonable to expect both flowering time and water-use physiology to experience episodes of stabilizing and/or directional selection.

In addition to the carbon cost, data suggest flowering also has a substantial water cost. Flowers are the least waterproof structures on most plants, evidenced by a positive correlation between water uptake and corolla area (Galen et al. 1999). In addition, because flower petals lack stomata, transpiration per area is much greater for petals than leaves during periods of high evaporative demand, when leaves respond by reducing stomatal conductance (McKay unpublished).

A positive genetic correlation between flowering time and $\delta^{13}\text{C}$ has also been shown in a number of agricultural species, including barley (Crauford et al. 1991), common and tepary bean (White 1993), cowpea (Menendez and Hall 1995), and wheat (Ehdaie et al. 1991). In cowpea, later flowering is often an unwanted correlated response to selection for reduced transpiration, suggesting pleiotropic effects of mutations that went to fixation while screening for reduced stomatal conductance or water loss (Menendez and Hall 1995). From a simple physiological argument, reduced stomatal conductance will result in a lower rate of carbon fixed during photosynthesis, and this alone may explain increases in the time required to acquire sufficient resources to flower. In *A. thaliana*, later flowering results in greater fecundity in some environments (Mitchell-Olds 1996). For *A. thaliana* plants exposed to drought stress, genetic differences, in mean phenotype and the degree of plasticity of flowering time, are important components of fitness (McKay, unpublished data).

Genetic and environmental contributions to covariance - All plants made the transition to flowering in our greenhouse conditions or growth chamber conditions without vernalization of the rosette, and at all day lengths. Not surprisingly we found a large effect of day length on flowering time. In the greenhouse, plants of a given genotype flowered much earlier than plants in the growth chamber. Since all of these plants were harvested at bolting, developmental age at sampling might cause differences in $\delta^{13}\text{C}$. We can estimate such environmental (non-genetic) contributions to trait covariance by looking within lines, where replicates within genotypes differ in both traits for environmental or ontogenetic reasons. If older plants discriminate less per se, then within genotypes, $\delta^{13}\text{C}$ should be correlated with later flowering. No such correlation was found within genotypes, in the natural accessions, EMS mutants or NILs. This is most clearly demonstrated in experiment 2 (late flowering mutants), where manipulation of day length had the largest effects on leaf number, flowering time, and leaf nitrogen, and the least effect on carbon isotope ratio (Table 5). Clearly, therefore, these observed patterns are determined primarily by genetic rather than environmental covariation.

We are primarily interested in genetic variation in $\delta^{13}\text{C}$. Assuming variation in $\delta^{13}\text{C}$ largely reflects variation in C_i/C_a , this may be due to genetic differences in photosynthetic capacity (A_{max}) and/or stomatal conductance. In our screening of flowering mutants we found mutational covariance between flowering time and $\delta^{13}\text{C}$ but not %N. However the NILs do show a strong negative genetic correlation between $\delta^{13}\text{C}$ and %N, which may reflect differences in photosynthetic capacity. However, stomatal conductance is not expected to be constant (Cowan and Farquhar 1977), and is influenced

by A (Wong et al 1979) in part because stomates are sensitive to C_i , being induced to open by low CO_2 (Leymarie 1998). Periods of maximal assimilation, A_{max} , which can be limited by stomatal conductance, will have the largest effect on $\delta^{13}C$, which integrates over time and over all tissue sampled. We are currently using measures of instantaneous gas exchange to examine differences among genotypes in stomatal conductance, transpiration, photosynthetic rates and WUE (Virgona and Farquhar 1996). These data indicate that NILs with a functional *FRI* and *FLC* have reduced transpiration (data not shown).

It is noteworthy that there seems to be a minimal (non-significant) effect of longer days increasing $\delta^{13}C$. One explanation is that longer days means a shorter nights, and thus may increase $\delta^{13}C$ (integrated WUE) by decreasing the amount of carbon respired at night (Farquhar et al. 1989). The situation may be more complicated as differences in tissue $\delta^{13}C$ may reflect fractionation due to: diffusion and biochemical discrimination during photosynthesis (Rubisco and PEP) as well as other forms of biochemical discrimination. For example, dark respiration has been shown to produce CO_2 enriched in ^{13}C in *Nicotiana sylvestris* and *Phaseolus vulgaris*, and in turn create a more negative $\delta^{13}C$ (Duranceau et al. 2001). If *A. thaliana* also releases CO_2 enriched in ^{13}C during respiration, this mechanism is sufficient to explain the (non-significant) effect of longer days (shorter periods of nighttime respiration) resulting in less negative $\delta^{13}C$.

Pleiotropy, Adaptation and the 2010 project - Mutant screening and large scale sequencing in *Arabidopsis* have identified many genes which are thought to play a role in drought adaptation. In *A. thaliana* it is now possible to identify all gene products that are

expressed when exposed to drought stress, indicating that they are involved in the biochemical pathways that regulate water stress responses (Seki et al. 2001). These assays of gene expression can be combined with a biochemical mutant screening approach to elucidate the function of these genes (Pesaresi et al. 2001). Identifying the genes involved in each biochemical pathways is a tremendous task, and an important component in attempting to understand the water relations of plants. However, mutant screens by necessity focus on a limited number of traits. In all of our mutant lines we found correlated effects on other traits. This phenomenon was also demonstrated recently by Boyes et al. (2001), where five biochemical pathway mutants resulted in substantial effects on life-history traits. In addition, QTL studies of nearly any trait in the Col x Ler or Ler X Cvi recombinant inbred lines have found some effect of the induced mutation *erecta* (personal communication). Other examples of pleiotropy can be found in almost every detailed description of mutants. This is expected as most mutant screens impose strong directional selection on particular traits, and minimal selection for fitness traits. Most mutations will have pleiotropic effects on fitness (Fisher 1958). In natural populations, the spectrum of possible mutational effects have been filtered of mutations with deleterious pleiotropic effects by natural selection.

In order to take full advantage of these candidate genes, it would be very informative to examine natural populations. This would allow us to determine which genes, out of all the possible genes in each biochemical pathway, have actually been selected in natural populations. By using this approach it is possible to find a subset of the estimated 25,000 loci in *A. thaliana*, which have changed without deleterious

pleiotropic effects on fitness. Secondly, identifying loci that contribute to trait variation and are polymorphic can provide data on the genetic basis of adaptation, information that is fundamental to our understanding of evolution and biodiversity.

Detailed work has shown that the *FRIGIDA* locus is responsible for naturally occurring variation in flowering time and leaf number at flowering. Although continuously distributed, flowering time is often analyzed as a categorical trait (late or early), in which case **FRI** behaves as a dominant allele for late flowering. Categories for the dichotomy early and late are arbitrary and vary across experiments, and mask the effects of other loci. A great number of QTL have been identified, but we are not aware of any besides *FRI* that have been mapped and cloned. Interestingly, out of over 50 loci shown to affect flowering time using a knockout approach, early flowering in natural accessions has evolved to a large degree by at least 3 independent loss of function mutations at the *FRI* locus. This suggests that mutations at the *FRI* locus may be less likely to have deleterious effects on fitness. Here we have shown that one of these naturally occurring alleles **FRI** (sf-2), also has a significant effect on $\delta^{13}\text{C}$ and %C. A significant effect of the *FLC* genotype on $\delta^{13}\text{C}$ further implies that this may be due to differences in *FLC* expression (Michaels and Amasino 2001).

Many population genetic models assume that most mutations with a large phenotypic effect will be deleterious due to pleiotropic effects at other traits influencing fitness. A recent mutation accumulation experiment in *A. thaliana* (Shaw et al. 1999), found the distribution of mutational effects symmetric (new mutations both increasing and decreasing fitness as measured in the lab). In addition they found positive mutational covariance, where mutations either increased or decreased all traits. We found a similar

pattern due to one naturally occurring alleles at the *FRI* locus. It would be interesting to determine if other functional or null alleles at *FRI* also have similar pleiotropic effects.

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Table 1- 42 Natural accessions used in Experiment 1. Data are accession number, name, geographic location, latitude North, longitude and elevation of collection site. Mean $\delta^{13}\text{C}$ and flowering time of each genotype in our greenhouse screening are also shown.

Acc #	ecotype	Collection Site	Lat N	Long	Elev (m)	delta 13 C	FlowerTime
900aa-0		Rhon, Germany	51	E 1	200	-29.7	22.7
901ag0		Argentat, France	45	E 1.	100	-30.5	21.8
902cvi-0		Cape Verde Islands	16	W 2	1200	-30.5	22.5
903kas-1		Kashmir, India	35	E 7	1580	-28.2	31.7
904mh-0		Muhlen, Poland	53.5	E 20.	100	-31.2	19.0
905ms-0		Moscow, Russia	55.5	E 3	100	-28.9	31.7
910di-g		Dijon, France	47.5	E	300	-30.5	20.4
911Est		Estland, Poland	n/a	n/	n/a	-30.180	19.3
915ws-3		Wassilewskija	52	E 3	n/a	-31.0	21.0
916Kondara		Tajikstan	n/a	n/	n/a	-28.9	29.3
917da(1)-12		Czechoslovakia	18	E 4	n/a	-30.6	20.0
922hodja-obi-garm		Tajikstan	39	E 70.	n/a	-29.9	25.7
924je54		Relichova, Czechoslovakia	n/a	n/	n/a	-30.5	22.5
926petergof		Russia	60	E 3	n/a	-31.0	23.0
927rubezhnoe-1		Ukraine	52	E 3	n/a	-29.8	26.7
930sn(5)-1		Czechoslovakia	n/a	n/	n/a	-30.6	19.8
931sorbo		Tajikstan	n/a	n/	n/a	-29.0	32.0
944an-1		Antwerpen, Belgium	51.5	E 4.	1	-29.8	19.8
958bch-3		Buchen, Germany	53.5	E 10.	100	-29.7	27.0
1064can-0		Canary Islands	28	W 15.	700	-28.9	32.0
1102db-1		Tenne, Germany	60	E 3	400	-30.4	20.8
1122edi-0		Edinburgh, UK	56	W	100	-29.1	32.0
1126ei-4		Eifel, Germany	50.5	E 6.	400	-29.4	25.7
1136en-1		Enkheim, Germany	50	E 8.	100	-30.2	19.5
1152et-0		Etraygues, France	44.5	E 2.	400	-28.7	32.0
1252jl-3		Vranov (Brna), Czech	n/a	n/	460	-30.4	18.7
1266ka-0		Kamten, Austria	47	E 1	900	-30.6	22.0
1374mrk-0		Markt, Germany	49	E 9.	200	-29.9	27.5
1454pi-0		Pitztal (Tirol), Austria	47	E 1	1000	-29.5	24.0
1482rd-0		Rodenbach, Germany	49.5	E 9.	500	-30.4	22.8
1494Rsch-4		Rschew, Russia	56.5	E 3	100	-30.1	22.5
1504sei-0		Seis Am, Italy	46.5	E 11.	1000	-30.6	19.5
1548ta-0		Tabor, Czecho	49.5	E 14.	400	-30.7	22.8
1630wl-0		Wilbad, Germany	n/a	n/	500	-30.6	23.3
1638ws-3		Wassilewskija	37.5	E 6	150	-30.1	25.0
1639wei-1		Weiningen, Switz	n/a	n/	n/a	-30.8	19.7
1640tsu-1		Tsu, Japan	34.5	E 136.	1	-30.6	25.5
1641rld-2		Rschew, Russia	56.5	E 3	n/a	-30.6	21.5
1643oy-1		Oytese, Norway	46.5	E 11.	1	-29.7	22.8
6175Condara		Tajikstan	n/a	n/	n/a	-29.5	26.0
6180shahdara		Tajikstan	37.5	E 6	3400	-30.6	20.5
PU2:24		n/a	n/a	n/	n/a	-28.9	31.7

Table 2 - List of mutants used in Experiment 2 to test for mutational covariance.

Access. number	Homozygous mutant allele	Genome	Described Phenotype	Predicted trait effect	Reference
6172	WT - Col	col	Early flowering		
86	WT - Ler	ler	Early flowering		
23	abi2-1	ler	abscisic acid sensitivity	delta	Koornneef et al. 1984
24	abi3-1	ler	abscisic acid sensitivity	delta	Koornneef et al. 1984
3101	aba1-4	ler	abscisic acid synthesis	delta	Koornneef et al. 1982
262	arc1	ler	50 % higher chloroplast density	delta	Pyke and Leech 1992
263	arc2	ler	50 % lower chloroplast density	delta	Pyke and Leech 1992
264	arc3	ler	7-8 x lower chloroplast density	delta	Pyke and Leech 1992
6169	hcs1	col	high CO2	delta	Artus 1990
6171	hcs2	col	high CO2	delta	Artus 1990
*	co-1	col	Late flowering, vernal insensitive	flowering	Redei 1962
*	gi	col	Late flowering, vernal insensitive	flowering	Michaels and Amasino 2001
*	ld-1	col	Late flowering, vernal responsive	flowering	Redei 1962
*	fve	col	Late flowering, vernal responsive	flowering	Koornneef et al. 1985

* - see methods

Table 3 - Near isogenic lines used in experiment 3.

Genotype	genome	Frigida	FLC	Expected phenotype	reference
Columbia	Col	0	1	Early flowering	
FRI[sf2]	Col	1	1	Late flowering	Lee and Amasino 1995
Ler	Ler	0	0	Early flowering	Lee and Amasino 1995
FRI[sf2] fci[Ler]	Ler	1	0	Early flowering	Lee and Amasino 1995
FRI[sf2] FLC[Col]	Ler	1	1	Late flowering	Lee and Amasino 1995

Table 4 - Analysis of variance for flowering time and $\delta^{13}\text{C}$ based on 141 plants representing 42 accessions (Experiment 1). Effects of genotype and block were considered random. Percent variance are based on variance components estimated using restricted maximum likelihood. The ratio of genetic to total phenotypic variation for the traits was flowering time $V_G/V_T=0.48$, $\delta^{13}\text{C}$ $V_G/V_T=0.59$

Flowering Time	df.	MS	%Var	F	P <
Geno (a)	41	40700.1	47.9	4.523	0.000000001
Block (b)	3	56430.0	7.6	6.270	0.001
Geno x Block (c)	95	9017.7	36.7	6.076	0.33
Error	1	1484.0	7.8		

$\delta^{13}\text{C}$	df	MS	%Var	F	P <
Geno (a)	41	1.7	58.7	5.836	0.000000000001
Block (b)	3	0.7	1.8	2.300	0.083
Geno x Block (c)	95	0.3	27.1	2.817	0.45
error	1	0.1	12.4		

a = 0.997 MS(GENO * BLOCK) + 0.002604 MS(Error)
b = 0.998 MS(GENO * BLOCK) + 0.002303 MS(Error)
c = MS(Error)

Table 5 Spearman's correlation coefficient (rho) of habitat variables versus $\delta^{13}\text{C}$ and flowering time. Significance tests are based on a two-tailed distribution and are not corrected for multiple tests.

EDAPHIC VARIABLE	N=	$\delta^{13}\text{C}$	P <	FLOWER TIME	P <
SPRING TEMP	27	-.135	.503	-.153	.445
FALLTEMP	27	-.194	.332	-.039	.845
SPRRAIN	17	-.526	.030	-.239	.355
FALLRAIN	16	-.180	.506	.051	.853
ELEV	37	-.013	.938	.006	.974
LATT	34	-.202	.251	-.312	.072

Table 6 - Phenotypic effects of four flowering time mutants (Experiment 2). Shown are means for each genotype in both photoperiods. Photoperiod effects are tested by comparing blocks within genotypes using 2-tailed t-tests, without assuming equal variance. P-values < 0.001 are represented by *** and remain significant after a strict Bonferroni controlling for 35 t-tests.

Trait	Germ time	Germ time	Flw time	Flw time	Leaf #	Leaf #	$\delta^{15}\text{N}$	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	$\delta^{13}\text{C}$	%N	%N	%C	%C
Day	11	13	11	13	11	13	11	13	11	13	11	13	11	13
Col	1.0	2.3	*	31.3	27.7	*	37	24	*	0.37	3.64	*	-33.8	-33.7
co-1	1.0	2.7	*	30.7	26.0	*	69	23	**	1.27	4.49	ns	-34.8	-33.7
Gi	1.0	3.0	*	40.0	31.7	*	35	29	-	1.06	4.69	**	-33.4	-33.7
Fve	1.0	3.0	*	35.3	28.5	*	40	26	***	1.29	3.71	***	-34.7	-34.5
ld-1	1.0	2.0	-	46.7	44.5	ns	78	34	-	1.36	2.85	**	-33.4	-32.8

* 0.1, **0.01, ***0.001

Table 7 - Statistical analysis of flowering time mutations (Experiment 3). All traits were analyzed in separate univariate ANOVAs, with genotype and block (=photoperiod) considered fixed effects. Bold P-values indicate significance following a strict Bonferroni controlling for multiple traits. Percent variance is based on variance components estimated using REML, assuming all factors to be random.

Genotype	df	Mean Square	% Variance	F	Sig (P <)
GERMTIME	4	0.261	0.0	0.7	0.622
FLWTIME	4	251.396	70.5	52.6	0.000000005
LEAF #	4	409.347	6.0	15.5	0.00005
N_DELTA	4	0.74	0.0	0.9	0.492
C_DELTA	4	2.019	41.6	6.5	0.005
PERC_N	4	8.174E-05	0.0	0.8	0.512
PERC_C	4	3.870E-04	0.0	1.7	0.200
Photoperiod					
GERMTIME	1	17.093	78.2	43.8	0.000005
FLWTIME	1	192.813	20.3	40.4	0.000005
LEAF #	1	2836.688	55.5	107.2	0.00000005
N_DELTA	1	56.297	78.8	51.0	0.000005
C_DELTA	1	0.876	4.2	2.8	0.108
PERC_N	1	5.381E-03	80.3	55.7	0.0000005
PERC_C	1	1.049E-02	70.6	44.8	0.000005
G x E					
GERMTIME	4	0.261	0.0	0.7	0.622
FLWTIME	4	8.554	1.9	1.8	0.170
LEAF #	4	375.822	32.9	14.2	0.0005
N_DELTA	4	0.943	0.0	0.8	0.508
C_DELTA	4	0.433	6.2	1.4	0.270
PERC_N	4	3.271E-05	0.0	0.3	0.848
PERC_C	4	4.015E-04	5.6	1.715	0.186
Error					
GERMTIME	19	0.390	21.8		
FLWTIME	20	4.775	7.4		
LEAF #	15	26.456	5.6		
N_DELTA	20	1.104	21.2		
C_DELTA	20	0.309	47.9		
PERC_N	20	9.652E-05	19.7		
PERC_C	20	2.341E-04	23.8		

Table 8 - Phenotypic effects of *FRIGIDA* and *FLC* loci, introgressed into isogenic Col and Ler backgrounds. Shown are genotype means for each genotype. The effects of NILs were tested separately in the Col and Ler background and considered fixed effects. Contrast indicates if NILs differ significantly from WT for each trait. Bold *P*-values indicate significance following a strict Bonferroni controlling for multiple-traits.

Background Genome NIL alleles	Germination Time (days)	Flowering Time (days)	Leaf #	$\delta^{13}\text{C}$	% C	% N
Columbia						
Col WT fri[col] FLC[col]	1.625	29.500	30.542	-33.76	40.1	3.35
FRI[sf2] FLC[col]	1.750	50.833	39.000	-32.56	43.2	1.56
	Contrast vs. WT	2-tailed P <				
FRI[sf2] FLC[col]	0.6	0.00001	0.06	0.01	0.01	0.005
Landsberg erecta						
Ler WT fri[col] flc[col]	2.500	27.708	27.375	-34.72	39.8	4.42
FRI[sf2] FLC[Col]	2.000	73.333	>>	-31.27	42.7	1.28
FRI[sf2] flc[Ler]	2.125	31.125	34.250	-34.27	41.3	3.84
	Contrast vs WT	2-tailed P <				
FRI[sf2] FLC[Col]	0.3	0.00000001		0.0000005	0.005	0.005
FRI[sf2] flc[Ler]	0.4	0.25	0.01	0.2	0.09	0.5

Table 9 - Percentage of variance in each trait explained by genetic differences. Frigida (FRI, FLC and FRIxFLC), genome (WT background) and total (Frigida + genome)

Trait	FRI-FLC	GENOME	TOTAL
germ	2.7	2.6	5.2
flwtime	70.2	27.0	97.2
Leaf #	27.2	0	27.2
delta_n	0	0	0
delta_c	71.9	17.7	89.5
perc_n	39.0	0	39.0
perc_C	21.8	0	21.8

Table 10 - Mixed-model ANOVA of Near Isogenic lines of *FRIGIDA* and *FLC* loci introgressed into isogenic Col and Ler backgrounds. The model used was the best-fitting 5 parameter model for the flowering time data. Genotype at *FRI* and *FLC*, and the interaction *FRI* \times *FLC* were considered fixed effects. Block and Genome (Ler or Col) are treated as random effects. All F-ratios represent the factor MS, tested over the error. Bold P-values indicate significance following a strict Bonferroni controlling for multiple-traits. Percent variances are based on variance components estimated using REML, assuming all factors to be random.

Factor	Trait	df	Mean Square	% Var	F	Sig (P <)
FRI	GERMTIME	1	0.14	0.0	0.29	0.6
	FLWTIME	1	979.08	4.1	40.54	0.000005
	Leaves	1	390.25	17.2	19.5	0.0005
	N_DELTA	1	7.05 x 10 ⁻²	0.0	0.03	0.869
	C_DELTA	1	4.53	5.1	12.38	0.002
	PERC_N	1	7.90 x 10 ⁻⁴	9.3	5.54	0.027
	PERC_C	1		18.4	9.28	0.005
FLC	GERMTIME	1	0.59	2.7	1.21	0.281
	FLWTIME	1	2954.88	57.0	122.34	0.00000000005
	Leaves	0		6.8		
	N_DELTA	1	1.08	0.0	.42	0.521
	C_DELTA	1	20.23	64.4	55.33	0.0000001
	PERC_N	1	8.60 x 10 ⁻⁴	25.1	6.03	0.021
	PERC_C	1	6.68	3.4	.20	0.657
FRI x FLC	GERMTIME	1	0.59	0.0	1.21	0.281
	FLWTIME	1	526.16	9.2	21.79	0.0001
	Leaves	1	74.952	3.2	3.75	0.070
	N_DELTA	1	0.35	0.0	.136	0.716
	C_DELTA	1	1.04	2.3	2.84	0.104
	PERC_N	1	2.35 x 10 ⁻⁴	4.6	1.65	0.211
	PERC_C	1	3.37 x 10 ⁻⁴	0.0	1.02	0.322
BLOCK	GERMTIME	1	33.43	76.4	68.25	0.00000001
	FLWTIME	1	24.86	0.0	1.03	0.320
	Leaves	1	1686.92	64.0	84.29	0.0000001
	N_DELTA	1	57.08	60.9	22.32	0.0001
	C_DELTA	1	3.60	3.9	9.85	0.004
	PERC_N	1	2.82 x 10 ⁻³	34.4	19.77	0.0005
	PERC_C	1	9.13 x 10 ⁻³	50.3	27.66	0.00005
Genome	GERMTIME	1	0.26	2.6	0.54	0.470
	FLWTIME	1	1266.28	27.0	52.43	0.0000005
	Leaves	0		0.0		
	N_DELTA	1	6.60 x 10 ⁻²	0.0	0.03	0.874
	C_DELTA	1	5.12	17.7	14.00	0.001
	PERC_N	1	1.48 x 10 ⁻⁶	0.0	0.01	0.920
	PERC_C	1	1.40 x 10 ⁻⁴	0.0	0.42	0.522
Error	GERMTIME	28	0.49	18.4		
	FLWTIME	25	24.15	2.8		
	Leaves	17	20.01	8.8		
	N_DELTA	25	2.56	39.1		
	C_DELTA	25	0.37	6.6		
	PERC_N	25	1.43 x 10 ⁻⁴	26.6		
	PERC_C	25	3.30 x 10 ⁻⁴	27.9		

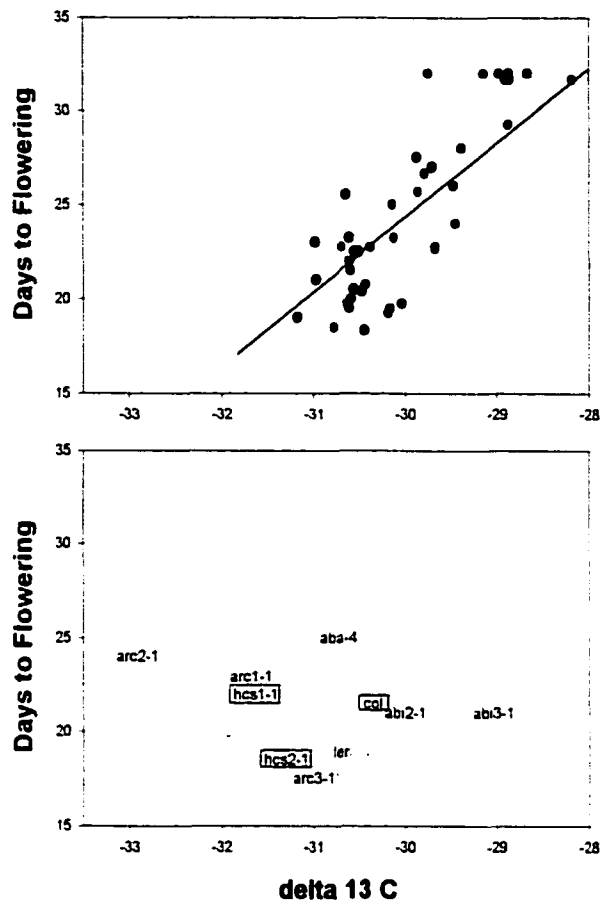


Figure 1 Variation and covariation in flowering time among natural populations (upper panel) and physiological mutants (lower panel). Large points and labels are genotype means. Small points are individual values, and shown to indicate the overall patterns of phenotypic and genetic (co)variance. Top panel shows the positive genetic covariance between days to flowering and carbon isotope ratios in 42 accessions. The genetic correlation, $r_g = 0.98$ (calculated as described in methods), is larger than the phenotypic correlation $r_p = 0.62$ and the correlation among genotype means, $r = 0.84$. All three were highly significant ($P < 0.00001$). The lower panel shows a lack of mutational covariance in physiological mutants, which have more negative $\delta^{13}\text{C}$, but do not differ in flowering time. Genotypes in boxes are in the Columbia background, all other are in the Landsberg *erecta* background. In this population of physiological mutants, the two traits are not significantly correlated phenotypically or genetically.

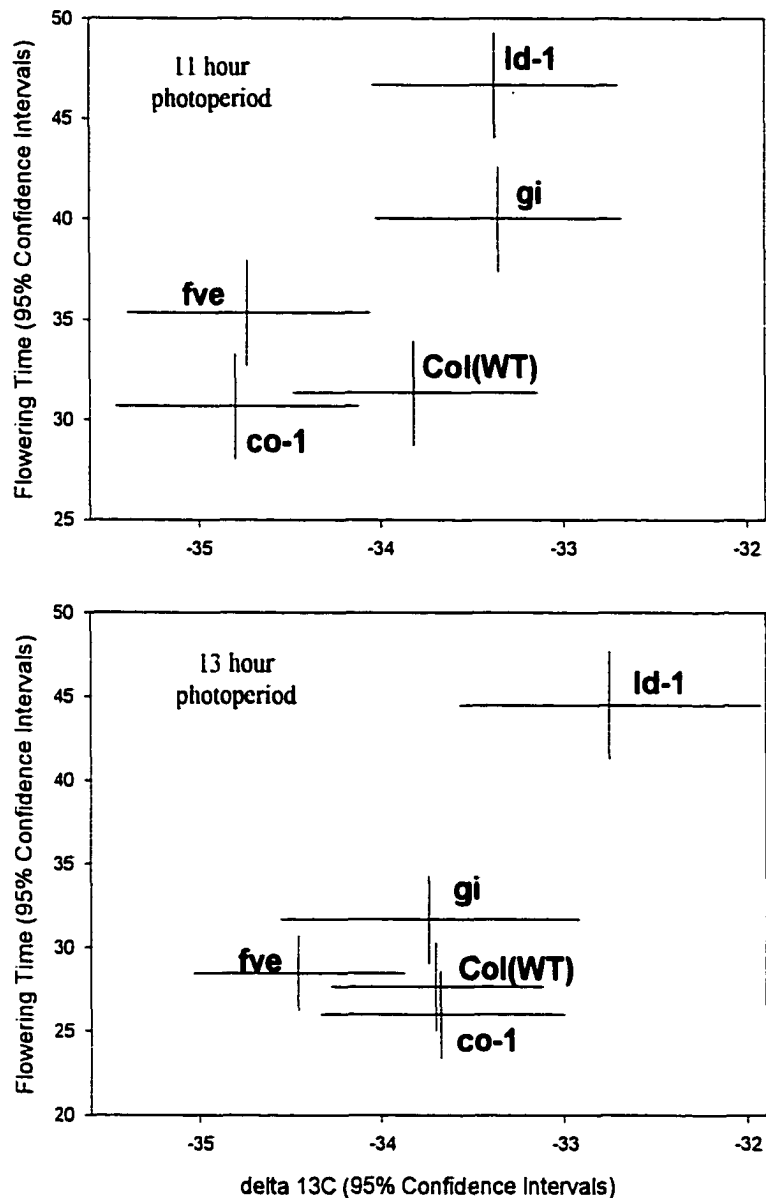


Figure 2 - Effects of EMS flowering time mutants on both flowering time and carbon isotope discrimination, in two environments differing in daylength. Bars represent individual 95% confidence intervals, based on the ANOVA. The top panel shows the phenotype effect of each mutant and the positive mutational covariance under 11/13 (day/night). The bottom panel shows the phenotypic effect and positive mutational covariance under slightly longer days, 13/11 (day/night). Comparing the phenotype of each mutant in upper to lower panel shows that longer days significantly decrease flowering time, and increase carbon isotope discrimination, although the latter effect is not significant (Table 7).

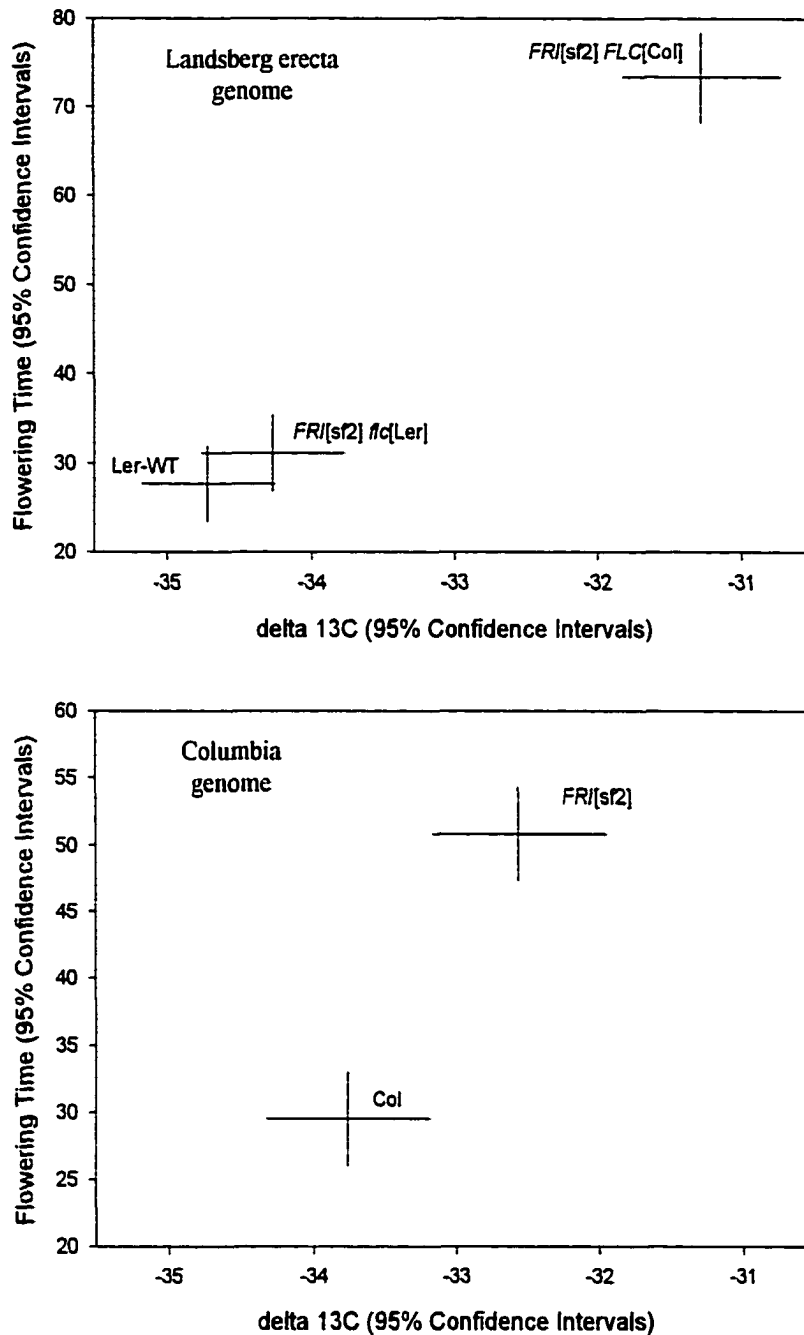


Figure 3 - Effects of *Frigida* and *FLC* genotypes on flowering time and $\delta^{13}\text{C}$, in two genetic backgrounds. Top Panel shows the phenotype of NILs in a *Landsberg erecta* background, where the effect of the late flowering *Frigida* allele depends on the genotype at the *FLC* locus (Michaels and Amasino 2000). The bottom panel shows the phenotypic effect of the *Frigida* allele in the Columbia background. Comparing the upper rightmost genotype across panels (at the same scale) shows that epistatic interaction between *FRI* and *FLC* has a larger effect on both traits in the Ler background.

**Genetic divergence among vertebrate populations is concordant
with the latitudinal gradient in species diversity.**

John K. McKay and Paul R. Martin

Abstract. – The increase in biological diversity from temperate to tropical regions may result from a greater number of speciation events at lower latitudes. If speciation events occur more frequently in the tropics, we predicted that genetic divergence of populations, an important component of cladogenesis, should be greater among lower latitude populations. We tested this prediction using within-species patterns of mtDNA variation across 31 vertebrate taxa. We found that genetic divergence of populations, controlling for geographic distance, is greater at lower latitudes, indicating a greater potential for future speciation. Results further suggest that evolutionary processes may differ between high and low latitudes.

Key words. – Latitudinal variation, species richness, genetic divergence, isolation x distance, speciation.

The spectacular increase in biological diversity from temperate to tropical regions is well known across taxa and spatial scales (Wallace 1878; Dobzhansky 1950; Fischer 1960; Pianka 1966; Rhode 1992; Rosenzweig 1995; Gaston 2000). This latitudinal gradient in species richness is present in the fossil record as far back as the Triassic Period (Jablonski 1993) and is evident across a diverse array of taxa and environments (Wallace 1878; Dobzhansky 1950; Fischer 1960; Pianka 1966; Rhode 1992; Rosenzweig 1995; Gaston 2000). It reforms after decimation by mass extinction events (Stehli et al. 1967), and is independently present across taxonomic levels, including family, genus, species and subspecies. Despite the robustness of this pattern, the latitudinal gradient in taxonomic richness remains one of the broadest but least understood patterns in biology (Rhode 1992; Futuyma 1998; Gaston 2000). Researchers have proposed many hypotheses for the origin and maintenance of tropical diversity (Pianka 1966; Rhode 1992; Gaston 2000), but hypotheses have generally neglected tests of microevolutionary processes that generate the pattern (but see Rhode 1992). Here we examine the hypothesis that speciation events occur more frequently at lower latitudes -that is to say, low latitudes are likely to have more speciation events per unit of time (Fischer 1960; Pianka 1966; Rhode 1992).

Allopatric speciation is thought to be the major mode of speciation for vertebrates and other taxa (Mayr 1963; Futuyma 1998; Barraclough and Vogler 2000; Coyne and Price;

Turelli et al. 2001). If species originate at a greater frequency in the tropics, we predicted, *a priori*, that populations within species should show greater genetic isolation at lower latitudes because genetic divergence is an important component of allopatric speciation (Mayr 1963; Slatkin 1987; Futuyma 1998; Barraclough and Vogler 2000; Turelli et al. 2001). By using this method we are examining cladogenesis resulting from populations or groups of populations diverging from one another. We are not concerned with the rate of lineage divergence, as is typically measured by phylogenetic studies examining the diversification of lineages (cite), although this may also contribute to latitudinal patterns of species richness.

METHODS

The great majority (80%) of phylogeographic studies are based on mtDNA (Avice 1998). We found that most of the published mtDNA phylogeographic studies contained insufficient data for our analysis (see also Leberg and Neigel 1999); in most cases the data missing were the precise sampling locations of individuals. We were able to find suitable data from a diverse array of vertebrate taxa (birds, fishes, mammals, and herps), environments (marine, freshwater, and terrestrial), continents and oceans across a broad span of latitudes (Table 1) to test the prediction that lower latitude populations within species exhibit increased genetic differentiation, after controlling for geographic separation. We reviewed studies on the population genetics of vertebrates that examined 200 or more base pairs of mtDNA, using either sequences or haplotypes. We limited our analysis to studies with at least 3 individuals per population and 4 populations per species,

spanning at least 8 degrees latitude, and where the necessary genetic data were publicly available. For each study that met our criteria, we separated populations into two groups - high latitude and low latitude - by dividing the latitudinal span of the populations by two. (In three cases, this resulted in only one population representing high or low latitudes. In these three cases, the lone population was compared to the population closest in latitude, with all other populations compared among each other in the opposite latitudinal group. Results are still significant if these species are excluded from the analysis.) We then calculated pair-wise estimates of genetic divergence [genetic distance or F_{ST} / geographic distance between populations (km)], and compared mean values between low and high latitude groups within each species. Comparing within species helped to control for variation in ecology, mating systems, taxonomy, and different metrics of genetic divergence that can confound across species and across study comparisons.

We excluded studies that described human-induced bottlenecks or reintroductions that could obscure population genetic patterns over latitude. Within studies, we excluded island populations from analyses. We only examined populations occurring on the same continents or within the same oceans (except for contiguous North and South Americas). In two cases, species were sampled from two oceans, each represented by many populations. In these cases, populations in different oceans were treated as independent taxa in the analyses. Individuals < 75 km apart were pooled into a single population for analyses. Otherwise we follow population designations from the original papers. We used either pair-wise genetic divergence data presented in each paper, or genetic data presented in the paper (or genbank) to calculate pair-wise genetic divergence (F_{ST}) among

populations using the Arlequin software package (<http://anthro.unige.ch/arlequin>).

Divergence parameters were the same for all populations within species, and thus our within-species comparisons were not influenced by variation in the parameters used. All parameter estimates controlled for within-population levels of genetic variation when calculating among-population differentiation.

We controlled for the geographic distance between populations in all analyses, because genetic differentiation of populations within species may be influenced by the distance separating those populations. We tested the strength and significance of this isolation by distance within each species using Mantel (1967) tests, implemented with the Arlequin software package.

RESULTS

Within species, we found greater genetic divergence of populations at lower latitudes in 23/31 (74%) of the species examined, including a diverse array of species such as Carolina chickadee, Dover sole, African buffalo, stinkpot turtle, and fin whale (Table 1, Figure 1). This greater genetic divergence per kilometer at lower latitudes was not explained by differences between latitudinal groups with respect to genetic distance (binomial exact test, $P = 0.72$) or geographic distance (binomial exact test, $P = 0.28$) alone. The pattern of greater genetic differentiation of low latitude populations, however, was not uniform across the globe. While 19 of 23 northern hemisphere species (83%) showed the predicted pattern, only two of five southern hemisphere species showed greater genetic divergence among lower latitude populations.

Overall the majority of taxa (28/31) showed a positive relationship (slope) between

genetic differentiation and the geographic distance separating the populations (i.e., “isolation by distance”, Wright 1943); Binomial Test, one-tailed $P < 0.000005$, Table 1). 16 showed individually significant isolation by distance (Table 1). The statistical significance of isolation by distance varied across species, and depended primarily on the number of populations sampled. A linear regression showed that number of populations included in the study was a significant predictor of (natural log transformed) Mantel P -values ($r^2=0.53$, $F=32.4$, $P < 0.000005$). We found no evidence that isolation by distance was influenced by the maximum distance separating populations ($r^2=0.004$, $F=0.1$, $P=0.73$).

DISCUSSION

Our results support previous evidence that the tropics may act as a “cradle” for new taxa (Dobzhansky 1950, Fischer 1960, Jablonski 1993), and are consistent with previous studies showing greater genetic structuring of populations in some tropical versus high latitude species (Seutin 1994, Braun 1996). Because we examined variation within species, our results are independent of species richness. If our results are a general phenomenon across taxa, then the greater number of species at lower latitude combined with greater divergence within species, suggests a much greater speciation potential at lower latitude.

Although genetic divergence of populations does not define a speciation event (Mayr 1963, Futuyma 1998), it is both a component and an influence on factors important in speciation (Slatkin 1987, Turelli et al. 2001). Accumulated genetic differences between

populations can lead to phenotypic divergence and reproductive isolation (Coyne and Orr 1989, Tilley et al. 1990), both of which play critical roles in speciation (Turelli et al. 2001). While traits important for reproductive isolation and speciation can evolve among connected populations (Barton and Bengtsson 1986), the relative genetic differentiation of populations provides an estimate of evolutionary independence and thus the relative probability of future speciation (Orr and Turelli 2001). Our results suggest that lower latitude populations are more likely to follow independent evolutionary trajectories, where mutation, recombination, selection and/or drift may lead to further divergence and speciation.

Thus, for a given time period, we expect a greater number of species to originate at lower latitudes, although this does not necessarily imply a difference in the rate at which individual lineages diverge. Differences among populations reflect both the rate of divergence and the time since isolation. The age of lineages may be greater in the tropics if extinction rates are lower, which seems a likely scenario given global patterns of glaciation. Regardless of how the differences arise, greater divergence among populations makes the probability of speciation higher at lower latitudes. Thus our results do not imply or assume faster rates of population divergence, but instead, higher likelihoods of future speciation.

While increased population divergence at lower latitudes may provide a mechanistic explanation for higher tropical species richness, we can further ask what environmental variation over latitude causes variation in population divergence. Our results are consistent with several hypotheses, which may account for reduced genetic

divergence of high latitude populations (Pianka 1966, Rohde 1992). For example, Janzen (1967) argued that increased seasonal variation in climatic conditions at higher latitudes resulted in greater adaptation of higher latitude organisms to conditions across spatially separated populations. Greater adaptation to conditions separating populations could reduce costs to dispersing between populations at higher latitudes, resulting in higher levels of gene flow and reduced genetic differentiation among high latitude populations. Alternatively, differences between high and low latitude groups may reflect historical influences on metapopulation structure. For example, glacial recession towards the poles following a glacial event may have (1) selected for high dispersal individuals at higher latitudes (Dennis et al. 1995), (2) resulted in greater mixing of high latitude populations during recolonization (Walter and Epperson 2001), and/or (3) resulted in repeated founder events and loss of genetic diversity during recolonization (Cwynar and MacDonald 1987). All three of these factors, together or individually, could explain the latitudinal variation in differentiation found in this study.

Teasing apart the historical influences on population structure is not possible with our data set, but is theoretically possible with a more ideal data set. In particular future studies may examine the influence of extinction and recolonization, as these may be more common at higher latitudes. Although extinction of local populations should increase variance among populations (Slatkin 1977), increasing recolonization will decrease differences among populations since the proportion of individual that are migrants increases. In the absence of extinction migration will affect the total diversity of the species, but not the variation within populations. When extinction exceeds migration both

within population and total species diversity will decrease (Maruyama and Kimura 1980) and F_{st} will be primarily influenced by colonization.

Latitudinal variation in population divergence has implications for other biogeographic patterns and evolutionary processes as well. For example, increased population divergence at lower latitudes can lead to the subdivision of large-ranged species into sister taxa occupying smaller, allopatric ranges. Thus, latitudinal variation in population divergence can provide a mechanistic explanation for the latitudinal increase in geographic range size across species ("Rapoport's Rule," Stevens 1989). Perhaps most importantly, our data showing greater divergence among low latitude populations suggest that the relative influence of the processes driving evolution (*e.g.* gene flow, genetic drift, and selection) may vary as a function of latitude (see Dobzhansky 1950, Fischer 1960). Given this variation, the bias towards temperate taxa in studies of evolution may obscure our understanding of how evolution and speciation occur in the majority of the earth's organisms.

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TABLE 1. Summary of results for vertebrate species examined in this study.

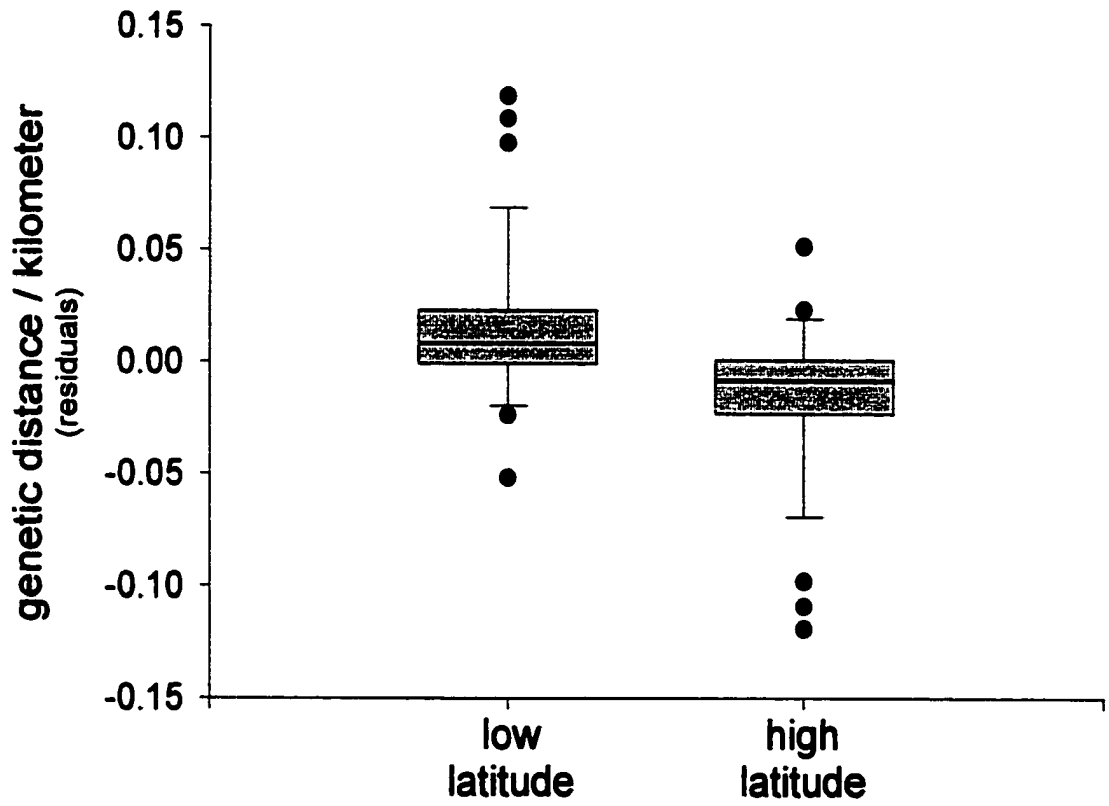
Species	Common Name	Continent	Environment	Taxa	Hemisphere	Mean Latitude	Lat span	No. of population	No. of individuals	Pattern	Mantel Z	h _{kd}	Ref
<i>Balaenoptera physalus</i>	Fin Whale	AO	m	m	N	51.0	26.5	5	237	+	0.897	0.008	1
<i>Brania canadensis</i>	Canada Goose	NA	vf	b	N	53.7	25.3	7	52	+	0.563	0.018	2
<i>Bufo marinus</i>	Marine Toad	NA/SA	t	a	N	10.8	11.7	7	27	-	0.436	0.024	3
<i>Chelonia mydas</i> -Atlantic	Green Turtle	AO	m	f	B	9.4	35.2	8	137	-	0.442	0.015	4
<i>Chelonia mydas</i> -Pacific	Green Turtle	PO	m	f	B	4.6	44.6	5	75	+	0.269	0.17	4
<i>Coregonus artedii</i>	Lake Cisco	NA	mf	f	N	55.4	8.5	7	139	-	-0.119	0.68	5
<i>Leuciscus cephalus</i>	Chub	EU	f	f	N	51.2	20.5	20	154	-	0.539	0.0000	6
<i>Macropus rufus</i>	Red Kangaroo	AU	t	m	S	-22.5	11.5	6	27	+	0.329	0.26	7
<i>Malaclemys terrapin</i>	Diamondback Terrapin	NA/AO	m	f	N	31.4	9.6	6	41	+	0.417	0.10	8
<i>Micropterus salmoides</i>	Largemouth Bass	NA	f	f	N	35.1	16.3	6	60	+	0.845	0.0095	9
<i>Microstomus pacificus</i>	Dover Sole	PO	m	f	N	41.0	25.3	6	112	+	0.540	0.084	10
<i>Nemadactylus pennsylvanicus</i>	Meadow Vole	NA	t	m	N	47.9	15.2	6	74	-	0.552	0.033	11
<i>Nemadactylus macropterus</i>	Jackass Morwong	PO	m	f	S	-39.0	12.5	9	>166	-	-0.179	0.70	12
<i>Odocoileus virginianus</i>	White-tailed Deer	NA	t	m	N	30.2	8.0	11	102	+	0.277	0.06	13
<i>Parus carolinensis</i>	Carolina Chickadee	NA	t	b	N	34.5	8.8	6	52	+	0.769	0.03	14
<i>Passerella iliaca</i>	Fox Sparrow	NA	t	b	N	48.0	31.6	31	253	+	0.442	0.0000	15
<i>Phoca vitulina</i> -Atlantic	Harbor Seal	AO	m	m	N	54.9	21.1	14	176	+	0.914	0.007	16
<i>Phoca vitulina</i> -Pacific	Harbor Seal	PO	m	m	N	44.3	24.8	7	34	-	0.734	0.028	16
<i>Pomastomus temporalis</i>	Grey-crowned Babbler	AU	t	b	S	-24.8	20.3	4	50	-	0.684	0.29	17
<i>Procavia capensis</i>	Rock Hyrax	AF	t	m	S	-28.5	12.3	9	54	+	0.270	0.09	18
<i>Salmo trutta</i>	Brown Trout	EU	f	f	N	45.3	18.4	12	87	+	-0.053	0.59	19
<i>Sclurus aberti</i>	Tassel-eared Squirrel	NA	t	m	N	35.4	10.1	15	86	+	0.420	0.0004	20
<i>Sternotherus minor</i>	Musk Turtle	NA	f	f	N	32.7	8.1	9	48	-	0.400	0.054	21
<i>Sternotherus odoratus</i>	Stinkpot Turtle	NA	f	f	N	33.4	9.9	13	88	+	0.196	0.13	22
<i>Sitix occidentalis</i>	Spotted Owl	NA	t	b	N	38.1	14.8	12	70	+	0.528	0.0001	23
<i>Syncerus caffer</i>	African Buffalo	AF	t	m	B	-4.9	21.8	9	227	+	0.448	0.015	24
<i>Tchecchus manatus</i>	West Indian Manatee	NA/SA	mf	m	N	15.1	21.5	7	79	+	0.170	0.21	25
<i>Uria aalge</i>	Common Guillemot	AO	m	b	N	57.2	25.1	4	130	+	0.612	0.09	26
<i>Ursus americanus</i>	Black Bear	NA	t	m	N	49.3	25.7	8	98	+	0.210	0.25	27
<i>Xenopus laevis</i>	African Clawed Toad	AF	f	a	S	-29.5	10.7	10	30	-	0.549	0.007	28
<i>Xerobates agassizi</i>	Desert Tortoise	NA	t	r	N	33.1	9.9	8	44	+	0.667	0.006	29

a- AF=Africa, AO=Atlantic Ocean, AU=Australia, EU=Europe, NA=North America, PO=Pacific Ocean, SA=South America
 b- f=freshwater, m=marine, t=terrestrial
 c- a=amphibian, b=bird, f=fish, m=mammal, r=reptile
 d- N=north, S=south, B=both
 e- +=taxa with increased genetic differentiation at lower latitudes
 f- see Appendix 1 for list of references

FIGURE LEGEND

FIGURE 1. The relative genetic distance per kilometer for low versus high latitude populations within 31 species of vertebrates (mtDNA). Values are residuals from an ANOVA, where genetic distance/kilometer (transformed) was the dependent variable and species was a random factor. Boxplots represent median values (middle line), 25-75 percentiles (box), 10-90 percentiles (bars), and outliers (circles). Figure does not reflect statistical analyses. The hypothesis that populations are genetically more divergent at lower latitudes was tested statistically by comparing populations within species using a one-tailed Binomial Test, because data did not conform to parametric assumptions before or after transformations. Results show significantly higher genetic divergence of low latitude populations within species (controlling for geographic separation, 17) (binomial exact test, one-tailed $P = 0.0053$).

McKay – Appendix 1
Fig. 1



Appendix I. References for data (Table 1).

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