Evolution and differentiation of edaphic races in the *Lasthenia californica* complex (Asteraceae:Heliantheae)

by

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

in

The Faculty of Graduate Studies
Department of Botany, The University of British Columbia

We accept this thesis as conforming
to the required standard

The University of British Columbia

December 2002

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ABSTRACT

"Recurrent evolution of taxa not only is possible, but may not be unusual in complexes that have undergone ecological diversification"

Donald A. Levin (2001)

The *Lasthenia californica* complex consists of two species, *L. californica s.s.* and *L. gracilis*, that include two races differing in flavonoid chemistry and edaphic tolerances. Populations of each race occur in each of the two species and the complex provides an ideal setting in which to examine the evolution of adaptations to soil conditions. Race A plants contain sulfated flavonoids and are found in habitats subjected to ionic stresses. Race C plants lack these sulfated compounds and are restricted to dry yet ionically-benign habitats. Studies described in this thesis show that the races are physiologically differentiated to deal with key environmental variables that are associated with their distinct habitats. Race A is better adapted to deal with ionic stresses, specifically with sodium and magnesium, ions that characterize their edaphic habitat. In contrast, race C is better adapted to drought, a feature that characterizes their edaphic habitat. Since both races achieve higher fitness under conditions that best match their natural environment, it is likely that the unique distribution pattern of the races has been achieved through differential adaptation. The edaphic races are also reproductively isolated via various means and it is likely that ecological selection has contributed to enhance isolation. The population genetic study conducted using RAPD markers strongly agrees with previous studies, supporting the notion of parallel occurrence of edaphic races in both *L. californica s.s.* and *L. gracilis*. It appears that race A is ancestral to race C and that edaphic specialization is an ancestral trait in the complex. Flavonoid features and traits

associated with tolerance to ionic stresses appear to have been lost in race C populations of both species found under ionically-benign habitats. The *L. californica* complex provides an ideal model to further test both the hypotheses of adaptive differentiation and parallel evolution and conduct much-needed studies on the genetics of ecological speciation in plants.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I wish to thank my committee, Drs. Bruce Bohm, Gary Bradfield, Anthony Glass, Les Lavkulich, and Jeannette Whitton for their advice and guidance during the course of my studies. A very special thank you to Dr. Whitton for her interest in my academic pursuits, within and outside my thesis work, and for her patient guidance throughout the course of my studies. I thank her for believing in my abilities and for always encouraging me to set higher goals. I also wish to thank Lesley Bohm, Nona Chiariello, Binney Girdler, Eduardo Jovel, Arlee Montalvo, Len Nunney, Molly Sullivan, Lori Valentine, Mike Vasey, and Ji Yang for their assistance in field collections; Administrative staff at Jasper Ridge Biological Preserve, Stanford University for their special hospitality; Kim Ryall for assistance in the laboratory; Marianne Hughes, Darin Bennett, Carol Dyck and Keren Ferguson for assistance in tissue and soil elemental analyses; David Ackerly, Bruce Baldwin, Raymund Chan, Andree Desrochers, Fred Ganders, Susan Harrison, Kent Holsinger, Art Kruckeberg, Massimo Pigliucci, Howard Rundle, Dolph Schluter, Yaeesh Siddiqi, Michael Whitlock, and Anne Worley for useful comments and advice on various manuscripts; Teika Olson, Kermit Ritland, Dilara Ally, and Mark Miller for assistance in genetic analyses; The Keeling Lab, especially Todd Harper, Brian Leander, John Archibald, and Matt Rogers for introducing me to the world of phylogenetic software; Teika Olson, Stacey Thompson, and Linda Jennings (Whitton Lab) for providing an enjoyable laboratory experience; Elaine Simons, Lebby Balakshin, Veronica Oxtoby, and Judy Heyes for taking care of all administrative issues; Shona Ellis for a wonderful partnership in teaching botany; and finally my wife, Anushka, for her continued support in all my academic endeavours. Last but not least, I would like to

gratefully acknowledge the financial support received from The University of British Columbia Graduate Fellowships (1998-2001), Department of Botany Teaching Assistantships, Natural Sciences and Engineering Research Council Grants (to Bruce Bohm, Anthony Glass, and Jeannette Whitton), as well as the generous support received in times of need from Elaine Simons, Department of Botany and Bruce & Lesley Bohm.

CHAPTER ONE

General Introduction

1.1. The edaphic factor in plant evolution

"The red-rock forest may seem hellish to us, but it is a refuge to its flora....it is the obdurate physical (and chemical) adversity of things such as peridotite (ultramafic) bedrock which often drives life to its most surprising transformations."

David Rains Wallace (1983)

Patchiness or discontinuity of edaphic phenomena leads to biological discontinuity. The most significant causes of localized or unusual distribution patterns of plants are traceable to isolating effects of discontinuities in geology and edaphics—geoedaphics (Kruckeberg, 1986). Geoedaphic isolates may range from distinct genotypes within species, through detectable local populations or races, to uniquely edaphically endemic species.

Extreme edaphic conditions such as guano deposits (Gillham, 1956; Ornduff, 1965; Vasey, 1985), vernal pools (Holland and Jain, 1977, 1981), salt marshes (Flowers *et al.*, 1986), granite outcrops (Wyatt and Fowler, 1977; Ornduff, 1986), serpentine soils (Kruckeberg, 1984; Brooks, 1987; Baker *et al.*, 1992) and mine tailings (Antonovics *et al.*, 1971, Shaw, 1990) provide ideal settings to examine the role of the edaphic factor in plant speciation. Populations of certain species may have the genetic preadaptedness to venture successfully onto soils that are edaphically extreme: a few preadapted genotypes could become founders of a tolerant population. Genetic accommodation to extreme edaphic conditions, particularly in the case of heavy metals, can take place quite rapidly, even within a few generations (Antonovics *et al.*, 1971; Bradshaw and McNeilly, 1981;

Liu and Godt, 1983; Shaw, 1990; Al-Hiyali *et al.*, 1988, 1990, 1993). Hence, edaphic conditions, when manifested in extreme form, can be potent agents of natural selection.

Several modes of origin of edaphic endemics have been proposed: biotype depletion, drift, catastrophic selection and saltational speciation, standard allopatric speciation with ecogeographic specialization, ecotypic differentiation, and hybridization with or without allopolyploidy are some modes of origin presented to explain edaphic endemism (Stebbins, 1942, 1980; Stebbins and Major, 1965; Proctor and Woodell, 1975; Raven and Axelrod, 1978; Kruckeberg, 1984; Kruckeberg and Rabinowitz, 1985). Using serpentine soils as an example of a challenging edaphic situation, Kruckeberg (1984, 1986) describes a set of stages that may lead to the establishment of an edaphically endemic species. Firstly, there exists preadaptedness for serpentine tolerance in nonserpentine populations. Then, disruptive selection, catastrophic selection (Lewis, 1962; Raven, 1964), or gradual divergence effectively separates the species into serpentine-tolerant and -intolerant gene pools. Further genetic divergence in structural and functional traits occurs within the serpentine-tolerant part of the effectively discontinuous populations. As a result, isolation between serpentine-tolerant and -intolerant populations becomes fixed and the two populations are unable to exchange genes. Further divergence of the serpentine ecotype leads to an edaphically endemic species. This sequence encompasses an evolutionary history from the initial tolerance of the habitat by certain preadapted variants to clear-cut species formation. These stages can be appropriately applied to other forms of geoedaphic challenges—mine soils with heavy metals (Antonovics *et al.*, 1971; Bradshaw *et al.*, 1990) and guano (Ornduff, 1965; Vasey, 1985)—also leading to the formation of edaphically endemic taxa.

While the stages illustrated by Kruckeberg (1986) may represent a more or less accurate description of the steps through which evolution proceeds from tolerant genotype to ecotype to an edaphic endemic, it does not indicate why some genotypes evolve through all the steps and others do not. The crucial step differentiating an ecotype from an endemic is likely to be the acquisition of complete reduction in gene flow between ancestral population and ecotype, allowing an independent gene pool to develop. How this occurs, in the absence of an extrinsic barrier to gene flow between two contiguous populations (i.e., races, ecotypes) is a fundamental question in speciation research.

Edaphically restricted species provide fascinating examples for the study of plant speciation. The study of factors contributing to the evolution of edaphic endemic species can shed light on the relationship between adaptation and reproductive isolation. Studies by Macnair and colleagues (Macnair and Christie, 1983; Christie and Macnair, 1987; Macnair and Gardner, 1998) suggest that reproductive isolation can result as a by-product of a physiological adaptation to unusual soil conditions. They have shown that the linkage block associated with copper tolerance in an ecotype of *Mimulus guttatus* Fischer ex DC. (Scrophulariaceae) also produces hybrid inviability; however, it is unclear if inviability is achieved via pleiotropy or hitchhiking. Subsequent work (Christie and Macnair 1984, 1987) has shown that both copper tolerance and hybrid inviability genes are commonly segregating in normal populations of this species, suggesting that hitchhiking is not improbable. Nevertheless, their work has clearly documented that natural selection for a clearly adaptive trait (copper tolerance) has caused a gene for post-

zygotic isolation to spread through the population (Macnair and Christie, 1983; Christie and Macnair, 1987).

With the advent of approaches such as the study of quantitative trait loci (QTLs), it is possible to identify and characterize candidate "speciation genes." Perhaps the best-known example in this regard is again in the genus *Mimulus* (Bradshaw *et al.*, 1998) where QTLs for floral traits associated with pollinator preference and reproductive isolation have been characterized, suggesting that loci of large effect can contribute to speciation. In *Aquilegia* (Ranunculaceae), a similar association between genes for pollinator preference and reproductive isolation has been recently established (Hodges *et al.*, 2002). Traits for pollinator preference are closely associated, indicating that either pleiotropy or linkage causes some of this integration. In both these QTL studies, reproductive isolation is a by-product of adaptation to pollinators, thus providing a direct link between adaptation and speciation.

1.2. *Lasthenia californica* as a case study for differentiation under edaphic influence

Lasthenia californica DC ex Lindl. (Asteraceae: Heliantheae) is the most widely distributed of the 18 species included in this mostly Californian genus (Ornduff, 1966, 1993). This obligately outcrossing, winter annual ranges from south-central Oregon throughout California, from the foothills of the Sierra Nevada to the coast, east into Arizona and in northern Baja California. The distribution of the species may be limited by its preference for a Mediterranean-type climate, characterized by mild, wet winters and long, hot, dry, summers. *Lasthenia californica* shows a high degree of

morphological, cytological (Ornduff, 1966), and biochemical diversity (Bohm *et al.*, 1974, 1989; Desrochers and Bohm, 1993, 1995) and is probably the most variable taxon in the genus.

Lasthenia californica has wide ecological tolerance: it occurs on coastal bluffs, in open grasslands, oak woodlands, alkali flats, chaparral, pastures and along roadsides, serpentine outcrops, and in the desert. Serpentine outcrops provide the most extreme growth conditions for this species. However, in these environments *L. californica* achieves dominance and is often restricted to the serpentine side of the serpentine and nonserpentine boundary. Thus, *L. californica* fits Kruckeberg's (1984) third category of plant distribution patterns on serpentine soils: species that are widespread on serpentine and nonserpentine soils but show regional prominence on serpentine.

Morphological and chemical studies conducted on plants collected from the entire range of *L. californica* demonstrated the existence of two genetically distinct geographical races (A, C) based on pappus shape, isozymes, and flavonoid patterns (Desrochers and Bohm, 1993, 1995). Race A is typically characterized by linear pappus scales, flavonoid pattern A (consisting of glucoronides, anthochlors, sulfated diglycosides, and eriodictyol 7-glucosides), b allele at the gene Nicotinamide Adenine Dinucleotide Dehydrogenase (NADHdh), and faster allozymes encoded by Phosphogluconate Dehydrogenase (6Pgd-1), while race C is typically characterized by lanceolate pappus scales, flavonoid pattern C (glucuronides, anthochlors) or B (glucuronides, anthochlors, and luteolin 7-glucosides), a allele at NADHdh, and slower allozymes encoded by 6Pgd-1 (Desrochers and Bohm, 1995). Preliminary crossing studies between the two geographical races indicated a low level of crossability

(Desrochers, 1992). Observations in the field also revealed differences in the flowering times between parapatric populations of the two races (Desrochers and Bohm , 1995).

Greenhouse experiments have indicated that the pattern of flavonoids seen among the populations of *L. californica* is controlled genetically. The flavonoid chemistry of an offspring matches that of its female parent (Bohm *et al.*, 1989). Further, cultivation of plants from seed in greenhouse potting soils does not affect the flavonoid pattern expressed in the offspring. Variation in flavonoid patterns between and within populations of plants is a well-documented phenomenon (Mears, 1980; Waser and Price, 1981; Levy, 1983; Bohm *et al.*, 1984, 1987; Bohm, 1987). However, no explanations have been provided for the variability seen in the flavonoid patterns in *L. californica*. The existence of different flavonoid and floral pigmentation patterns in plants seems to be influenced by soil type (Horovitz, 1976; Mears, 1980; Menadue and Crowden, 1983; Heywood, 1986; Reid, 1995; Rosenthal and Human, 1997; Schemske and Bierzychudek, 2001; Rajakaruna , pers. observations). It is uncertain, however, if this reflects a direct cause and effect relationship or merely a correlation. Interestingly, populations of race A collected under serpentine or other ionically-harsh substrates show the greatest intensity of the sulfated flavonoid on thin layer chromatography, suggesting that the edaphic condition may correlate with the quantity of these compounds produced (Rajakaruna, pers. observations), although sulfated flavonoids have not been quantified in *L. californica*.

The two geographical races of *L. californica* show a north-south distribution pattern within the species' range. Race C is found predominantly from southern Oregon to northern and north-central California while race A is found predominantly in the

southern parts of California , Arizona, and Baja California (Desrochers, 1992). In the central parts of California, there exist several populations with both races. The largest of the mixed populations so far encountered occurs in the Jasper Ridge Biological Preserve, Stanford University, San Mateo County. The preserve is located west of Palo Alto, in the Santa Cruz Mountains, at approximately $37^0$25' north and $122^0$2.5' west. Here, both races of the species coexist in a population found on an extensive serpentine outcrop that runs in a west-northwesterly to east-southeasterly direction. This population lies at an elevation of approximately 180 meters.

The climate at Jasper Ridge is Mediterranean, with mean summer temperatures of 20.1 °C, mean winter temperatures of 9.2 °C, total annual precipitation of 622.4 mm, and a dry season of approximately six months (May-October, precipitation < 50 mm/month) (Ackerly *et al.*, 2002). *Lasthenia* plants germinate after the first significant rains in the late fall and flower and set seed mostly by April or early May. The distribution pattern of the two races on this serpentine ridge has been studied for almost 20 years (Bohm *et al.*, 1989; Rajakaruna, pers. observations). Ridge top is always populated by race C plants, and ridge bottom, by race A plants. Year after year, the two races maintain a distinct boundary (Bohm *et al.*, 1989; Desrochers and Bohm 1993, 1995), with a sharply-defined transition zone which varies by no more than a meter from year to year.

Previous studies have documented boundaries between species or genetically distinct races of the same species on two geologically very different substrates — serpentine and nonserpentine (Kruckeberg, 1951, 1954, 1967, 1992; Proctor and Woodell, 1975; Brooks, 1987; Mayer and Soltis, 1994a,b) and calcareous and noncalcareous (Snaydon, 1962; Kruckeberg, 1969; Heywood, 1986). Another well-

studied example of sharply demarcated boundaries in nature involves areas of heavy metal contamination (Antonovics *et al.*, 1971; Bradshaw, 1972; Shaw, 1990). Here, the vegetation boundary possibly exists on the same geologic substrate and is due to obvious contamination of one side of the boundary with heavy metals. Studies have shown that distinct populations, with different levels of metal tolerance, can be found within distances as small as a meter (Snaydon, 1963, 1970; Jain and Bradshaw, 1966; Antonovics, 1978; Snaydon and Davies, 1976; Al-Hiyaly *et al.*, 1993) to several meters (McNeilly, 1968; Antonovics and Bradshaw, 1970; Watson, 1970). The distinct distribution pattern observed at Jasper Ridge is unique since the transition zone is maintained on essentially the same edaphic substrate, serpentine, a phenomenon previously undocumented for any species (Kruckeberg, personal communication).

A detailed ecological study (Rajakaruna, 1998; Rajakaruna and Bohm, 1999) documented that the soils on the serpentine outcrop at Jasper Ridge vary along an elevational gradient and that the two races are restricted to distinct edaphic environments within the ridge. Such differentiation within a serpentine outcrop had not been previously reported (Jurjavcic *et al.*, 2002). A survey of 22 other populations showed that the previously described geographical races within *L. californica* are edaphically-differentiated throughout their range with race A predominating in habitats subject to edaphic stress. Race A occurs on serpentine outcrops, coastal bluffs, in vernal pools, and alkaline flats, while race C is found only in ionically-benign sites. The pH and ionic strength of the soil solutions of the sites where the two races grow are significantly different, along with concentrations of specific ions such as Na^{+}, Mg^{+2}, as well as Ca^{+2}/Mg^{+2} ratios (Rajakaruna and Bohm, 1999). At Jasper Ridge, where the two races

occur in parapatry, race A is restricted to the ionically-harsh bottom reaches (Rajakaruna and Bohm, 1999). Plant tissue concentrations of various elements differed between the two races at Jasper Ridge and throughout the species' range, with race A accumulating significantly higher concentrations of Na^{+} and Mg^{+2} than race C plants. Based on these findings Rajakaruna and Bohm (1999) described the geographical races of Desrochers and Bohm (1995) as edaphic races within *L. californica.*

My Ph.D. thesis examines various factors and mechanisms involved in the process of divergence among populations of *L. californica sensu* Ornduff (1993), specifically emphasizing adaptation to edaphic conditions, reproductive isolation and genetic differentiation. Speciation is a challenging puzzle to untangle especially because it involves a process complicated by a variety of past influences. Hence, studies of speciation should ideally be inter-disciplinary, especially if one is to attempt to unravel the many pieces of this complex process. The studies I have conducted, which draw upon several different disciplines, are inter-related in that they are central to understanding key aspects of plant speciation and making informed inferences regarding the origins and patterns of diversity within *L. californica.* A recent phylogeny for *Lasthenia* suggests that *L. californica sensu* Ornduff is not monophyletic (Chan *et al.*, 2001, 2002). Chapter Two examines the relationship of the edaphic races to these recently described phylogenetic clades within *L. californica sensu* Ornduff. This chapter places my studies within the context of comprehensive phylogenetic work conducted at The University of California, Berkeley and sets the stage for chapters that follow on adaptation (Three and Four), reproductive isolation (Five) and divergence (Six). Chapter Three examines ion physiology of edaphic races in order to determine if the two races are distinct in their

tolerance to various ions that predominate in their natural habitats. The chapter emphasizes the role of differential tolerance to these ions in the ecological differentiation of the two races. Chapter Four looks at differential responses to water stress in order to determine the role of water in the observed distribution pattern and divergence of the two races at Jasper Ridge. Chapter Five examines the extent of reproductive isolation between the two races and species in the *L. californica* complex and describes various mechanisms likely responsible for reproductive isolation observed between species and races within the complex. Chapter Six describes a population genetic study conducted to estimate the extent of divergence within and between races and speculates on a plausible mode of divergence for the two races. Finally, Chapter Seven, summarizes my findings on *L. californica* and emphasizes the use of *Lasthenia* as a model genus for studies in evolutionary ecology.

CHAPTER TWO

Edaphic races and phylogenetic species in the *L. californica* complex: A hypothesis of parallel evolution

2.1. Introduction

The genus *Lasthenia* Cass. (Heliantheae:Asteraceae) has been the subject of intense study for nearly half a century. Ornduff's monograph for the genus (1966) recognized 17 species of predominantly herbaceous annuals, 16 of which are endemic to western North America, while one species, *L. kunthii* (Less.) Hook. and Arn., is endemic to central Chile. Despite its relatively small size, the genus is taxonomically challenging, with considerable inter- and intra-specific morphological, secondary metabolic, cytological, macromolecular, and ecological diversity (Bohm *et al.*, 1974; Crawford *et al.*, 1985; Vasey, 1985; Crawford and Ornduff, 1989; Chan, 2000; Chan *et al.*, 2001).

Lasthenia californica sensu Ornduff, the common goldfields of California, is considered the most variable taxon in the genus and has been the subject of numerous investigations (Bohm *et al.*, 1989; Desrochers and Bohm, 1993, 1995; Rajakaruna and Bohm, 1999; Chan, 2000; Chan *et al.*, 2001, 2002). Recent molecular phylogenetic studies (Chan *et al.* 2001, 2002; Desrochers and Dodge, in press) indicate that *L. californica sensu* Ornduff is not monophyletic. Based on a comprehensive ITS/ETS/cpDNA phylogenetic study, Chan *et al.* (2001, 2002) found that *L. californica sensu* Ornduff represents two geographically-based, non-sister clades. Chan (2001) and Chan *et al.* (2002) recognized the clades as two cryptic taxa, *L. californica* subsp. *californica* representing the northern clade (populations previously treated as *L. macrantha* (A. Gray) Greene constitute two other subspecies of *L. californica*) and *L.*

gracilis DC. (Greene) representing the southern clade. *Lasthenia californica* subsp. *californica* comprises plants with clear to brown linear/subulate awns, which are restricted to northern California and Oregon; *L. gracilis* plants possessing opaque, white, ovate-lanceolate scales, each tapering to an awn, which have a wider distribution spanning California, Baja California, and Arizona. While pappose and epappose plants may be found together in individual populations of both species (Ornduff 1966; Chan 2000; Chan *et al.*, 2002), some populations may comprise only epappose plants, which are difficult if not impossible to identify to species based on morphology alone (Chan 2000; Chan *et al.*, 2002). It is interesting to note that Desrochers and Bohm (1995) and Rajakaruna and Bohm (1999) also employed pappus features to recognize their races (Chapter One). In the edaphic races, epappose cypselae are almost always found in race A plants occurring along the coast, in salt flats, and on several serpentine outcrops (e.g., Rattlesnake Rock, San Mateo Co., CA), making the absence of pappi a fairly good predictor of race when populations are found in these edaphically-harsh environments.

In the remainder of the thesis, I adopt the treatment of Chan (2001), referring to the northern clade as *L. californica* subsp. *californica* and to the southern clade *as L. gracilis*. When referring to the previous treatment of *L. californica*, I use the designation *L. californica sensu* Ornduff or the *L. californica* complex.

The molecular phylogeny has led us to ask about correspondence of the edaphic races (A and C; See Chapter One) to the phylogenetically distinct taxa. If ecological selection has played a role in the origin of edaphic races, as seems plausible, then similar edaphic tolerances may have evolved in parallel within one or both species. Indeed, a previous study of allozyme variation (Desrochers and Bohm, 1995) indicated that single

populations of race A and race C cluster with sets of populations belonging to the opposing race. To address this intriguing issue, several specimens from *L. californica* subsp. *californica* and *L. gracilis* in Chan *et al.* (2001, 2002) were tested for their flavonoid-pigment profiles, and nuclear ribosomal DNAs (rDNA) of several representatives from the two edaphic races were characterized to determine their placement in the context of Chan *et al.*'s molecular phylogeny.

2.2. Materials and Methods

Two heads from each of three populations belonging to *L. gracilis* and three populations of *L. californica* subsp. *californica* (Chan *et al.*, 2001, 2002) were tested for their flavonoid profiles employing one-dimensional thin-layer chromatography, as described earlier in Desrochers and Bohm (1993). The location and species of the populations tested are described in Table 1 (populations 1-6).

In order to characterize variation in ITS, genomic DNAs were isolated from approximately 1 g of plant tissue following the modified CTAB protocol of Doyle and Doyle (1987). Samples were further purified using the Elu-Quick DNA purification kit (Schleicher and Schuell, Keene, NH). The rDNA internal transcribed spacer (ITS) region was sequenced, as described previously by Chan *et al.* (2001), from DNA isolates of two plants from each of three race C and six race A populations (Table 1, populations 7-15). Although the phylogenetic study of Chan *et al.* (2001, 2002) was based on analysis of ETS, ITS and cpDNA regions, 26 nucleotide differences, and a single 11 bp indel distinguished the ITS sequences of all populations of the two phylogenetic species sequenced thus far. Therefore ITS variation is sufficient for assigning populations to one or the other species.

The 11 bp indel described above is located in the ITS I region, flanked by the 18S and the 5.8S ribosomal genes. This size difference was used as a marker for assignment of 18 additional populations of known flavonoid type to phylogenetic lineage (Table 1, populations 16-33). Populations 7-15, sequenced above were used as size controls. The ITS I region was amplified using primers ITS2 and ITS5 (White et al., 1990). Amplifications were carried out in 25 µl volumes including 10 ng of template DNA, 30mM Tris-HCl, 50 mM KCl, 2mM $MgCl_2$, 0.1 mM each dNTP, 10pmoles of each primer, 5% acetamide and 1.5 units of DNA polymerase. Amplification was carried out on a 60-well PT-100 thermal cycler (MJ Resreach, Waltham, MA), programmed for an initial denaturation of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, with a final extension of 7 min at 72°C. An aliquot of 2 µl of each PCR product was separated by electrophoresis on 2% (w/v) SeaPlaque agarose (BMA, Rockland, ME) in 0.5X TBE buffer. After staining in ethidium bromide, the gel was visualized under UV light with an AlphaImager 1200 gel documentation system (AlphaInnotech Corporation, CA).

2.3. Results

Examination of flavonoid profiles and ITS sequences reveals that edaphic races are not strictly concordant with *L. californica* subsp. *californica* and *L. gracilis*. Flavonoid typing (Table 1, populations 1-6) indicates that two of the populations recognized as *L. gracilis* are race A (RO10079 and RC98011), while the third is race C. The three *L. californica* subsp. *californica* populations are race C. Although the flavonoid profiles do not correspond to the two clades, both populations with flavonoid profile A were found in alkaline flats, supporting the correlation between flavonoid profile and edaphic habitat.

Table 2.1. Populations of the *L. californica* complex (*L. californica* subsp. *californica* and *L. gracilis*) sampled in this study. Populations 1-6 of known phylogenetic species, were tested for flavonoid profile. Populations 7-15, of known edaphic race, were sequenced to confirm the phylogenetic status. Genbank accession number for these new sequences are included in parentheses next to population designations. Populations 16-33 of known edaphic race, were assigned to phylogenetic lineage by assessing the relative size of the ITS 1 amplicon. Vouchers for populations 1-6 are deposited in the Jepson Herbarium, UC Berkeley; those for populations 7-33 are at the UBC Herbarium.

No	Population	Locality	Race	Species
1	RO10079	Alkali flats W and N of Byron Hot Springs Road, E of the airport S of Byron, Contra Costa Co., CA	A	*L. gracilis*
2	RC 98011	Alkaline field beyond E corner of Hartford Ave., SW of Livermore, Alameda Co., CA	A	*L. gracilis*
3	RC98010	Grassy Hillside ~ 50 m E of Hwy 33, 0.25 miles N of its junction with Palmer Ave., N of Coalinga, Fresno Co., CA	C	*L. gracilis*
4	RO10160	Parking area at Point Reyes Lighthouse, Marin Co., CA	C	*L. californica*
5	RC98017	Grassy Field ~ 100m W of Hwy 29, 2.4 miles S of State Hospital, Napa Co., CA	C	*L. californica*
6	RC98020	Grassy pasture NW of Hwy 120, SW of road leading to Two-Mile Bar Rec. Area, Knights Ferry, Tuolumne Co., CA	C	*L. californica*
7	RS (AF550682-83)	Serpentine soils of north-facing slope of Rattlesnake Rock, Jasper Ridge Biological Preserve, San Mateo Co., CA	A	*L. californica*
8	JRA (AF550680-81)	Bottom reaches of the serpentine outcrop, Jasper Ridge Biological Preserve, San Mateo Co., CA	A	*L. californica*
9	AVEQ (AF550689)	Pasture along Avenue Q, across from Holiday Inn, Palmdale, Los Angeles Co., CA	A	*L. gracilis*
10	COA (AF550690)	Pasture, 1 km E of Coalinga Springs Road on Route 198, Fresno Co., CA	A	*L. gracilis*
11	CA3 AF550691	Serpentine hillside, 4 km E of Paskenta Bridge, Paskenta/Covelo Road, Paskenta, Tehama Co., CA	A	*L. gracilis*
12	TEHA (AF550688)	Along Tehachapi Willow Springs Road, 3.3 km S of intersection with Highline Road, Kern Co., CA	A	*L. gracilis*
13	JRC (AF550686-87)	Upper reaches of the serpentine outcrop, Jasper Ridge Biological Preserve, San Mateo Co., CA	C	*L. gracilis*
14	TR (AF550684)	Andesite deposit, summit of Lower Table Rock, Jackson Co., OR	C	*L. californica*
15	OR1 (AF550685)	Roadside, Kirtland Road, 0.5 km E from intersection with Table Rock Road, near Water Treatment Plant, Jackson Co., OR	C	*L. californica*
16	SPS	On coastal bluff, Salt Point State Park, Sonoma Co., CA	A	*L. californica*
17	PR	Along roadside on way to Light House, Point Reyes. 4 km from Drake Beach. Marin Co., CA	A	*L. gracilis*
18	MT	On serpentine substrate at Mount Tamalpais State Park. Marin Co., CA	A	*L. californica*
19	KCN	On serpentine substrate, N-facing slope of Kerby Canyon, W of Coyote Ridge, Santa Clara Co., CA	A	*L. californica*
20	25	Roadside pasture along Route 25, 8.5 km S of Hollister, San Benito Co., CA	A	*L. gracilis*
21	PV	Pasture along Route 198, 4 km NW of County line of Monterey and Fresno Counties, Priest Valley, Monterey Co., CA	A	*L. gracilis*

No	Population	Locality	Race	Species
22	AS	Hillside N of 16-M. Road on trail to stream, ~ 4 km from Arroyo Seco Campsite. Los Padres Nat. Forest, Monterey Co., CA	A	*L. californica*
23	CASR	Roadside ditch. Intersection California Ave. & Stowe Rd. W of Stetson & Warren Rd. Near Hemet, Riverside Co., CA	A	*L. gracilis*
24	MR	On soils derived from granite. Motte Rimrock Reserve, Riverside Co., CA	A	*L. gracilis*
25	AZ	Sandy soil along dry streambed. N of Apache Junction on Route 88, near M. 203, on left (W) side of road. Maricopa Co., AZ	A	*L. gracilis*
26	ARZ	Sandy ridge near a sidetrack ~ 1.5 miles along Dripping Springs Road, N of Mile 153 marker on AZ Route 77. Gila Co., AZ	A	*L. gracilis*
27	OR2	0.5 km from intersection Kirtland Road and Table Rock Road. Roadside pasture, Water Treatment Plant, Jackson Co., OR	C	*L. californica*
28	OR3	On gravelly pasture across road from population OR2. Jackson Co., OR	C	*L. californica*
29	44	Near Millville, 2 km from Old 44 Road/Route 44. On oak woodland at Route 44/A17, Shasta Co., CA	C	*L. californica*
30	44A	Near Millville, 1 km from population 44 heading towards Redding, Shasta Co., CA	C	*L. californica*
31	RB	Pasture along Route 36, 1.7 km from intersection with Highway 99. Near Red Bluff, Tehama Co., CA	C	*L. californica*
32	R20	Roadside pasture along Route 20 between Colusa and Clear Lake, Colusa Co., CA	C	*L. gracilis*
33	R29	Roadside pasture, 4 km N of Calistoga along Route 29. Napa Co., CA	C	*L. californica*

New ITS sequences of the three race C and six race A populations revealed a similar pattern (Table 1, populations 7-15). Here, populations of each race nest within each species, further verifying that edaphic races do not correspond to the two molecular clades.

Finally, PCR-amplified ITS 1 fragments assigned 6 of 7 race C populations to *L. californica*. Of 11 race A populations, 4 had the longer fragment characteristic of *L. californica*, and 7 displayed the shorter fragment characteristic of *L. gracilis* (Table 1, populations 16-33). While the longer ITS 1 product is also characteristic of *L. californica* subsp. *macrantha* (formerly *L. macrantha*), this taxon is distinguished by morphological and flavonoid features, and thus we are confident in assigning all samples here to *L. californica* subsp. *californica*. Note that the size difference was also scored for the 9 sequenced samples (populations 7-15) and was concordant in each case with phylogenetic assignment based on the complete ITS sequence.

Thirty-three populations were characterized as part of this study. Of the sixteen populations of *L. gracilis*, 13 are race A and 3 are race C. Of the 17 populations of *L. californica* subsp. *californica*, 11 are race C and 6 are race A (Table 1). Both edaphic races occur in parallel in both taxa.

2.4. Discussion

The flavonoid and DNA results revealed that the two edaphic races do not correspond to *L. californica* subsp. *californica* and *L. gracilis,* and that both races occur in both taxa. The pattern is further confirmed by carefully assessing the results from the allozyme analysis of Desrochers and Bohm (1995) and the ITS phylogeny of Desrochers and

Dodge (in press), where the edaphic races do not always correspond to the geographical clades.

Although race C populations generally nest within *L. californica* subsp. *californica* and race A generally corresponds to *L. gracilis*, the pattern is reversed at Jasper Ridge as well as in several other populations. In many such cases, soil features seem to be a better predictor of flavonoid profile (Rajakaruna and Bohm, 1999; Rajakaruna, unpublished) than either the allozyme data (Desrochers and Bohm, 1995) or macromolecular phylogenies (Chan *et al.* 2001, 2002; Desrochers and Dodge, in press). For example, in every instance where a plant from an extreme serpentine exposure (JRA, RS) or alkaline flat (RO10079 and RC98011) was tested, the plant had the flavonoid profile of race A, although the plants belonged to different geographical clades, and thus to different species. This intriguing finding suggests parallel evolution of edaphic races; racial features may have evolved secondarily in response to the contrasting soil conditions under which these plants are found.

Race A plants predominate in habitats of ionic stress while race C plants grow in more benign soils. The primary feature that distinguishes these edaphic races is the flavonoid pigment profile; race A contains sulfated compounds, namely sulfated kaempferol and quercetin diglycosides plus prominent eriodictyol glycosides (Bohm *et al.*, 1974, 1989; Desrochers and Bohm, 1993) not found in race C plants. Ecological roles for flavonoid pigments, such as protection against U.V. damage, pathogens and herbivores, have often been postulated (Bohm, 1987; Clegg and Durbin, 2000; Bohm and Steussey, 2001). The case for a correlation with habitat and sulfated flavonoids has previously been noted (Harborne, 1975, 1977), for example, a large number of taxa found

in habitats with waterlogged and saline conditions contain sulfated flavonoids (Harborne, 1975; Barron *et al.*, 1988). Marine, alkaline, and serpentine habitats are high in sulfates and it is tempting to suggest that sulfation of flavonoids may be beneficial in such sulfate-rich environments, as a means of detoxifying excess sulfate. For example, the more hydrophilic sulfated flavonoids would be better contained within vacuoles, and furthermore, by reducing the chemical activity of inorganic sulfate, precipitation of compounds such as calcium sulfate that are characterized as having low solubility products may be avoided. Further, these negatively charged sulfated flavonoids may play a role similar to that of sulfur containing glucosinolates in providing tolerance to excess metal ions (Mathys, 1977) or in providing ionic balance in light of excess cations such as those found in race A habitats. A study by Nissen and Benson (1964) showed that over 50% of radioactive sulfate fed to *Zostera* (sea grass) was later found in the flavonoid fraction. In addition, limited work also suggests that sodium can act as the counter cation for these negatively charged sulfated flavonoids (Ahmed and Mabry, 1987; Tomas-Barberan *et al.*, 1987; De Beck *et al.*, 1998; Mann *et al.*, 1999), although potassium is usually regarded as the predominant counter cation. Interestingly, race A plants are sodium accumulators (Rajakaruna and Bohm, 1999) and it is intriguing to find that sulfated flavonoids occur in these sodium-accumulating plants, which in turn are found in sulfate- and sodium-rich environments.

The above findings have led to eco-physiological studies (Chapter Three) to determine experimentally if putatively parallel edaphic races from the two distinct taxa are similar in sodium-ion physiology, which is likely to be of adaptive significance in the

ionically-extreme habitats where race A grows. Such an examination will be critical for testing further the hypothesis of parallel evolution of edaphic races in the *L. californica* complex.

CHAPTER THREE

Differential responses to Na^+/K^+ and Ca^{2+}/Mg^{2+} in two edaphic races of the *L. californica* complex: A case for parallel evolution of physiological traits

3.1. Introduction

The distribution of many plant species is strongly influenced by edaphic conditions. The significance of the edaphic factor (Mason, 1946a,b) in patterns of plant diversity has long been recognized (Jenny, 1941; Kruckeberg, 1986; Brooks, 1987); the classic generalizations on the distribution of plants (Cain, 1944) place the edaphic factor second only to climate as the major environmental determinators of plant distribution. Extreme edaphic conditions provide sharp discontinuities in edaphic features leading to intriguing patterns of plant diversity (Chapter One). Under such extreme cases, a particular factor or a suite of factors can be identified to be of paramount importance, either because a particular species is restricted to soils with a particular edaphic status (i.e., edaphic endemic) or because it is excluded from such soils. Although plant species largely avoid edaphic extremes, genetic accommodation to stressful edaphic conditions has been shown to take place quite rapidly in some species (Shaw, 1990).

Members of *Lasthenia* have wide edaphic tolerance; species are found in habitats such as coastal bluffs, guano deposits, vernal pools, salt and alkaline flats, serpentine outcrops, deserts, grasslands, and open woodlands (Ornduff, 1966, 1993). *Lasthenia californica sensu* Ornduff has the widest edaphic tolerance within the genus, with populations spanning all but guano habitats. The ecological survey of the *L. californica* complex (Rajakaruna and Bohm, 1999) documented that race A plants predominate in

habitats subject to ionic stress, specifically, coastal bluffs, alkaline flats, serpentine outcrops, and salt flats, suggesting cross-resistance to harsh environments. Race C plants appear to be restricted to relatively "benign" habitats such as inland pastures, roadsides, and open fields. The relatively higher ion concentrations in race A soils have led to significant differences in ionic strength of the soil solutions (ranges for race A and C are 2.23 - 111.7 mM and 1.4 - 26.8 mM, respectively) of the sites where the two races grow (Rajakaruna and Bohm, 1999), suggesting that race A plants are adapted to growing in soils of greater ionic strength and/or in soils where concentrations of specific ions may inhibit the growth of race C plants. Race A plants are often found in osmotically extreme soils where electrical conductivities can reach values as high as 7.49 $mScm^{-1}$ (Rajakaruna and Bohm, 1999); the highest value obtained for a race C soil is 1.79 $mScm^{-1}$. Electrical conductivity above 4 $mScm^{-1}$ is highly toxic to most plants (Brady, 1990). In some extreme sites (for example, near Soda Lake in San Luis Obispo Co., CA) where race A plants occur, the soil surface is crystalized with salts.

The study by Rajakaruna and Bohm (1999) suggested that the concentrations of Na^+ and Mg^{2+} may be important in delimiting the distribution of these races, particularly race C. Exchangeable Na^+ and Mg^{2+} in race A soils showed a much wider range than for race C soils (Rajakaruna and Bohm, 1999). The highest Na^+ concentration encountered in race A soils was 13-fold higher than the highest concentration recorded for race C soils. Further, race A plants appeared to predominate in Mg^{2+}–rich serpentine habitats where Ca^{2+}/Mg^{2+} ratios were considerably below 1. Low Ca^{2+}/Mg^{2+} ratios in serpentine soils are often thought to restrict species from growing in these soils (Walker *et al.*, 1955; Kruckerberg, 1984; Tibbetts and Smith, 1992).

Previous studies (Rajakaruna and Bohm, 1999) have also consistently shown that the two edaphic races are physiologically distinct with respect to ion accumulation: race A plants accumulate 3-4 times the concentration of Na^+ found in race C plants. For example, at Jasper Ridge Biological Preserve, where the races occur in parapatry on a serpentine ridge, race A accumulates approximately 4 times more Na^+ than race C, although the exchangeable soil Na^+ for race A is only slightly higher than that for race C at this site (Rajakaruna and Bohm, 1999). This differential accumulation is also evident when examining populations of the two races throughout the range of *L. californica sensu* Ornduff (Rajakaruna and Bohm, 1999). At certain extreme habitats, Na^+ accounts for 5.2% of the dry weight of race A plants (Rajakaruna, unpublished), approaching levels found in halophytic plants (Flowers *et al.*, 1986; Welch and Rieseberg, 2002). By contrast, the highest Na^+ concentration ever recorded for race C plants was 0.2%. Thus, under extreme conditions, race A plants had accumulated 26 times as much Na^+ as race C plants. Interestingly, a detailed field study involving 22 populations of the *L. californica* complex (Rajakaruna and Bohm, 1999), revealed a strong correlation between soil Na^+ and tissue Na^+ in the case of race A plants (r=0.87: $P < 0.001$) but not race C plants (r=0.23: NS). In comparison to Na^+, tissue concentrations of K^+ and the relationship between soil and tissue K^+, did not differ significantly between race A and C plants. Thus, while tissue Na^+ appears to vary widely according to soil conditions in race A plants, K^+, an essential element, is maintained within relatively narrow limits in both races. Given the essential nature of potassium (Epstein, 1972; Glass 1988), this finding is not surprising. Calcium and Mg^{2+} concentrations in plant tissue were also rather similar between the two races. However, the field survey by Rajakaruna and Bohm (1999)

indicates differential response to these ions by the two races. For both ions, there was a strong correlation between soil and race A tissue concentrations (Ca^{2+} r= 0.78; Mg^{2+} r= 0.81: $P < 0.05$) while there was no such correlation for race C (Ca^{2+} r= 0.36; Mg^{2+} r= 0.31: NS). The observations made in the field clearly suggest that the two races are physiologically distinct entities, especially with respect to Na^+, Mg^{2+} and Ca^{2+} uptake and tolerance.

In the study presented in this chapter, I grew plants from two populations from each of the two edaphic races under controlled hydroponic conditions, in order to determine if the physiological differences observed in the field are under genetic control. I addressed the following specific questions:

1) Are the races distinct in their Na^+/K^+ and Ca^{2+}/Mg^{2+} ion physiology, i.e., do the observations made in the field accurately reflect genetic differences in Na^+/K^+ and Ca^{2+}/Mg^{2+} physiology between the two races? I determined ion uptake rates and accumulation of the four cations and examined germination, survivorship and tolerance responses to potentially toxic Na^+ and Mg^{2+} levels in the medium.

2) Has the ion accumulating behavior responsible for tolerance to Na^+ evolved in parallel in race A plants belonging to *L. californica* subsp. *californica* and *L. gracilis* ? i.e., is there evidence to suggest parallel evolution of this physiological trait?

3.2. Materials and Methods

Cypselae of *L. californica* subsp. *californica* and *L. gracilis* were obtained from field collections made during 1996-2002. The populations used represented both edaphic races from the two phylogenetic species (Table 3.1). Race A plants from *L. californica* subsp.

californica and *L. gracilis* are hereafter referred to as A_C and A_G while race C plants from the two species are referred to as C_C and C_G. Note first letter represents race while the subscript refers to the specific epithet. The two Jasper Ridge populations are A_C and C_G.

3.2.1. Experiment 1—Differential responses to Na^+ and K^+: ion uptake and accumulation

3.2.1.1. Germination and plant growth—Approximately 200 cypselae from each population were dipped in 1% bleach, washed three times with deionized distilled water (DDW), placed on moist filter paper in a Petri dish, and moved to a dark, cold room (5°C) for 3 days. Five germinating cypselae each were then placed in washed sand contained in germination tubes. Each germination tube was an open plastic cylinder (1 cm diameter x 1 cm depth) fitted with nylon mesh (1 mm) at the bottom. The germination tubes were fitted onto a styrofoam raft (7 tubes per raft) and rafts were placed randomly in a plastic tub (45cm x 45cm x 20cm) containing 8 L of aerated nutrient solution. Each of the four populations was replicated six times, each replicate consisting of approximately 35 plants. For each population, plants were grown in three extra styrofoam rafts in order to estimate relative growth rates during the experimental period. The edges of the tubs were covered with aluminum foil to limit the entry of light and restrict the growth of algae.

The nutrient solution was prepared based on soil solution concentrations. Soils from the four populations were analyzed for chemical features at field capacity using a method outlined by Proctor *et al.* (1981). Table 3.1 lists the soil Na^+, K^+, Ca^{2+}, and Mg^{2+}

Table 3.1. Race, species, and field soil solution features of the populations used in the hydroponic study. C_G: Upper reaches of the serpentine ridge, Jasper Ridge Biological Preserve, Stanford University, San Mateo Co., CA (same as JRC in Table 2.1); A_C: Bottom reaches of the serpentine ridge, Jasper Ridge Biological Preserve (same as JRA in Table 2.1); C_C: Andesite deposit on summit of Lower Table Rock, Jackson Co., OR (Same as TRC in Table 2.1); A_G: Roadside across from Holiday Inn, Avenue Q, Palmdale, Los Angeles Co., CA (Same as AVEQ in Table 2.1). Soil features include pH, ionic strength (*I*) in mM, Na^+, K^+, Mg^{2+}, and Ca^{2+} under field capacity (μM) analyzed using one 500 g sample of soil per population.

Population	*Race*	*Species*	*pH*	*I*	Na^+	K^+	Mg^{2+}	Ca^{2+}
C_G	C	*L. gracilis*	6.4	5.7	245	101	2480	192
A_C	A	*L. californica*	6.6	9.5	263	29	2701	89
C_C	C	*L. californica*	5.5	2.7	87	203	222	306
A_G	A	*L. gracilis*	6.1	17.5	291	136	378	409

concentrations, total ionic strength and pH from sites supporting these populations. Since the populations came from fairly distinct habitats, an average value for each element was obtained to make the final solution. A few plants from each population were initially grown in this solution to confirm suitability for growth for plants originating from the various populations.

The composition of the nutrient solution used in the current study is listed below. Macronutrients were added as (mM): 0.1 KH_2PO_4; 1 $MgSO_4.7H_2O$; 0.5 $CaCl_2.2H_2O$; 0.5 NH_4NO_3. Micronutrients were added as (μM): 20 FeEDTA; 50 H_3BO_3; 12 $MnSO_4.H_2O$; 1 $ZnSO_4.7\ H_20$, $CuSO_4.\ 5H_20$, $NiSO_4$; 0.2 $Na_2MoO_4.2H_2O$; 62.5 $Na_2\ SO_4$. The pH of the nutrient solution was adjusted to approximately 6.0 using 0.5 $Ca(OH)_2$. Thus, the final concentrations of the ions of interest, i.e., Na^+ and K^+, in nutrient solution were 125 and 100 μM, respectively.

The nutrient solution was changed once in 10 days initially and every week once the plants were approximately 1 cm tall. Plants were maintained in a walk-in environment room at 26°C (day) and 18°C (night) cycles; photo-period 16 hr (light) and 8 hr (dark) cycles. Light was provided by 8 Duro-Test Vita Lite (40 W) fluorescent tubes with a spectral composition similar to sunlight (380 $\mu mols^{-1}cm^{-2}$ at plant level). Plants were grown under these conditions for 40 days from germination before the ion depletion study was initiated.

3.2.1.2. Net uptake rates—Each styrofoam raft was removed from the tub and placed in fresh solution for approximately 15 minutes to equilibrate roots to the fresh uptake solution. Subsequently, each flat was placed in 200 mL of solution, from which 1 mL was

withdrawn immediately after transferring the plants (T_0) to measure the initial concentration of Na^+ and K^+. One mL of DDW was then added back to restore the initial volume. Each container was then weighed in order to compensate for any loss of volume due to transpiration by corresponding additions of DDW. Subsequently, 1 mL of the medium was taken 1 hour after the transfer, and then every 3 hr for 24 hr. At every measurement, 1 mL of DDW was added back to the container; also, the container was weighed and DDW was added to replace water lost due to transpiration. Net uptake rates expressed are means (± SE) of six replicates (n=6). The experiment was repeated on three consecutive days. At the beginning and end of the depletion study, several plants were harvested from each of the extra rafts to determine relative growth rates.

Concentrations of Na^+ and K^+ were measured using IL 443 and IL 943 Flame Photometers (Instrumentation Laboratory Inc., Lexington, Massachusetts). After the depletion study, flats containing the plants were placed in 0.5 mM $CaSO_4$ solution for 5 minutes to remove Na^+ and K^+ from the free space. Plants were then harvested, separating the roots and shoots and then centrifuged to remove surface water prior to determining fresh weight. The plant material was then dried in a forced draft oven at 70°C to a constant weight and dry mass determined. Roots and shoots were then ashed in a muffle furnace at 450°C overnight, and the ash suspended in 10 mL of DDW to estimate total Na^+ and K^+ in shoots and roots.

3.2.2. Experiment 2— Differential response to Mg^{2+} and Ca^{2+}: ion uptake and accumulation

A_C and C_G plants (Jasper Ridge) were grown using the conditions described previously for Experiment 1. Due to limitation in available seed, differential responses

to $Mg^{2+}$and Ca^{2+} were determined only for plants from Jasper Ridge. The concentrations of the ions of interest, i.e., Ca^{2+} and Mg^{2+}, in nutrient solution were 0.5 and 1 mM, respectively. Net uptake rates of Ca^{2+} and Mg^{2+} were determined after 66 days from germination as described above. Uptake rates expressed are means ± SE for three replicates (n=3), each replicate consisting of approximately 10 plants. The experiment was repeated on three consecutive days. At the end of the third day of measurements, plants were harvested after washing the roots in DDW for 1 minute. Roots and shoots were separated and ashed to evaluate total concentrations of the two ions in the roots and shoots. Concentration of Mg^{2+} and Ca^{2+} were determined by inductively coupled plasmaspectroscopy (ICP) using a *Varian* Vista-Pro CCD Simultaneous ICP-OES instrument (Victoria, Australia).

3.2.3. Experiment 3—Differential response in germination, survivorship and tolerance to Na^+ and Mg^{2+}

3.2.3.1. Sodium: Cypselae from the two races at Jasper Ridge (A_C and C_G) were dipped in 1% bleach for 1 minute, washed three times with DDW, and then placed on Petri dishes containing filter paper soaked with 0.1, 10, 50, 100, and 200 mM NaCl solutions, in a dark, cold room (5 °C) for 3 days. Ten cypselae each were placed on five Petri dishes per treatment per race. On the third day, dishes were moved to a walk-in environment room (conditions as in Experiment 1) and germinants counted on day 8 from sowing. Cypselae showing emergence of radicles were considered successfully germinated. The NaCl concentration at which germination was reduced by 50% (I_{50} value) was calculated for both races using either a linear or exponential regression analysis.

Seedlings were then placed on a mesh (0.5mm) attached to a floating device and placed in plastic containers (6 x 6 x 7 cm) containing nutrient solutions (identical in composition to Experiment 1) supplemented with the appropriate NaCl concentration. The initial number of seedlings per treatment per race ranged from 25-40. On day 14 from transplant, the seedlings were counted to estimate survivorship.

For each surviving seedling, the length of root was measured. Using either linear or exponential regression analyses, tolerance indices or I_{50} values (NaCl concentration at which root length was reduced by 50 percent) were estimated for the two races (Macnair, 1983; Ashraf *et al.*, 1989).

3.2.3.2. Magnesium: Using a similar protocol as for Na^+, germination, survivorship, and tolerance were estimated for plants from Jasper Ridge (A_C and C_G) exposed to 1, 5, 10, 20, and 50 mM $MgSO_4$.

3.3. Results

3.3.1. Experiment 1— Differential responses to Na^+ and K^+: uptake and accumulation

Table 3.2 lists the mean ion uptake rates ($\mu mol\ h^{-1}g^{-1}$ fwt) for the two races from the two species. Rates of Na^+ uptake by edaphic race C of both species (C_C and C_G) were barely detectable even during the light period, while they absorbed K^+ at relatively high rates (Table 3.2). By contrast, edaphic race A of both species (A_C and A_G) absorbed Na^+ at rates comparable to their K^+ uptake rates. Race A differed from race C in two other respects: firstly, in A_C and A_G uptake of Na^+ continued during the dark period albeit at

lower rates (18% in A_c and 65% in A_G, of the rates during the light period), while in C_C and C_G net uptake was almost non-existent during the dark period. During the light period, unlike Na^+, rates of K^+ were similar between the two edaphic races of the same species (A_C and C_C) but interspecific differences were significant, e.g. K^+ uptake rate of A_G was > 2-fold that of A_C (Table 3.2).

Accumulation and root/shoot partitioning patterns of Na^+ differed markedly between the edaphic races, irrespective of the species. A_C and A_G accumulated 2-3 times more Na^+ in the shoot than C_C and C_G (Figure 3.1a). In all populations, root Na^+ concentrations were much higher than the shoot concentrations (Figure 3.1a,d). Interestingly, the differences observed between the two races in rates of uptake and accumulation of Na^+ persisted in older (70 d) plants (data not shown). Potassium concentrations of roots and shoots were not significantly different between the edaphic races A and C; however, A_G accumulated significantly more K^+ than the other three populations (Figure 3.1b). In contrast to the situation for Na^+, K^+ concentration in the shoot was higher than that in the root in all populations; but the differences between root and shoot K^+ were relatively small (Figure 3.1b,d) compared to the differences observed for Na^+ (Figure 3.1a,d). Shoot weight/root weight ratios (~ 4) were not significantly different between the species or between the races. When shoot weight/root weight ratio of these species is taken into account, it is clear that > 85% of the total K^+ absorbed is translocated to the shoot, compared to < 50% in the case of Na^+. It is noteworthy that in both race A populations (A_C and A_G), a significantly greater proportion of Na^+ (48 and 49%, respectively) was translocated to the shoot than in the race C populations (30% in

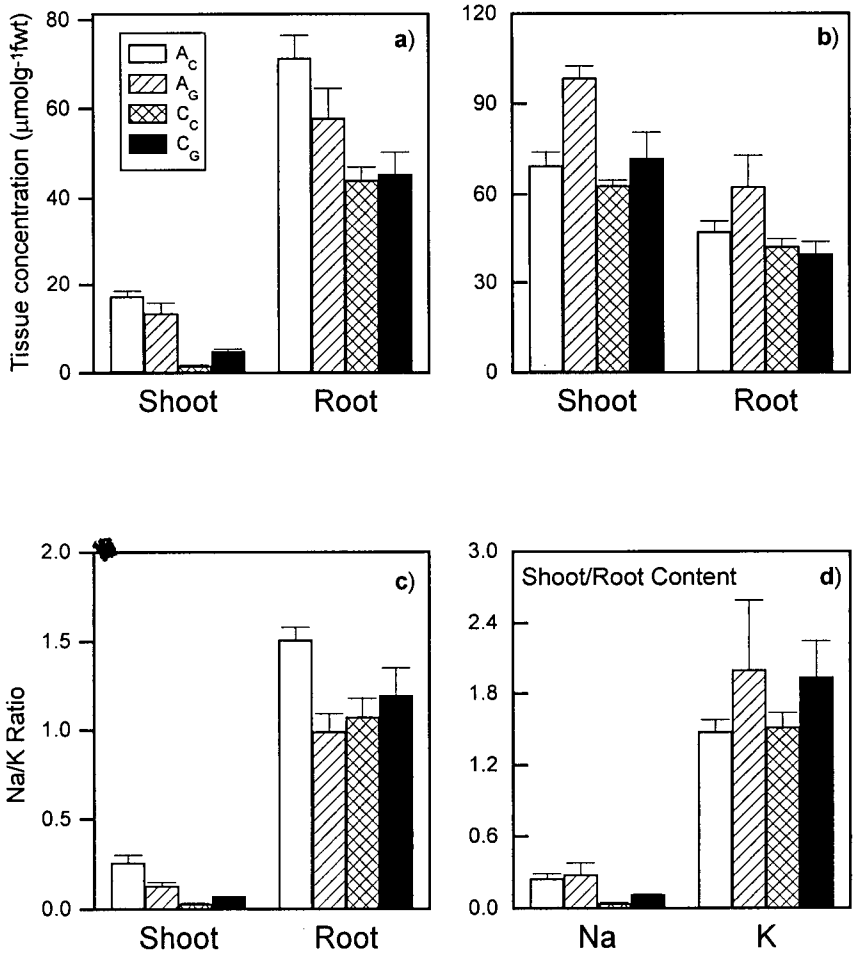


Figure 3.1. Figure 1. Total tissue Na^+ (a), K^+ (b) and Na^+/K^+ ratio (c) and Shoot/Root content ratio for Na^+ and K^+ (d). Bars represent means (± SE). Multiple comparison of means (Tukey Test) suggest significant mean differences for shoot Na^+: A_C, $A_G > C_C$, C_G ($P < 0.001$); root Na^+: $A_C > C_G$, C_C ($P < 0.001$); shoot K^+: $A_G > A_C$, C_G, C_C; root Na^+/K^+: $A_C > A_G$ ($P < 0.05$); shoot Na^+/K^+: $A_C > C_G$, A_G, C_C ($P < 0.001$) and $A_G > C_C$ ($P < 0.01$); and shoot/root Na^+: A_C, $A_G > C_C$ ($P < 0.05$).

Table 3. 2. Mean ion uptake rates (μmol h^{-1} g^{-1} fwt) ± SE during Day (8:30-20:30) and Night (20:30-8:30) for race A and C populations of *L. californica* and *L. gracilis*. Means denoted by different superscripts within a column are significantly different (t-Test or Tukey multiple comparison test: $P < 0.05$). Day and Night values are analyzed separately. For Na^+ and K^+, F (ANOVA) values and probabilities are given while for Ca^{2+} and Mg^{2+} t values and probabilities given. n=18 for Na^+, K^+ and n=9 for Ca^{2+}, Mg^{2+}. ND = not determined.

Population		Na^+	K^+	Ca^{2+}	Mg^{2+}
A_C	Day	0.76^a (± 0.11)	1.17^a (± 0.08)	0.61^a (± 0.37)	1.18^a (± 0.29)
	Night	0.37^x (± 0.11)	0.21^x (± 0.06)	0.32^x (± 0.22)	0.914^x (± 0.11)
A_G	Day	1.84^c (± 0.5)	2.67^b (± 0.27)	ND	ND
	Night	0.57^x (± 0.66)	1.75^y (± 0.24)		
C_G	Day	-0.01^b (± 0.06)	1.27^a (± 0.1)	0.32^b (± 0.34)	0.55^b (± 0.7)
	Night	0.02^x (± 0.09)	0.09^x (± 0.04)	-0.06^x (± 0.02)	-0.22^y (± 0.13)
C_C	Day	0.06^b (± 0.03)	1.05^a (± 0.14)	ND	ND
	Night	0.04^x (± 0.02)	0.01^x (± 0.01)		
F/t	Day	8.07	15.53	10.06	4.65
P		0.001	0.001	0.005	0.02
F/t	Night	0.439	32.04	1.65	6.65
P		>0.05	0.001	>0.05	0.02

C_G and 12% in C_C). The racial differences between Na^+ and K^+ accumulation patterns are well illustrated by Figures 3.1a-d.

3.3.2. Experiment 2— Differential responses to Mg^{2+} and Ca^{2+}: ion uptake and accumulation

Table 3.2 shows ion uptake rates for Ca^{2+} and Mg^{2+} in race A and C plants from Jasper Ridge (A_C and C_G). Uptake rates for both ions are two-fold greater in A_C than C_G. During the dark period of the diurnal cycle, rates of Mg^{2+} and Ca^{2+} uptake declined in A_C by 23% and 48%, respectively; compared to C_G where uptake had ceased completely.

Figure 3.2a,b show total tissue concentrations for Ca^{2+} and Mg^{2+}, respectively, for 70-day old A_C and C_G plants. A_C had ~ 127 fold higher Ca^{2+} and ~ 28 fold higher Mg^{2+} in its shoot than C_G, however, in the roots concentrations of both ions were similar in the two populations (Figure 3.2a,b). The populations also showed marked differences in their ion allocation patterns: A_C freely translocated the two cations to the shoots while C_G localized the two ions in its root (Figure 3.2d). Further, the shoot Ca^{2+}/Mg^{2+} ratio (Figure 3.2c) was significantly higher in A_C (~1) compared to C_G plants (~0.2). It is noteworthy that A_C translocated > 95% of the two cations to its shoot while C_G translocated only 36% of Ca^{2+} and 54% of Mg^{2+} to its shoot.

3.3.3. Experiment 3— Differential responses in germination, survivorship and root elongation to Na^+ and Mg^{2+}

Germination, survivorship and root elongation responses of 14-d old race A (A_C) and C (C_G) plants (seed source: Jasper Ridge) to varying external Na^+ or Mg^{2+} concentrations are shown in Figure 3.3a-f. For each parameter, the results were subjected

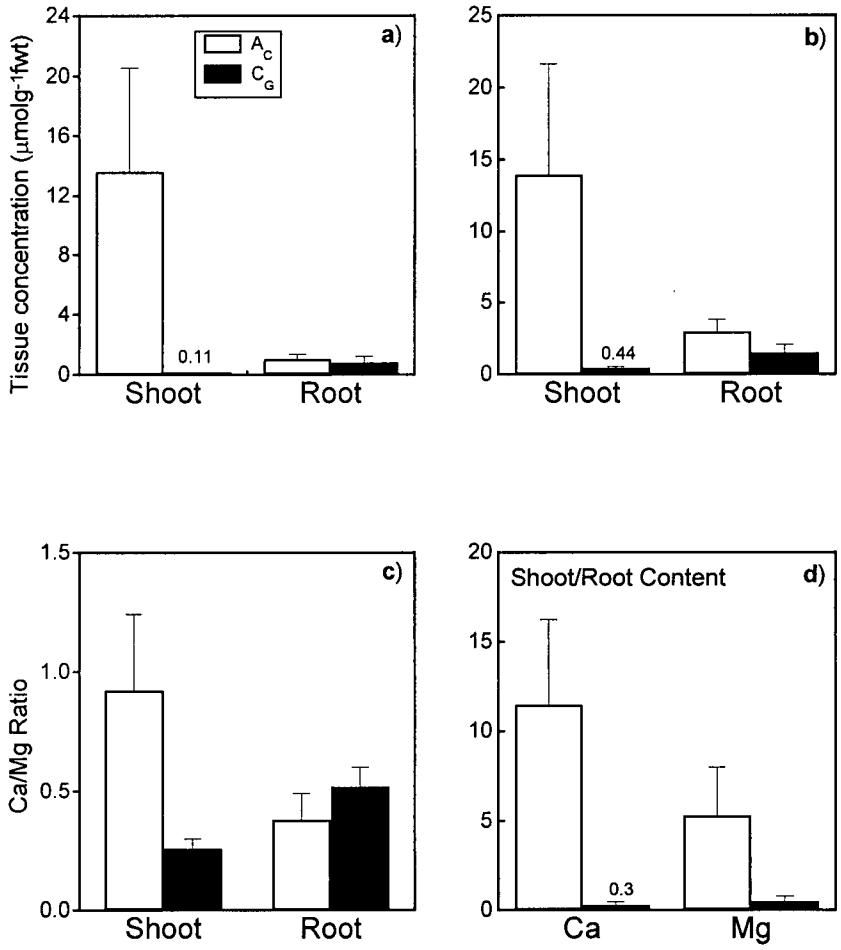


Figure 3.2. Total tissue Ca^{2+} (a), Mg^{2+} (b) and Ca^{2+}/Mg^{2+}ratio (c) and Shoot/Root content ratio for Ca^{2+} and Mg^{2+} (d) for Jasper Ridge plants. Bars represent means (± SE). Significant mean differences (t-Test) observed for shoot Ca^{2+}: $A_C > C_G$ ($P < 0.03$); shoot Mg^{2+}: $A_C > C_G$ ($P < 0.05$); shoot Ca^{2+}/Mg^{2+}: $A_C > C_G$ ($P < 0.05$); and shoot/root Ca^{2+}, Mg^{2+}: $A_C > C_{CG}$ ($P < 0.05$).

to a Two-Way ANOVA separately for Na^+ and Mg^{2+} (Table 3.3). Note that in the 200 mM Na^+ and 50 mM Mg^{2+} treatments both races showed virtually no germination in most replicates, and root elongation measurements in these treatments represent the few surviving seedlings; thus these treatments were excluded from statistical analyses. In all cases, genotype (G) x treatment (T) interactions for both Na^+ and Mg^{2+} were significant. Treatment had significant independent effects in all cases; however, a significant independent G effect was only observed in the case of root elongation under Mg^{2+}. The most notable feature of the data was that at low concentrations of both ions, according to all three parameters examined, race C performed significantly better than race A. Race C, however, was more susceptible to increases in Na^+ and Mg^{2+} concentrations ($\geq$ 50 and 10 mM, respectively) and, at these higher concentrations, performance of race A was superior to race C. For both Na^+ and Mg^{2+}, in germination as well as root elongation, I_{50} for race A was > 2 times that for race C (Table 3.4).

All three measures described above suggest considerable differences between Jasper Ridge A (A_C) and C (C_G) plants in their response to NaCl and $MgSO_4$, with A_C clearly appearing to be the more tolerant of the two populations. The significant G x T interaction for both cations confirms that the two edaphic races from Jasper Ridge are differentiated in their tolerance responses. Interestingly, at both 100 and 200 mM concentrations of NaCl and the 20 and 50 mM treatments of $MgSO_4$, shoots of A_C plants showed signs of toxicity and the plants resembled the phenotype (stunted, succulent) of those race A populations found on some coastal bluffs (Rajakaruna, pers. obs.).

Table 3.3. Tolerance Index (I_{50} values in mM) for germination and root length for the two races from Jasper Ridge. Linear[1] and exponential[2] models utilized to obtain R^2 values. * No R^2 value for root length in A_C / $MgSO_4$ since a significant change was only observed between two (20-50 mM) treatments.

Population/Treatment	*Germination*	*Root length*
A_C / NaCl	139 mM	71.7 mM
	(R^2 =0.99; $P < 0.001$) [1]	(R^2 =0.97; $P < 0.01$) [2]
A_C / $MgSO_4$	27.8 mM	46.2 mM*
	(R^2 =0.99; $P < 0.001$) [2]	
C_G / NaCl	38.2 mM	29.4 mM
	(R^2 =0.99; $P < 0.001$) [2]	(R^2 =0.94; $P < 0.01$) [2]
C_G / $MgSO_4$	12.3 mM	19.2 mM
	(R^2 =0.99; $P < 0.001$) [2]	(R^2 =0.99; $P < 0.01$) [2]

Table 3.4. Results of Two-Way ANOVA for germination and root length (tolerance) for Jasper Ridge A (A_C) and C (C_G) plants grown under NaCl and $MgSO_4$ treatments. Values given are sum of squares (SS) and significance levels; ns = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.001$.

Treatment Variable	*Genotype (G)*	*Treatment (T)*	*G x T*
NaCl Germination	1.55 ns (*df*=1)	7.49* (*df*=3)	5.32* (*df*=3)
$MgSO_4$ Germination	0.32 ns (*df*=1)	359.5** (*df*=4)	24.3* (*df*=4)
NaCl Tolerance	0.06 ns (*df*=1)	5.5** (*df*=2)	1.07** (*df*=2)
$MgSO_4$ Tolerance	1200.5** (*df*=1)	38022.7** (*df*=4)	3101.5** (*df*=4)

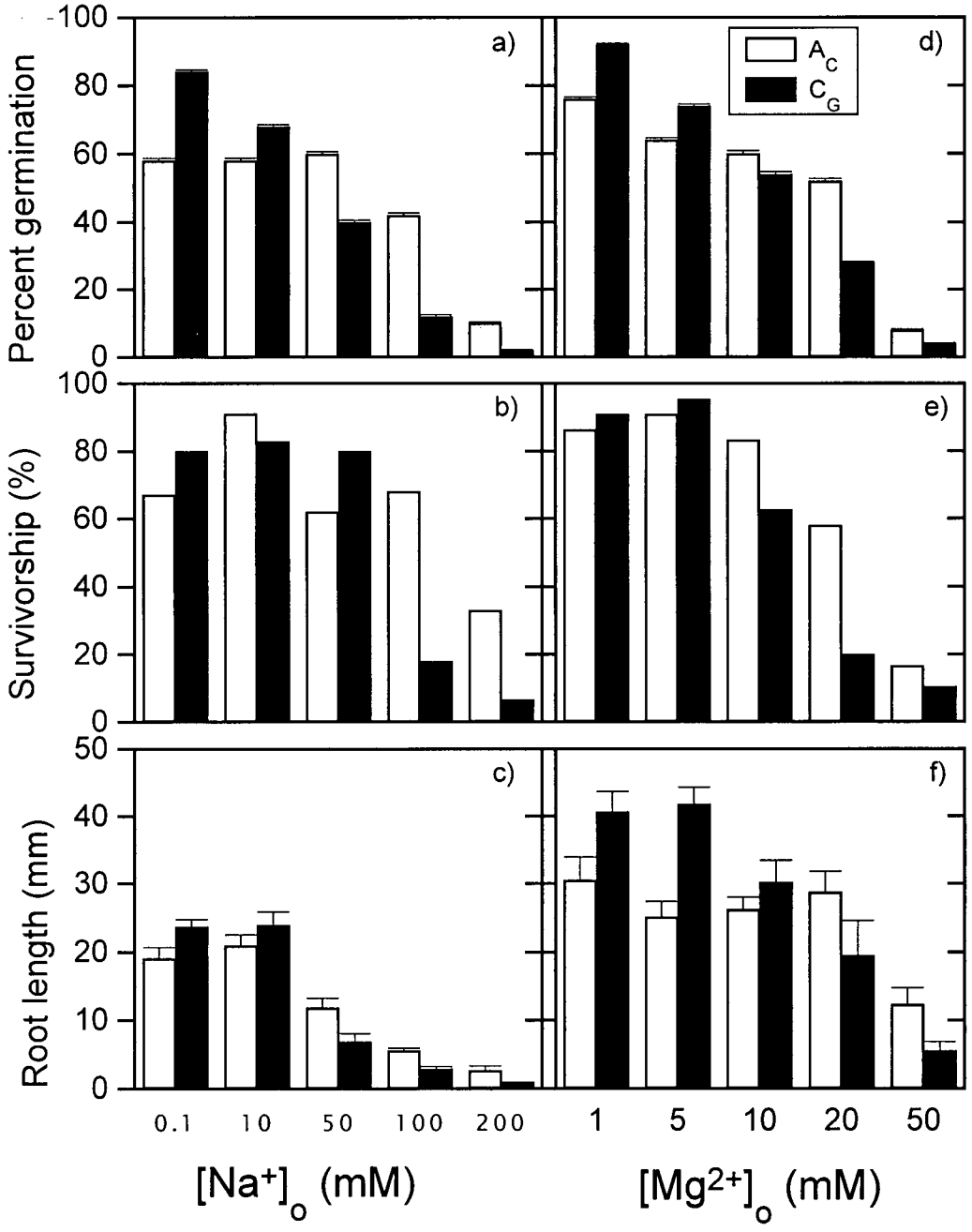


Figure 3.3. Measures for tolerance to NaCl (a. germination; b. survivorship; c. root length) and $MgSO_4$ (d. germination; e. survivorship; f. root length) for race A (A_C) and C (C_G) plants from Jasper Ridge.

3.4. Discussion

Previous studies have suggested cross-resistance to harsh edaphic conditions such as serpentine and saline habitats (Kruckeberg, 1954; Ferreira, 1963; Proctor, 1971; Goodwin-Bailey *et al.,* 1992) and serpentine and mine tailings (Baker *et al.*, 1992; Macnair and Gardner, 1998; Brooks, 1998; Gardner and Macnair, 2000) by several species. Salt flats and serpentine habitats are characterized by harsh environmental conditions, such as high soil surface temperatures, poor soil structure, low osmotic potentials and high concentrations of specific ions, including Mg^{2+} (Wu, 1981; Kruckeberg, 1984; Flowers *et al.,* 1986; Fitter and Hay, 1987; Brooks, 1987). Jenny (1980) suggests that successful colonization of harsh environments such as serpentine habitats requires tolerance to the *serpentine syndrome* characterized by multiple stresses. Once accommodation to one harsh environment has been achieved, however, a plant can successfully venture onto another similarly harsh environment (i.e. preadaptedness), although adaptations to specific local conditions will determine the ultimate success in the new habitat.

The findings of the present study confirm that racial differences in ion accumulation, previously observed under field conditions, are not exclusively the outcome of differences of soil ionic composition, since they are sustained even under identical growth conditions in the laboratory. This strongly suggests a genetic basis for the observed patterns of ion accumulation. Race A plants, regardless of the species, are clearly more tolerant of potentially-toxic Na^+ concentration. In the case of Mg^{2+}, race A plants from Jasper Ridge (A_C) are more tolerant than the race C plants (C_G) from this site and it is tempting to speculate that Mg^{2+} tolerance is also a feature common to race A

plants, irrespective of the species. Both Na^+ and Mg^{2+} are prevalent in some or all of the habitats where race A is found and our measures for germination, survivorship, and root length have shown that race A from Jasper Ridge is better adapted to grow in Na^+- and Mg^{2+}- rich habitats. For both cations, there was a significant G x T interaction, suggesting that the two races are differentiated in their tolerance responses. This tolerance is not based upon ionic exclusion, because root absorption and shoot accumulation of Na^+ and Mg^{2+} by race A plants was substantially higher than by race C plants.

Previous studies on plant-soil relations provide examples of intra-specific differences of accumulation of and/or tolerance to Na^+ (Ashraf *et al.*, 1986a, 1986b, 1989), K^+ (Siddiqi and Glass, 1983a,b; Flowers and Lauchli, 1983), Ca^{2+} (Snaydon and Bradshaw, 1961, 1969; Snaydon, 1962; Ramakrishnan and Singh, 1966), Mg^{2+} (Main, 1974), and heavy metals (Antonovics *et al.*, 1971). The case with *Lasthenia* is intriguing, however, because we find parallel physiological changes in two closely-related cryptic species. Schat *et al.* (1996) provide one of the best examples to date of parallel genotypic changes in tolerance to an edaphic extreme within a plant species. Studies of copper tolerance in *Silene vulgaris* Garcke (Caryophyllaceae) show population specific alleles for copper tolerance in geographically isolated populations, suggesting independent evolution of the same genetic loci at different localities. My studies add to this list whereby physiological traits (i.e., tolerance to Na^+ and Mg^+) responsible for adaptation to extreme edaphic conditions such as saline/serpentine habitats appear to have evolved in parallel in two closely related species. It is unclear at this stage whether identical genetic changes are also responsible for these differences. However, the putatively parallel physiological changes in edaphic races of *L. californica sensu* Ornduff. raises questions

about the intriguing correlation previously discussed between ion physiology and flavonoid chemistry (Chapter Two).

Plants avoid ionic toxicity by either excluding toxic ions or by accumulation and sequestration (Fitter and Hay, 1987). In the case of Na^+, some tolerant species are able to maintain higher concentrations in the shoot and sequester the ions in the vacuole (Binzel *et al.*, 1988; Amtmann and Sanders, 1999; Maathuis and Amtmann, 1999) via Na^+/H^+ antiporters (Apse *et al.,* 1999; Blumwald *et al.,* 2000). This necessitates the generation of compatible solutes within the cytosol to compensate for adverse osmotic effects of high vacuolar Na^+ concentrations (Flowers *et al.,* 1977, 1986). While glycophytic (i.e., non-halophytic) species depend primarily on the exclusion of Na^+ at the plasma membrane, halophytic species accumulate large amounts – up to 700 mM – in the vacuole (Flowers *et al.,* 1977) by the use of the Na^+/H^+ antiporter (Barkla and Blumwald, 1991; Barkla *et al.,* 1994). In *Plantago*, the vacuolar Na^+/H^+ antiporter activity is only present in the salt-tolerant *P. maritima* L., but not in the glycophytic *P. media* L. (Staal *et al.,* 1991) and this difference is thought to be critical in the ecological divergence of these two species. Although both races in our study restricted a greater proportion of internal Na^+ to their roots, race A plants were clearly able to accumulate greater concentrations of Na^+ in the above-ground tissues than race C plants (~5 fold) and translocate ~ 50% of Na^+ to the shoot, showing greater tolerance to this ion. Whether the presence/absence or level of expression of the Na^+/H^+ antiporter gene is responsible for the differences in uptake and accumulation is an area worthy of investigation. Such a study is essential in order to address the underlying biochemical/genetic basis of these traits.

Serpentine substrates are characterized by high concentrations of Mg^{2+}, a factor that appears to have a major influence on the ecology of serpentine vegetation (Proctor and Woodell, 1975; Brooks, 1987; Baker *et al.*, 1992). Chelation of Mg^{2+} by soluble carboxylates appears to play an important role in vacuolar accumulation and sequestration of this ion (Woolhouse, 1983) and has been shown to be an important mechanism for tolerance in *Sedum anglicum* Hudson (Crassulaceae) found in serpentine and other Mg^{2+}-rich soils (Tibetts and Smith, 1992). Race A plants from Jasper Ridge translocated greater concentrations of Mg^{2+} to the shoots than race C plants (> 95% in A compared to 54% in C) and it is possible that tolerance mechanisms such as those documented for *Sedum* also exist in race A plants, allowing them to accumulate higher concentrations of potentially cytotoxic Mg^{2+} in their shoots.

Of the other two cations tested, K^+ and Ca^{2+}, K^+ is typically the major cation in plant tissues (Glass, 1988). This fact may account for the relatively similar uptake rates and accumulation of K^+ in the two races. However, for Ca^{2+}, the uptake rate and the accumulation level in the shoots were 2-fold and 124-fold higher, respectively, in race A plants from Jasper Ridge. It is noteworthy that race A translocated > 95 % of total internal Ca^{2+} to its shoot compared to only 36% in race C. Higher Ca^{2+} uptake and accumulation levels have been previously reported for other taxa found in Ca^{2+}-poor serpentine soils (Madhok and Walker, 1969), suggesting that race A is perhaps better equipped to deal with serpentine soils.

Plants typically have highly selective uptake systems, capable of distinguishing physicochemically similar pairs of ions such as Na^+/K^+ and Ca^{2+}/Mg^{2+}. At low K^+ concentrations (normally under 0.5 mM), Na^+/K^+ selectivity of barley roots is high, but at

high concentrations, this selectivity is only found in halophytes (Epstein, 1969). The primary effect of high Na^+ is in its negative interaction with K^+, an ion that is required by all plants (Flowers and Lauchli, 1983; Maathuis and Amtmann 1999; Schachtmann, 2000). One of the key elements in salinity tolerance is the capacity to maintain a high cytosolic K^+/Na^+ ratio regardless of the external concentrations of these two ions (Yeo, 1998). Similarly, at low Ca^{2+}/Mg^{2+} ratios in soils, such as those found in serpentine habitats, excess Mg^{2+} accumulates in tissues of non-tolerant plants with toxic effects (Proctor, 1971). The general paucity of species in serpentine habitats is thought to result from this condition (Baker and Walker, 1990) although various other features, biotic and abiotic, (Kruckeberg, 1984; Walker, 2001) also contribute to exclusion of species from serpentine habitats. Limited studies show that species that are found in serpentine habitats take up Ca^{2+} and Mg^{2+} to a greater extent in proportion to the external concentration than do susceptible genotypes, suggesting that the resistance mechanism is internal (Walker, 1954; Walker *et al.*, 1955; Madhok and Walker, 1969; Lyon *et al.*, 1971; Main, 1974). This is clearly the pattern seen in race A plants from Jasper Ridge, the race that predominates in serpentine habitats. Race A has ~ 127 fold higher Ca^{2+} and ~ 28 fold higher Mg^{2+} in its shoot than race C and is able to maintain a favorable Ca^{2+}/Mg^{2+} ratio (~1) despite the higher external Mg^{2+} concentrations. Extensive field collections also suggest that race A is clearly more tolerant of adverse Ca^{2+}/Mg^{2+} ratios (Rajakaruna and Bohm, 1999). In other species, for example in *Secale* "rye" (Olsen, 1971), ability to withstand low Ca^{2+}/Mg^{2+} lies in the ability to discriminate in favor of Ca^{2+}; this same phenomenon is applicable to *Helianthus bolanderi* subsp. *exilis*, the serpentine endemic sunflower (Madhok and Walker, 1969). These few studies to date show that differences

observed between the serpentine and non-serpentine populations may not be due to a single mechanism but, rather, a combination of several possible mechanisms, i.e., differences in root morphology, uptake, translocation, and interactions between cations. It will be important to look at K^+/Na^+ and Ca^{2+}/Mg^{2+} selectivity and tolerance under different external concentrations of these ions to determine if the edaphic races from both species differ in their capacity to maintain favorable ion ratios.

An important aspect of my eco-physiological studies that needs further investigation is the ability of the races to withstand low osmostic potentials. Although I have not determined water potential for soils where the races grow, it is clear from other parameters (Rajakaruna and Bohm, 1999) and field observations that race A plants grow in soils with a lower osmotic potential. Ability to accumulate and tolerate greater concentrations of cations such as Na^+, Mg^{2+}, and Ca^{2+} will undoubtedly allow race A plants to survive under osmotically-harsh conditions in which they are often found. Race C plants generally are not found in habitats such as coastal bluffs, serpentine soils, and alkaline flats (Rajakaruna and Bohm, 1999). It is possible that in addition to the lack of tolerance to specific ions and/or ability for osmotic adjustment may be influential in this clear-cut distribution.

Chapter Four further examines ecophysiological differentiation in the *L. californica* complex, seeking evidence for differences in reproductive strategies in response to water stress, an environmental variable as critical as ionic stresses in the natural habitats of these plants.

CHAPTER FOUR

Adaptive divergence in response to water stress in two edaphic races of the *L. californica* complex

4.1. Introduction

Fitness depends on an interaction of the phenotype with the environment. When closely related sets of populations (e.g., species, subspecies, races) overlap in geographic distribution and occupy distinct habitat types, it is reasonable to hypothesize that each may achieve its distribution through differential relationships of relevant environmental variables with fitness, i.e. through differential adaptation (Wade and Kalisz, 1990; Dudley, 1996a, b; Sultan, 2001). Reaction norm data can be pertinent to studies of adaptation if it can be shown that populations differ in their fitness response to variables that are known to distinguish their habitats (Marshall *et al.,* 1986; Zhang and Lechowicz, 1994; Schmitt *et al.,* 1992; Schmitt, 1993; Dudley, 1996a). An adaptive hypothesis remains tenable if individuals achieve higher fitness under the environmental conditions that best match their natural environment. Once candidate environmental variables are found to be associated with differences in fitness, more detailed analysis of selection gradients can be used to infer the role of natural selection in achieving fitness differences under different environmental conditions (Wade and Kalisz, 1990; Sultan, 1995; Dudley, 1996a, b; Pigliucci and Schlichting, 1996).

Species that occur under a range of water availability regimes may exhibit intra-specific differences associated with temporal or spatial heterogeneity in soil environments. A classic example of this phenomenon is wild oats, *Avena barbata* L., where different genotypes are characteristic of contrasting soil water environments

throughout the Californian Floristic Province (Clegg and Allard, 1972; Hamrick and Allard, 1972). On a single hillside where conditions range from mesic to xeric, genotypes are found associated with particular habitats, and selection acts to maintain this distribution despite considerable gene flow (Hamrick and Holden, 1979). Differential responses to water availability are thought to play a role in the ecological divergence of closely related taxa (Roy and Mooney, 1982; Farris, 1987, 1988; Aronson *et al.,* 1993; Sultan and Bazzaz, 1993).

Water availability is critical to the timing and extent of reproduction in chaparral communities (Chiariello, 1989; Girdler, 1999), and this is particularly acute on serpentine soils, where the extreme ionic composition further complicates water uptake. In serpentine environments, physiological stress results from low availability of water, high ionic content of the soil solutions, or a combination of both (Proctor and Woodell, 1975; Kruckeberg, 1984; Baker *et al.,* 1992; Proctor, 1999). These effects are exacerbated by high soil surface temperatures and the shallow, rocky nature of serpentine soils. On the serpentine outcrop at Jasper Ridge, the two races of *L. californica sensu* Ornduff occupy distinct habitats that differ in water holding capacities (Rajakaruna and Bohm, 1999). Race C plants (assigned to *L. gracilis*) occupy the upper reaches of the outcrop, an environment that is ionically more moderate than the lower reaches, but tends to dry out more rapidly. Race A plants (assigned to *L. californica* subsp. *californica*) occur at the bottom of the outcrop, which is ionically more extreme but only dries out at the end of the growing season. Reciprocal growth studies conducted in the greenhouse using field-collected soils have shown that race C plants are unable to grow to reproductive maturity in the ionically-extreme soils of race A plants (Rajakaruna and Bohm, 1999). Field

observations and greenhouse studies have indicated that the races may respond differentially to water stress. For example, race C plants consistently germinate 2-3 days and flower 7-10 days prior to race A plants. Faster growth to reproductive maturity may allow race C plants to avoid drought (Fox, 1990; Aronson *et al.*,, 1992). Observations have also revealed that the races differ in their biomass allocation patterns; race A appears to allocate more mass to roots than shoots, consistent with patterns often seen in plants growing under nutrient-poor, ionically-stressed conditions (Kramer, 1980; Grime, 1994). Further, towards the end of the growth season, race A plants are also exposed to drought and the greater root/shoot ratio may be advantageous during the latter part of its life cycle.

In this study, I used populations of the two edaphic races collected in distinct microhabitats at Jasper Ridge to explore the relationship between fitness and other phenotypic traits under differing water regimes. While ionic stresses of serpentine soils are generally emphasized in the literature (Baker *et al.*, 1992), I chose to explore the effects of water stress in isolation because available water for uptake is known to be critical in ionically harsh environments (Fitter and Hay, 1987; Hughes *et al.*, 2001). I hypothesize that adaptation to the differing water availabilities experienced by the two races will have produced differentiated responses to water stress. I predict that the two races will show differentiation in reproductive strategies related to their response to water stress. Specifically, I expect race C plants to be better adapted to severe water stress and to achieve higher fitness in this environment than race A plants. As race A plants generally experience severe water stress only at the end of the growing season, I predict that they will be less well adapted to long-term drought and will have lower reproductive

fitness under these conditions. In order to test this hypothesis, I have performed a greenhouse experiment to characterize the reaction norms of the two races exposed to a gradient in water availability.

4.2. Materials and Methods

4.2.1. Experimental design

Cypselae were collected randomly from plants along three previously established transects (Rajakaruna and Bohm, 1999) and from plants found between the transects in order to sample plants growing in the range of microhabitats represented on the ridge. The area of seed collection represented approximately 60 x 100 m of the serpentine outcrop. At least two flower heads from approximately 100 individual plants from both races were targeted and the cypselae were pooled in large envelopes. From this pool of approximately 10,000-20,000 (each flower head has ~ 50-100 cypselae) mixed cypselae, 10 were randomly chosen to be sown on each pot. Thirty pots (4x4x4 inches) were filled with potting mix (Terra Lite Soil Mix, W. R. Grace and Co. of Canada Ltd., Ajax, Ontario, Canada) and watered with tap water until the soils were saturated. The pots were moved to a dark, cold room for three days (5°C), after which they were moved to random bench locations in a growth chamber (18°C/day, 13°C/night; humidity 55-75%; light 382 $\mu Mols^{-1}cm^{-2}$). Soils were brought back to saturation by adding 100 mL of tap water per pot and pots were watered every three days with 100 mL of tap water. On the 10^{th} day from sowing, the pots were thinned to five plants approximating field density. On the 17^{th} day from sowing, three water treatments were begun, with five pots per treatment for each of the two races. In the high water treatment, pots were watered every three days

with 100 mL of tap water maintaining soils at or near saturation. In the medium and low water treatments, pots received no further water for a period of six days from saturation, at which time the soils were brought to field capacity by adding 100 mL of tap water per pot. From then on, the medium water treatment consisted of adding 75 mL of water per pot every three days maintaining soils at or near field capacity for the duration of the experiment. In the low water treatment, pots received 100 mL of water every six days to bring the soil to field capacity. The low water treatment appeared to have a similar effect on both races, since a few plants from both races showed signs of wilting at the time of watering. On a few occasions, early in the experiment, low water treatment pots required water on the fifth day to avoid severe wilting and mortality of plants. On the third week from germination, the temperature in the growth chamber was increased to 22°C by day and 18°C by night to correspond more closely with field conditions. The experiment was initiated on June 21, 1998 and was run for a period of two months, until all plants had completed flowering. The experiment was terminated when plants of both races had completed flowering and had reached senescence. Plants were then harvested, separating roots, shoots, and flower heads. Plants were not fertilized during the experiment.

Responses of five variables to the water treatments were recorded for each pot: survivorship, days to flowering, root and shoot dry weights (used to derive total dry weight and root/shoot dry weight ratio), and a measure of reproductive fitness, number of flower heads. Shoot and root dry weights were determined after drying at 80°C for 24 hours in a forced draft oven. The values obtained for each pot were then divided by number of survivors per pot to obtain an average value per individual.

4.2.2. Data analysis

Tests for normality and homogeneity of variance suggested that no special transformations of the data were needed. Statistical analyses of the effects of race, treatment, and race x treatment interaction on the measured response variables were conducted using two-way analysis of variance (ANOVA) implemented in SYSTAT version 10 (SPSS Inc., Chicago, IL). Reaction norm plots were produced to assist in visualizing responses to the different water treatments and Tukey's test was used to compare treatment means of the two races for each phenotypic variable. To test further for differences between the two races in survivorship, Kaplan-Meier Survival Analysis was conducted for each treatment with race as the predictor variable.

In order to explore the relationship between phenotypic traits and fitness, I used selection analysis (Lande and Arnold, 1983) implemented in JMP version 3.2.1 (SAS Institute Inc., Cary, NC). For each race, I performed simple regressions of relative fitness (number of flower heads/mean number of flower heads) in each of the three treatments against each of the non-reproductive variables. The slopes of the regression lines for the two races were compared using analysis of covariance (ANCOVA). Unequal regression slopes indicate a significant interaction between race and the phenotypic trait, suggesting that the relationship between the phenotypic trait and fitness differs between the two races (Kleinbaum *et al.*, 1988). Multivariate selection analysis was precluded by the small sample size per treatment. In an attempt to examine the possible effects of correlation among phenotypic variables on observed trends, I examined Pearson correlation coefficients among traits for each race within each water treatment.

4.3. Results

Except for survival, which was high throughout the experiment, all plant growth variables showed significant ($P < 0.05$) race effects (Table 4.1), indicating that the races are differentiated in the measured traits. Race A plants were generally larger, allocated relatively more biomass to roots, and flowered later than race C plants. Race A plants also produced fewer flower heads, especially under medium and low water treatments (Figure 4.1, Table 4.1).

All variables except survivorship also showed plasticity in response to the water treatments, and trends were mostly similar for the two races (Table 4.1). Both races responded to more severe water restriction by shifting their first flowering earlier, by accumulating less biomass, and allocating relatively more of their biomass to roots (Figure 4.1, Table 4.1). Mortality was low during the course of the experiment with the highest mortality found in the low water treatment for both races (3 for race A; 2 for race C). Kaplan-Meier Survival Analysis suggests that the races do not differ ($P > 0.05$) in survivorship in any of the water treatments.

Only number of flower heads showed a significant Race x Treatment interaction, indicating that the races are differentiated in their plastic response for this trait (Table 4.1). The two races show non-parallel reaction norms for number of flower heads with race C producing significantly greater number of flower heads than race A under the low water treatment (Figure 4.1; Table 4.2).

Table 4.1. Sum of squares values from two-way ANOVAs of race and watering treatment effects on five response variables in *Lasthenia californica*. All analyses based on sample sizes of 30 pots. Significance levels: *ns* = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Variable	Race (*df*=1)	Treatment (*df*=2)	Race x Treatment (*df*=2)	Total R^2
Survival	0.033 ns	1.067 ns	0.267 ns	0.19
Days to flowering	93.63 *	416.27 ***	3.47 ns	0.62
Total dry weight (g)	5.513 **	26.043 ***	0.738 ns	0.73
Root/Shoot dry weight ratio	12.04 ***	10.33 **	0.6 ns	0.64
Number of flower heads	760.03***	2412.2 ***	525.27***	0.87

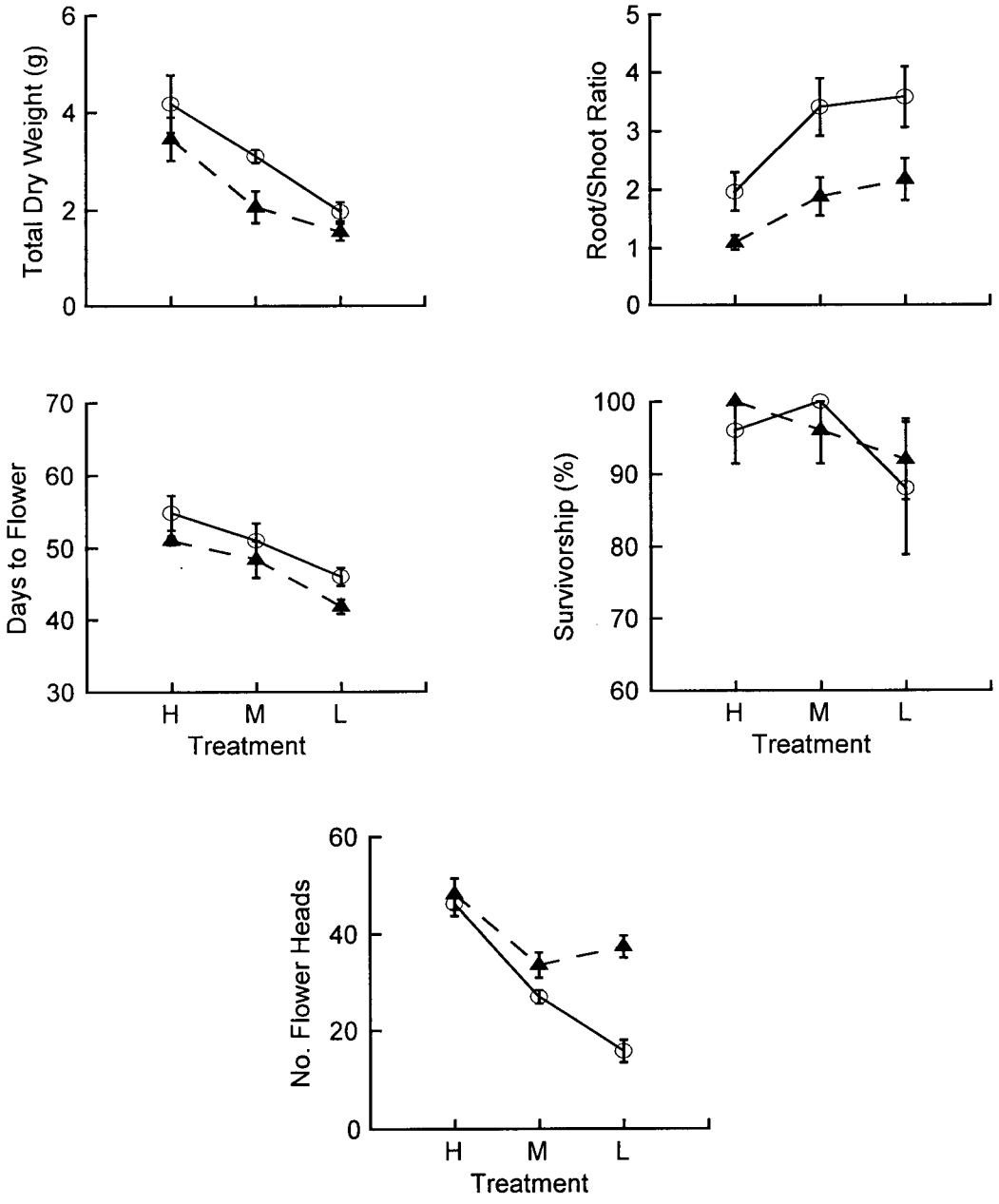


Figure 4.1. Reaction norm plots for the two races of *Lasthenia californica* grown under high (H), medium (M) and low (L) watering treatments. Race A = O; Race C = ▲. Values plotted are means and standard errors. Number of flower heads was the only variable with a highly significant ($P < 0.001$) race x treatment interaction effect.

Univariate selection analysis only detected a significant relationship between relative fitness and survivorship in one treatment level for each race. In the medium water treatment for race C, survivorship was positively correlated with relative fitness (R^2 0.94; $P< 0.05$) while the correlation was non-significant for race A ($P > 0.05$). In the low water treatment, relative fitness was significantly positively correlated with survivorship for race A (R^2 0.9; $P< 0.05$) while the correlation was non-significant for race C ($P > 0.05$). None of the remaining phenotypic variables was significantly correlated with relative fitness. The comparisons of the regression slopes between the two races were non-significant in all cases.

Correlations among phenotypic variables were also mostly non-significant. Significant Pearson correlation coefficients were detected only between root/shoot dry weight ratio and total weight for race A in the low water treatment, and for race C in the medium water treatment.

4.4. Discussion

The patterns observed in the reaction norms, along with knowledge of the environmental conditions under which the two races grow, suggest a straightforward interpretation of the results of this study. I observed that under low water treatments, both race A and race C plants achieve higher root/shoot ratios and tend to flower earlier. However, in race A, these phenotypic shifts are associated with a decrease in reproductive output. Race C plants, however, do not sacrifice flower production under low water treatments (Figure 4.1). These results suggest that race C plants are adapted to tolerate drought conditions; they are able to maintain their reproductive fitness under low water availability. As the

Table 4.2. Multiple comparison of means for the response variables using Tukey's Test. Bars in second column indicate race-treatment combinations not significantly different ($P > 0.05$). H, M, L subscripts are high, medium, and low watering treatments, respectively. Last column shows direction of response for all variables with a significant racial effect; ns = non significant.

Variable	*Race-Treatment (means decreasing ⇨)*	*Race Effect*
Total Dry Weight (g)	$\underline{A_H\ C_H}\ \underline{A_M\ C_M\ A_L\ C_L}$	A > C
Root/Shoot Dry Weight Ratio	$\underline{A_L\ A_M\ C_L}\ A_H\ C_M\ C_H$	A > C
Number of Flower Heads	$\underline{C_H\ A_H\ C_L}\ \underline{C_M\ A_M}\ A_L$	C > A
Days to Flowering	$A_H\ \underline{A_M\ C_H\ C_M\ A_L}\ C_L$	A > C
Percent Survivorship	$\underline{A_M\ C_H\ A_H\ C_M\ C_L\ A_L}$	ns

phenotype of race A is impacted by drought, reproductive fitness declines, as one would predict for plants that rarely experience drought in their natural environment. Because this experiment was performed on seed collected from the wild, some of the differences we observed may be due, in part, to maternal effects (Mousseau and Fox, 1998 but see Lawrence, 1964; Thomas, 1967; Roach and Wulff, 1987 for exceptions). However, these effects are likely minimized by our attempts to sample seeds from throughout the range of microhabitats present at Jasper Ridge. Further, all areas of the ridge, including where race A is found, dry out during seed set and maturation, when maternal effects are most likely to be strongest (Roach and Wulff, 1987). Maternal effects are also most likely to influence seedling characteristics and diminish over time (Thomas, 1969; Hayward and Nsowah, 1969; Schmitt and Antonovics, 1986) and our variables were all measured on adult plants. Hence I assume in the discussion of results that maternal influence is either minimal or uniform in its effect on the two races.

The relationship between phenotype and fitness is mitigated by the environment in which organisms occur, and thus it is critical to attempt to relate phenotypic variation and variation in fitness to the environmental conditions experienced in nature. In this study, the responses that I have observed to varying levels of water availability can clearly be related to the ecology of the two races of the *L. californica* complex at Jasper Ridge, and extended to the conditions experienced throughout the species' range. The two races of *L. californica sensu* Ornduff occur in distinct sets of habitats that can be classified on the basis of water availability and ionic stresses (Rajakaruna and Bohm, 1999). Race A occupies edaphically extreme environments, occurring primarily on coastal bluffs, vernal pools, alkaline fields, serpentine outcrops, and salt flats. Although

the soils in these environments are ionically harsh, the percent clay content is generally high, increasing the water-holding capacity of the soil. Race A plants are often found restricted to moist or even saturated soils in such environments. In contrast, race C populations are found in more ionically "benign" inland environments, along roadsides, in rocky open fields, and pastures. The soils found in these environments are often sandy and shallow, drying out early in the growing season. The conditions at Jasper Ridge mirror the trends seen across the range of the species, with the two races again occupying distinct microhabitats; race A occupies the wet, yet ionically harsh, soils at the bottom of the ridge, while race C occupies the fast-drying yet ionically less stressful upper reaches (Rajakaruna and Bohm, 1999).

The significantly higher root/shoot ratios observed in race A plants in this experiment are typical of plants found under nutrient poor and edaphically harsh environments (Kaufmann, 1972; Kramer, 1980; Crick and Grime, 1987; Grime, 1994). The combination of higher root/shoot ratios and delayed growth relative to race C plants suggest that race A plants are well adapted to growing under nutrient poor and ionically-stressed conditions (Parsons, 1968). The significantly lower reproductive output under the low water treatment may indicate that race A plants are not well-adapted to growing under drought conditions. Such declines in reproductive effort, in response to water stress, have been shown for other drought-intolerant herbaceous annuals (Aronson *et al.*, 1993).

Race C plants have a more restricted regional distribution and, at Jasper Ridge, occupy soils that are drier than those where race A is found (Rajakaruna and Bohm, 1999). The faster growth to reproductive maturity observed under the low water

treatment in our experiment is consistent with observations at Jasper Ridge. In the field, Race C plants are often past anthesis when race A plants begin flowering. Race C appears to be avoiding extreme water stress by completing its life cycle faster, using a strategy often referred to as phenological escape (Mulroy and Rundle, 1977; Fitter and Hay, 1987). This strategy has been documented in many other herbaceous annuals found growing in dry habitats (Monson and Szarek, 1981; Fox, 1990; Aronson *et al.,* 1992). This experiment also revealed that race C plants allocate relatively more of their biomass to reproduction under water stress, which is again consistent with a strategy employed by drought- tolerant herbaceous species (Baker, 1972; Gaines *et al.,* 1974; Monson and Szarek, 1981; Aronson *et al.,* 1990, 1993; Sultan and Bazzaz, 1993). Thus, race C is able to avoid as well as tolerate drought.

I suggest that the failure to detect significant selection gradients within treatments should be interpreted cautiously, as my sample size within treatments was likely insufficient to obtain statistical significance in some of these cases. Similarly, the two significant regressions that were observed between survivorship and relative fitness should also be interpreted conservatively, as few data points may have been important in determining the significance of these relationships. However, the fact that I have detected plasticity in most of the phenotypic traits measured and variation for plasticity in flower production, the estimate of fitness, suggests that a hypothesis of adaptive differentiation is reasonable. As such, I intend to build on these results and seek evidence of significant differences in the direction or intensity of selection in further experiments to be conducted next year.

CHAPTER FIVE

Reproductive character displacement in *Lasthenia*: Building a case for reinforcement and ecological selection

5.1. Introduction

Characterizing the contribution of natural selection to the evolution of reproductive isolation remains a key focus of evolutionary studies in both plants and animals. The emphasis on systems that suggest a role for selection in the evolution of reproductive isolation arises because such systems allow us to probe the mechanisms and stages critical in speciation. However, even when selection is implicated in the development of reproductive barriers, the precise agent of selection can remain intractable (Levin, 1985). The historical, and ongoing, debate surrounding the demonstration of reinforcement serves as a case in point. Selection is commonly invoked to account for reproductive character displacement (*sensu* Howard, 1993, i.e. "a pattern of greater divergence of an isolating trait in areas of sympatry between closely related taxa than in areas of allopatry"). While character displacement is a predicted outcome of selection against hybridization (i.e., reinforcement), it is possible for character displacement to arise in response to other forms of selection including direct selection on parental fecundity, or as a byproduct of adaptation to differing ecological conditions (Howard, 1993; Noor, 1999). This recognition has raised the bar for studies of reinforcement, and recent work has, for example, attempted to examine the potential role of ecological selection in producing patterns of character displacement (Rundle and Schluter, 1998; Higgie *et al.*, 2000).

While a number of examples of reproductive character displacement have been documented in plant and animal systems (see Howard, 1993 for a compilation), additional supportive evidence for reinforcement is less common (but see Thoday and Gibson, 1962; Waage, 1979; Levin, 1985; Rundle and Schluter, 1998; Noor, 1999; Higgie *et al.,* 2000). Examples of reproductive character displacement in plants focus on traits that reduce or prevent interspecific pollen transfer (i.e., premating mechanisms), such as divergence in flower colour and shape that affect pollinator behaviour (Levin and Kerster, 1967; Kruckeberg, 1957; Grun and Radlow, 1961; Grant, 1966; Levin, 1985), mechanisms affecting the ability of pollen to subsequently fertilize ovules (Searcy and Macnair, 1990; Diaz and Macnair, 1999), and on divergence in flowering times (Antonovics, 1968, McNeilly and Antonovics, 1968; Stam, 1983). While reinforcement has been suggested in some of these cases, ecological selection is postulated in others. For example, *Phlox drummondii* has red flowers where it occurs sympatrically with *P. cuspidata*, whereas both species have pink flowers in allopatry. The two species hybridize in the zone of overlap and hybrids are almost completely sterile. Levin (1985) showed that rates of hybridization are reduced in artificial interspecific populations that differ in flower colour compared with interspecific populations with the same flower colour, and postulated that reinforcement is responsible for the observed patterns. McNeilly and Antonovics (1968) demonstrated that flowering time differences reduce hybridization between copper tolerant and non-tolerant parapatric populations of *Agrostis tenuis*. These authors also postulate that reinforcement was involved in enhancing flowering time differences in parapatry, though other authors favor ecological selection as a likely explanation for divergence in flowering time (Macnair and Baker, 1994).

The *L. californica* complex provides an ideal system in which to examine the question of reproductive character displacement. Previous studies at Jasper Ridge suggest that the two species (then considered races) are at least partially reproductively isolated. Isozyme data (Desrochers and Bohm, 1995) indicate low gene flow between the two species at Jasper Ridge (G_{st} = 0.417; Nm = 0.35). There is also evidence suggesting strong prezygotic isolation at the Jasper Ridge site. Firstly, based on a limited number of crosses, Desrochers (1992) observed generally low crossability of the two species at Jasper Ridge. Secondly, flowering times of the two species differ in the parapatric location, further reducing the potential for gene flow (Desrochers and Bohm, 1995; Rajakaruna and Bohm, 1999). Taken together, these data suggest a strong barrier to reproduction in the parapatric location. The extent of reproductive isolation between the species at other localities has not been directly examined. Given evidence of low crossability and strong ecological selection at Jasper Ridge, I undertook a crossing study to look for evidence of reproductive character displacement in this system. I use data from a greenhouse crossing study including data on pollen incompatibility and seed set to isolate the effect of post-pollination, prezygotic isolation on variation in seed set between interspecific crosses involving parapatric and allopatric populations of *L. californica* subsp. *californica* and *L. gracilis*. To my knowledge, no previous studies have considered the pollen incompatibility reactions as evidence for reproductive character displacement.

5.2. Materials and Methods

5.2.1. Crossing study

Seeds collected in 1997 from seven source populations were used to produce plants for the crossing study. These represent the two recently described species of Chan *et al.* (2001) and the two previously described edaphic races (Rajakaruna and Bohm, 1999). In addition to collections of the two species from Jasper Ridge ($C1_A$ - *L. californica*; $G1_C$ - *L. gracilis*) populations representing the northern ($C2_C$ - *L. californica*), central ($G2_A$ - *L. gracilis*; $G3_A$ - *L. gracilis*; and $C3_A$ - *L. californica*) and southern ($G4_A$ - *L. gracilis*) parts of the ranges of the taxa were included. The populations, their location, race, and species designation are listed in Table 5.1.

Twenty cypsellae from each population were sown on Whatman Number 42 filter paper placed in plastic petri dishes (100 x 15 mm) and soaked with distilled water. The dishes were then stored in the dark at 5 ° C for four days. Ten geminants from each population were transferred to pots (10 x 10 x 8 cm) filled with potting soil (Terra Lite Soil Mix, W. R. Grace and Co. of Canada Ltd., Ajax, Ontario, Canada). Pots were then moved to a growth chamber and maintained at 18 ° C day and 15 ° C night for one week. A second set of $_{GIC}$ seeds was started a week later to compensate for known difference in flowering times between the two species at this site. The temperature was then increased to 20 ° C night (10 h) and 25 ° C day (14 h), 70-80% humidity, and maintained at this level for the remainder of the experiment. Plants were watered three times per week. Fertilizer was not added during the growth period.

Plants originating from the various populations were randomly placed in the chamber. The plants were kept separate from each other in order to avoid contact

between flower heads. Initiation of flowering was noted for all plants as the day (from germination) that the first inflorescence was fully open. Inflorescences first appeared at the beginning of the 7th week from germination and flowering continued for approximately 5 weeks.

The number of crosses attempted ranged from a minimum of 4 (e.g., $G2_A$ x $C1_A$; $G3_A$ x $G1_C$) to a maximum of 38 ($C1_A$ x $G1_C$). Differences in the number of crosses resulted from variable germination, survivorship to flowering, as well as from differences in the timing of anthesis. Plants from Jasper Ridge had the highest germination (approx. 70 % for $C1_A$ and 90 % for $G1_C$) and survival rates (100 % for JR plants) while plants from the other populations showed lower germination rates (app. 50-70 %) and lower survival rates (app. 40-80 %). The numbers of plants per population used for crossing were 10 ($C1_A$, $G1_C$), 8 ($C2_C$), 7($C3_A$), 6 ($G4_A$), 5 ($G2_A$), and 4 ($G3_A$).

Flower heads at anthesis were pollinated by rubbing heads up to 3 times a day for about 2 days. Although there were differences in flowering times among the populations, there was enough overlap in flowering to cross flower heads that were at the same stage of maturity. The total number of crosses performed is given in Table 5.2. Pollinated flowers were marked using color-coded wires indicating intra- and inter-population crosses. The flower heads were then left intact to set seed and harvested after the ray florets had completely withered (7 - 10 d after crossing). Care was taken to harvest flower heads prior to the shedding of cypselae. Flower heads were then placed in small paper envelopes and stored for later counts of seed set. Seed set per cross was estimated by counting the number of cypselae that were full and dark (prior germination studies from field-collected cypselae have shown that the two features are good indications of

Table 5.1. The populations of the *L. californica* complex used in the crossing study.

Population ID	Locality	Race	Species
$G1_C$	Upper reaches of serpentine outcrop, Jasper Ridge Biological Preserve, San Mateo County, CA	C	*L. gracilis*
$C1_A$	Bottom reaches of serpentine outcrop, Jasper Ridge Biological Preserve, San Mateo County, CA	A	*L. californica*
$C3_A$	Rattlesnake Rock, north-facing slope of a serpentine outcrop, Jasper Ridge Biological Preserve, San Mateo County, CA	A	*L. californica*
$C2_C$	Andesite deposit on summit of lower Table Rock, near southern end of mesa, Jackson County, OR	C	*L. californica*
$G3_A$	Hillside, 4km E of Paskenta Bridge, Paskenta/Covelo Road, Tehama County, CA	A	*L. gracilis*
$G2_A$	Pasture, 1km E of Coalinga Springs Road on Route 198, Fresno County, CA	A	*L. gracilis*
$G4_A$	Pasture, Avenue Q across from Holiday Inn, Palmdale, Los Angeles County, CA	A	*L. gracilis*

viability) and then averaging the number from the two flower heads used in each reciprocal cross. This average of seed set was used in subsequent data analysis.

Insects were absent from the chamber and the amount of pollen flow due to air movement was considered minimal. However, a few flower heads from several plants from each population were left without artificial pollination while others were self-pollinated (rubbed with a flower head from the same plant) to determine the effects of extraneous pollination and/or selfing.

5.2.2. Seed viability

Fertilized cypselae from each cross were pooled in an envelope and left in the dark at room temperature for eight months. Forty fertilized cypselae per cross were randomly selected from several crosses involving Jasper Ridge, for assessment of viability. The crosses used were $G1_C$ x $G1_C$, $G1_C$ x $C1_A$, $G1_C$ x $C2_C$, $G1_C$ x $G4_A$, $C1_A$ x $C1_A$, $C1_A$ x TR_C, $C1_A$ x $G4_A$, $C2_C$ x $C2_C$, $C2_C$ x $G4_A$, $G4_A$ x $G4_A$. Cypselae were germinated using the methods outlined above and germinants counted after 7 - 10 d on the basis of emergence of the radicle. The fate of germinants was not followed beyond the 10-d period as many germinants died as a result of a fungal infection at about 8 d after germination.

5.2.3. Pollen tube growth

The growth of pollen tubes in inter- and intra-population crosses was examined by epifluorescence microscopy. Five plants from each of the two species at Jasper Ridge and the northern and southern populations of *L. californica* ($C2_C$) and *L. gracilis* ($G4_A$)

were grown in a growth chamber as above. Prior to flowering, individual plants were placed inside wooden frame cages (16.5 x 16.5 x 23 cm) covered with clear plastic on top and two layers of nylon monofilament bolting cloth (Nitex, B.S.&H. Thompson and Co. Ltd., Montreal) on the four sides. The pore diameter of the mesh (53μ) was greater than the pollen grain diameter (15-21μ) reported for the genus (Ornduff, 1966), and thus two layers of mesh were used to minimize pollen movement. The temperature inside the cages was within 1 ^{0}C of the rest of the chamber.

Flower heads reaching anthesis were marked with color-coded tags then artificially pollinated by rubbing flower heads. Five crosses each were made within and between plants of the four populations. Five to 10 individual disk florets were then removed from each flower head using sharp forceps at 1, 6, 12 and 24 h after pollination. Florets were then prepared for observation of pollen tube growth using a slightly modified version of a method developed by Martin (1959) and described in Kearns and Inouye (1993). Florets from each flower head were placed separately in small glass vials and then fixed in 1:3 acetic acid: ethanol (v/v) for 24 h. Florets were then transferred to vials containing 70 % ethanol and stored in a refrigerator (5 ^{0}C) until further processing. Prior to making observations, florets were removed from the vials, dipped in distilled water and immediately transferred to vials containing 5 M NaOH for 1 h. This step is necessary to soften perianth tissue and to facilitate removal of the pistil from the floret. Florets were then transferred to vials containing distilled water for 1/2 h. Pistils were then carefully removed from each floret, placed on a glass slide with a drop of 0.1 % Aniline Blue (in 50 mM K_3PO_4) and squashed using a cover slip. Slides were then viewed under epifluorescence using a Zeiss Axioplan 2 Imaging Microscope (Carl Zeiss,

Inc., Thornwood, New York) at the Bio-Imaging Facility of the University of British Columbia. In addition to noting the presence of pollen tubes, the nature of the callose plugs was recorded. Various features of callose are often used as indicators of pollination. At least half the florets observed in preliminary crosses lacked pollen grains on the stigmatic surface, indicating that the pollination technique may not have been adequate. Thus, only florets observed with pollen grains on the stigmatic surface were used in this analysis in order to avoid including florets that were not successfully pollinated. It is possible that the absence of pollen grains actually represents very early incompatibility (Richards, 1993). Preliminary observations revealed that pollen tubes were clearly visible as early as 6 h after pollination and that they continued to be visible in florets fixed 12 or 24 h after pollination. A few florets (mostly from $C2_C$) showed pollen tube growth as early as 1 h after pollination. However, florets fixed 12 h after pollination seemed to show the highest density of pollen tubes and were used for the final observations.

5.2.4. Data analysis

The data set for seed set was tested for normality and homogeneity of variance using the Shapiro Wilks and the Levene tests, respectively. Once the assumptions of normal distribution and equal variances were found to be applicable, one-way and nested analyses of variance (ANOVA), two-sample *t*-tests, and Tukey's Test for multiple comparison of means were used to determine whether the means differed within and among populations. All tests were conducted using the statistical packages SPSS (Norusis, 1993) and SYSTAT (Wilkinson *et al.*, 1992) in the University of British Columbia Botany Department Computing Facility.

5.2.5. Ecological differentiation

To determine the extent to which the various sites used in the crossing study differed in their edaphic environments, I reanalyzed soil data from Rajakaruna and Bohm (1999). An array of soil features (cation exchange capacity, exchangeable Mg, Ca, Na, K, Ca/Mg, extractable Co, Cr, Ni, Cu, Fe, Mn, Zn, and pH) was included in a Principal Components Analysis (PCA) in order to visualize the extent to which edaphic conditions of the parapatric populations at Jasper Ridge differed from other populations used in the study (note that soil data were not available for $G4_A$). Five additional populations were included to allow a clearer assessment of how the conditions at Jasper Ridge compare with those throughout the range of the two taxa. Mahalanobis Distances were calculated to estimate ecological distances among populations used in the PCA. For the four populations with complete data sets for both edaphic conditions and seed set ($C1_A$, $G1_C$, $C3_A$, $C2_C$), a Mantel Test was conducted to explore any correlations between the extent of ecological differentiation and reproductive isolation.

5.3. Results

5.3.1. Flowering time differences

There was no consistent pattern in phenology observed for plants originating from the various populations. The northernmost population of $C2_C$ -*L. californica* was the first to flower (day 1), closely followed by $G1_C$ -*L. gracilis* and the southernmost population of $G4_A$ –*L. gracilis* (day 3), $G3_A$-*L. gracilis* (day 5), $C3_A$ -*L. californica* (day 7), $C1_A$-*L. californica* (day 8), and finally $G2_A$-*L. gracilis* (day 12). *L. californica* and *L. gracilis* from Jasper Ridge maintained the flowering time separation observed in the field (Desrochers and Bohm, 1995; Rajakaruna and Bohm, 1999) with $G1_C$-*L. gracilis*

flowering 5 d prior to $C1_A$-*L. californica* plants. In the field and in earlier experiments up to a 10-day difference in flowering has been observed (Rajakaruna, unpublished observations).

5.3.2. Crossing study

As I found no significant differences in the success of reciprocal crosses (two-sample *t*-test: $P > 0.05$ in all cases), total seed set was determined by averaging the counts from both flowers used in each reciprocal cross.

Seed set by either selfing or by airborne pollination was uniformly low for all populations, with means of 2.8 % (± 0.6) and 1.9 % (± 0.7), respectively. This level of seed set was less than seed set by any of the actual crosses (Table 5.2) and did not differ significantly (ANOVA: $P > 0.05$) among the various populations. Hence, seed set by selfing or by airborne pollen was considered to have little effect on the results of the crossing study. The results indicate that there are significant differences in seed set between species and among populations. The key results are highlighted below.

5.3.3. Species effects

The mean % seed set in intra-specific crosses (*L. californica* x *L. californica* 54.1± 2.4; *L. gracilis* x *L. gracilis* 49.5 ± 3.5) was significantly greater (ANOVA $P < 0.001$; Tukey's Test $P < 0.001$) than the mean % seed set for inter-specific crosses (17 ± 1.6). Further, intra-population seed set was significantly greater (ANOVA $P < 0.001$; Tukey's Test $P < 0.001$) than inter-population seed set for both species (Figure 5.1). The mean % seed set for intra-population crosses within *L. californica* was 63.9 ± 2.1 while

seed set for inter-population crosses is 37.1 ± 4.1. A similar pattern exists for *L. gracilis*. Mean % seed set for intra-population crosses within *L. gracilis* was 60.9 ± 3.4 while seed set for inter-population crosses was 25.4 ± 5.2. Based on a large number of artificial hybridizations, Ornduff (1966) concluded that there is generally prevalent low crossability among plants originating in separate populations. Approximately two-thirds of the inter-population crosses conducted by Ornduff produced less than 30 % seed set agreeing with the level of seed set observed in my studies from inter-population and inter-species crosses (Figures 5.1, 5.2). The mean % seed set between *L. californica* x *L. gracilis* was significantly different from all means (ANOVA $P < 0.001$;Tukey's Test $P < 0.05$) except the mean for inter-population crosses of *L. gracilis* (Tukey's Test $P > 0.05$).

5.3.4. Isolation between races

Results suggest that racial identity also may contribute to patterns of reproductive isolation within each of the phylogenetic species. Intra- and inter-racial seed set was further examined using a nested ANOVA with race nested within clade. The results for clade and race (clade) were both highly significant ($P < 0.001$) indicating that the races also contribute to patterns of reproductive isolation among populations. Within *L. californica*, mean % seed set in race A x A was 57.7 ± 2.8; in race C x C 69 ± 2.4 (Note: C x C represents seed set from intra-population crosses only) and A x C 38.4 ± 4.7. The inter-racial cross was significantly different (ANOVA $P < 0.001$) from race A x A crosses. Since only one race C population was used from each clade, I am unable to conclude if a similar pattern also exists between race C x C and the inter-racial crosses. Similarly, for *L. gracilis*, mean % seed set in race C x C was 69.8 ± 2.5 (Note: C x C represents seed set from intra-population crosses only); in A x A, 44.6 ± 5.3, and in A x C

22.5 ± 6.2. Again, the inter-racial cross was significantly different (ANOVA; Tukey's Test $P < 0.001$) from race A x A crosses. While the comparisons made for A x A and A x C are supported by multiple populations the comparisons for C x C represents only one population of each race from each phylogenetic clade. Hence, further studies are clearly needed to clarify the role of race in the patterns observed in seed set.

5.3.5. Seed viability

As in the case of seed set, seed germination was highest for within population crosses for both species (Figure 5.3). For *L. californica*, mean % germination was 56.3 (±10.2) for crosses within populations and 12.5 (±9.5) for inter-population crosses. A similar pattern was found in *L. gracilis*: mean % germination was 68.7 (±7.9) for crosses within populations and 12.5 (±9.5) for inter-population crosses. For both species there was a significant difference for germination of seed between intra- and inter-population crosses (ANOVA, Tukey's Test $P < 0.001$). Germination of the seed obtained from the inter-species cross (17.5 ± 3.8) was low, however, did not significantly differ (ANOVA; Tukey's Test $P > 0.05$) from the mean obtained from inter-population crosses for either species (Figure 5.3).

5.3.6. Pollen tube growth

Florets with clearly visible pollen tubes were considered successfully pollinated while florets with excess callose on stigmatic surface and partially grown pollen tubes were considered failures. The remaining florets had pollen grains on the stigmatic surface; however, the pollen grains showed no sign of germination and there was no callose

Table 5.2. Percent seed set, as determined by the setting of full and dark cypselae, in the various crosses made within and between populations of *L. californica* and *L. gracilis*. The mean total seed set (± SE) was determined by averaging the seed count from both flower heads used in a cross. n represents number of pairs of reciprocal crosses.

	$C1_A$	$G1_C$	$C2_C$	$G4_A$	$G3_A$	$G2_A$	$C3_A$
$C1_A$	62.0 (±2.93) n=35	7.8 (±1.54) n=38	34.5 (±4.9) n=14	36 (±5.5) n=9	26 (±7.3) n=4	44.4 (±1.7) n-4	34.5 (±7.8) n=10
$G1_C$		69.8 (±2.5) n=26	22.6 (±5.4) n=10	17.7 (±5.3) n=8	7.8 (±2.7) n=4	46.9 (±18.7) n=4	16 (±3.8) n=10
$C2_C$			69 (±2.4) n=9	12.9 (±2) n=5	No cross	26.4 (±13.7) n=4	46.2 (±11) n=7
$G4_A$				42.4 (±8.7) n=8	No cross	36.8 (±6.4) n=4	16.5 (±2.4) n=5
$G3_A$					42.8 (±17.1) n=4	No cross	10.8 (±2.1) n=5
$G2_A$						58.6 (±10.8) n=4	No cross
$C3_A$							65.8 (±3.7) n=10

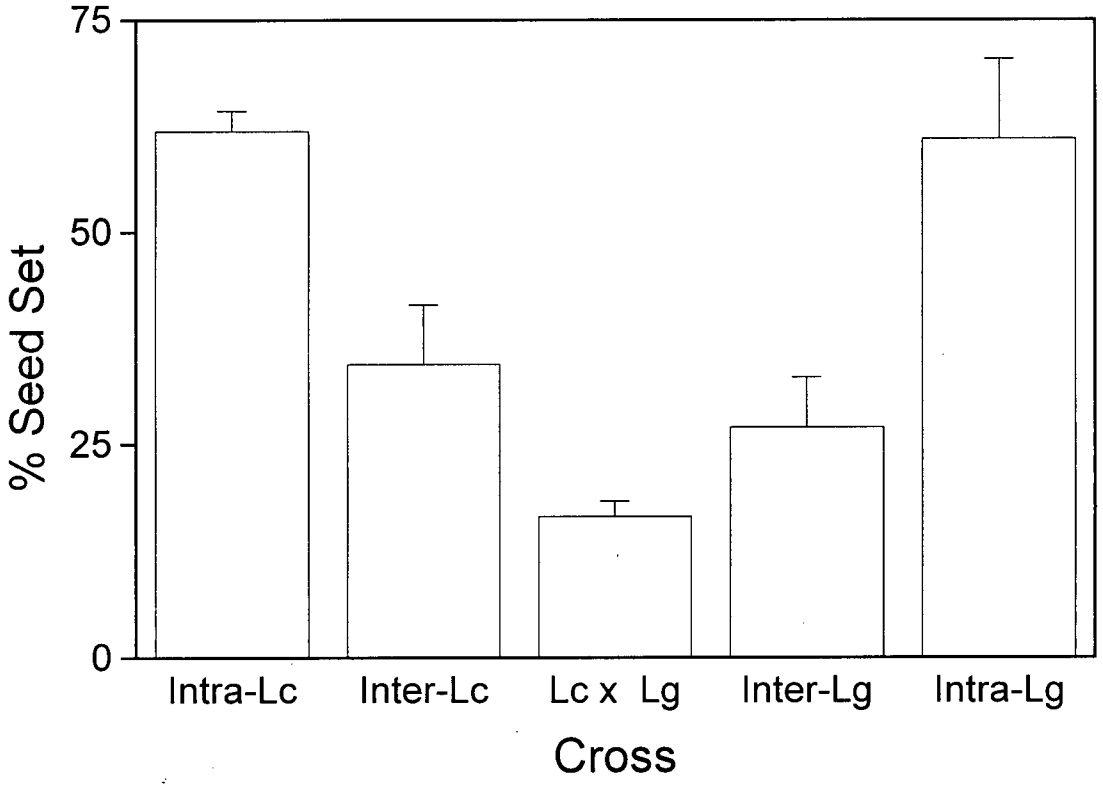


Figure 5.1. Mean percent seed set (± SE) for intra- and inter-specific crosses within and between *L. californica* (Lc) and *L. gracilis* (Lg). All columns but Lc x Lg and Inter-Lg significantly different from each other (ANOVA and Tukey test $P < 0.001$).

Table 5.3. Observations made on success and failure of pollen tube growth in the various crosses. n represents number of florets observed.

Cross	% Success	% Florets with partially-germinated grains and excess callose	% Florets without pollen tubes or excess callose	% Failure (total)
Intra-Populational				
$C1_A$ x $C1_A$ (n=20)	70	10	20	30
$G1_C$ x $G1_C$ (n=26)	73	0	27	27
$C2_C$ x $C2_C$ (n=10)	80	0	20	20
$G4_A$ x $G4_A$ (n=10)	100	0	0	0
Inter-Populational				
$C1_A$ x $G1_C$ (n=30)	10	17	73	90
$C2_C$ x $G4_A$ (n=10)	40	0	60	60
$C2_C$ x $G1_C$ (n=10)	40	0	60	60
$C2_C$ x $C1_A$ (n=10)	0	20	80	100
$G4_A$ x $G1_C$ (n=10)	10	50	40	90
$G4_A$ x $C1_A$ (n=10)	30	40	30	70

formation. These florets were considered as another category of incompatibility (according to Richards, 1993). Table 5.3 summarizes the observations of pollen tube germination and growth.

Intra-population crosses were clearly the most successful in terms of the total number of florets successfully pollinated, density of pollen tubes, and growth rates of pollen tubes. The mean % of florets successfully pollinated in the intra-population crosses was 80.8 ± 6.8, while the % success in the inter-populational crosses was 21.7 ± 7.0 (two-sample *t*-test: $P < 0.001$). Further, the density of the tubes was generally higher (at times, covering the entire style cortex) in the intra-populational crosses. The growth rates of the tubes were also significantly different: overall, the intra-populational crosses had a higher percentage of tubes reaching the bottom of the style/tip of ovary than florets used in inter-populational crosses (personal observations). One of the most striking observations involves a set of crosses with Jasper Ridge populations: $C1_A$ x $G1_C$, $C2_C$ x $C1_A$, $G4_A$ x $G1_C$, and $G4_A$ x $C1_A$ where the majority of the germinated tubes remained on the stigmatic surface without any apparent growth. The region of the stigma where the retarded tubes were present was highly fluorescent, indicating excess callose.

Pollen germination and tube growth pattern did not differ significantly within and between the species. Percent success for *L. californica* was 50 ± 25.2; *L. gracilis* 61 ± 26.6; and *L. californica* x *L. gracilis* 30 ± 7.1. The means are not significantly different due to the large standard error (ANOVA and Tukey's Test $P > 0.05$), although the lowest success was for the inter-species cross.

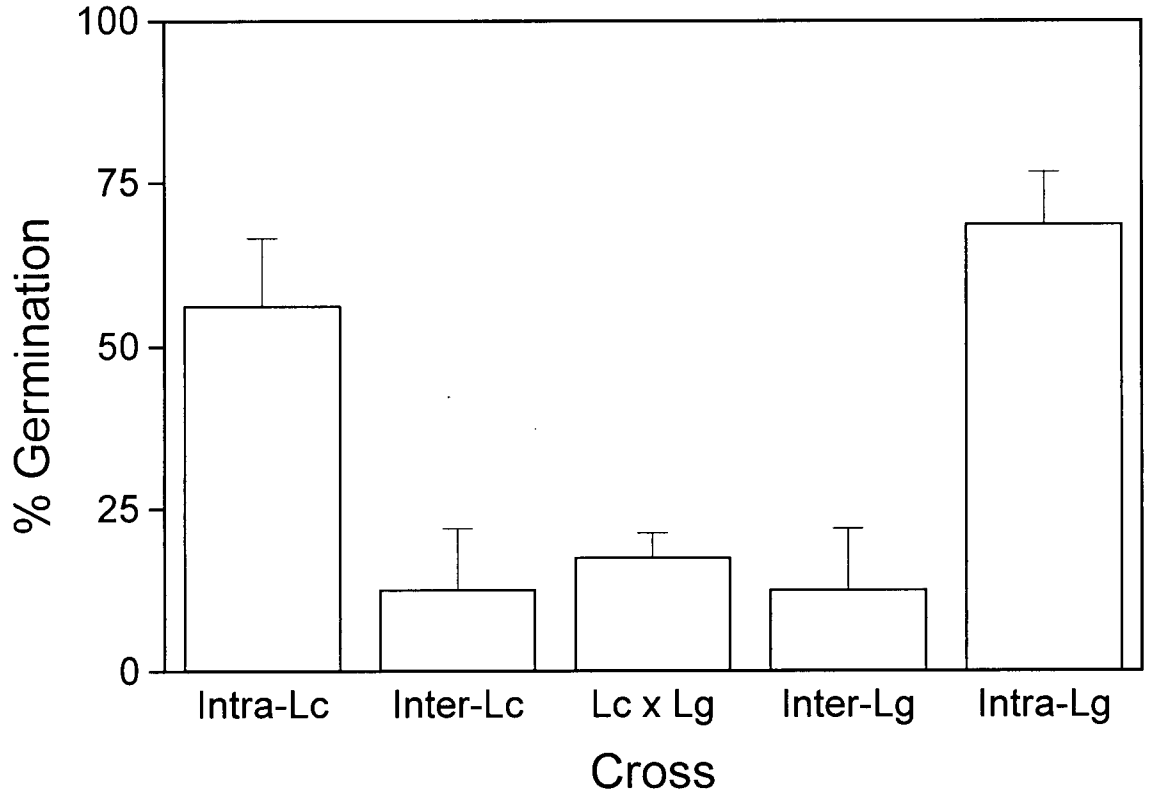


Figure 5.2. Mean percent germination (± SE) for intra- and inter-specific crosses within and between *L. californica* (Lc) and *L. gracilis* (Lg). All columns but Inter-Lc, Lc x Lg, and Inter-Lg significantly different from each other (ANOVA and Tukey test $P < 0.001$).

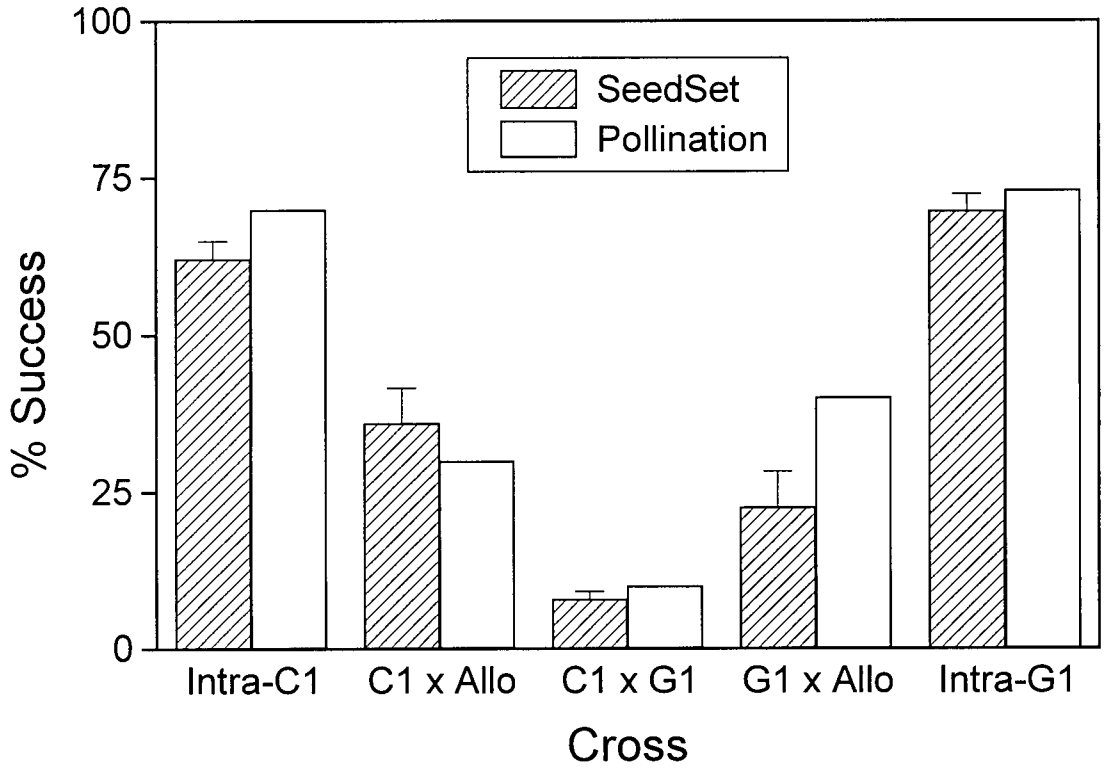


Figure 5.3. The percentage successful seed set (hatched columns) and pollination (open columns) in crosses involving Jasper Ridge populations of *L. californica* ($C1_A$) and *L. gracilis* ($G1_C$). C1 x Allo and G1 x Allo refer to interspecific crosses made between $C1_A$ and allopatric *L. gracilis* populations and $G1_C$ and allopatric *L. californica* populations, respectively. C1 x G1 refers to the interspecific (and interracial) cross at Jasper Ridge.

5.3.7. Reproductive isolation in parapatry versus allopatry

In addition to the trend noted above for flowering time differences, a number of the results suggest greater divergence of *L. californica* and *L. gracilis* in parapatry. Both species at Jasper Ridge had significantly greater (ANOVA and Tukey's Test; $P < 0.001$) mean seed set in interspecific crosses with allopatric populations than with each other at Jasper Ridge. Mean % seed set for *L. californica* (Jasper Ridge) x *L. gracilis* (allopatric) is 35.2 ± 3.6 and *L. gracilis* (Jasper Ridge) x *L. californica* (allopatric) was 22.6 ± 5.4. Both these means are significantly greater than 7.8 ± 1.5, the mean % seed set for the two species at Jasper Ridge (Figure 5.4).

While the crossability data indicate stronger isolation at Jasper Ridge, it should be noted that data on seed set potentially confound prezygotic and postzygotic isolation, as seed set can be diminished as a result of failure of fertilization (prezygotic), embryo abortion (postzygotic) or both. Figure 5.4 compares the effects of intrapopulation, interspecific parapatric and interspecific allopatric crosses on both seed set and successful pollination for populations included in the pollen tube growth study. Patterns of pollen tube growth help elucidate the contributions of pre- and post-zygotic isolation to the observation of decreased seed set in parapatry, and suggest that the patterns seen in seed set are mostly due to differences in prezygotic isolation in parapatry versus allopatry. Note that seed set was reduced by 78% (from 36 to 7.8) when $C1_A$ - *L. californica* was crossed with allopatric versus parapatric *L. gracilis*. This closely corresponds with the reduction in pollination success in allopatry versus parapatry (67%) for crosses involving $C1_A$. Similarly, $G1_C$ experienced a 66% reduction in seed set (from

22.6 to 7.8) when crossed with parapatric versus allopatric *L. californica*, while pollination success decreased by 75%.

5.3.8. Ecological differentiation

The Principal Components Analysis of soil characteristics indicates greater ecological differentiation of the Jasper Ridge locality (Figure 5.5; Table 5.4). The only other location that falls within the edaphic environment of Jasper Ridge is $C3_A$, another race A *L. californica* population growing on the serpentine substrate. The only population of *L. gracilis* (and race C) known to occur on serpentine is at the Jasper Ridge ($G1_C$) location. This population appears to be experiencing vastly different edaphic conditions than the rest of *L. gracilis* (and race C). The Mantel Test revealed a fairly high r value (0.7855; $P = 0.077$) for the relationship between extent of seed set (i.e. reproductive isolation) and ecological distance among the four populations.

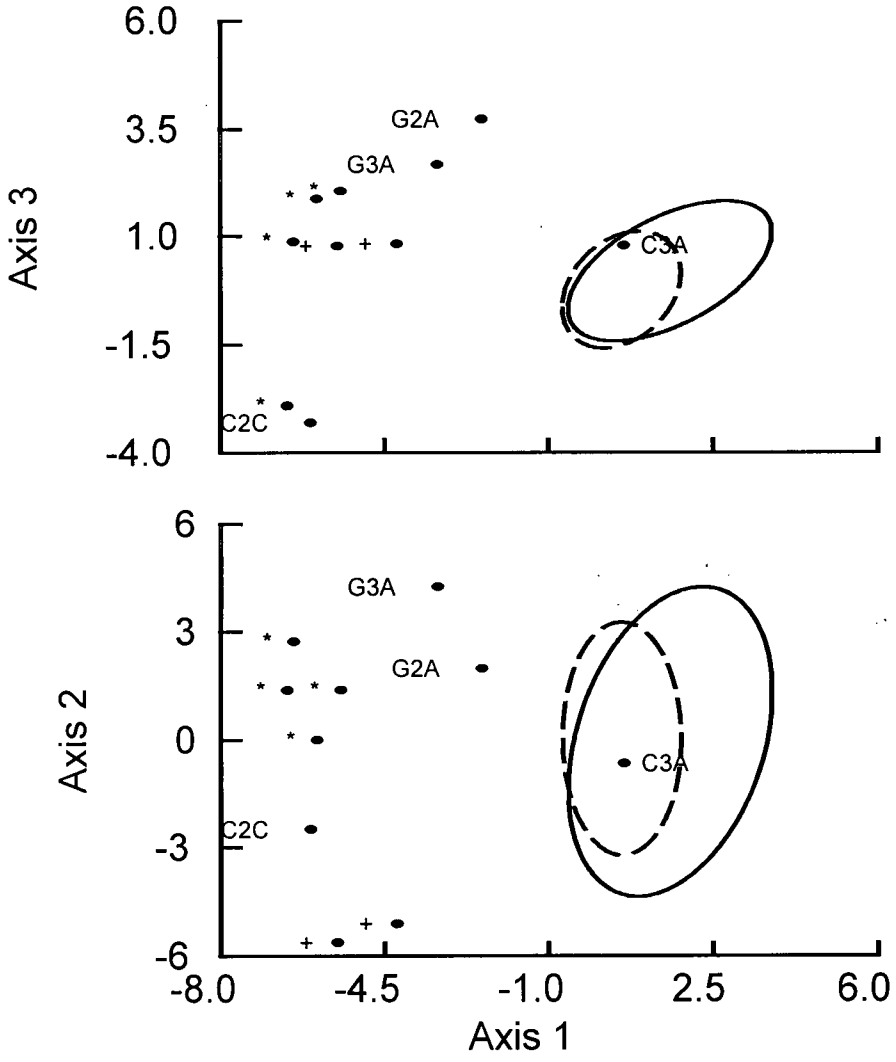


Figure 5.4. Principal Components Analysis for 15 soil features for all populations but $G4_A$ used in the crossing study. Solid elipse represents $G1_C$ (n=93) and dotted elipse represents $C1_A$ (n=30). $C1_A$, $G1_C$, and $C3_A$ are serpentine populations. + = *L. gracilis* (race A) and * = *L. californica* (race C) are additional populations. Percent of total variation explained; Axis 1 38%, Axis 2 27%, Axis 3 13%.

Table 5.4. Correlations of soil variables with the three PCA axes. Soil variables are listed in order from strongest positive to strongest negative correlations along PCA Axis 1. The total variance explained by each axis is listed under axis title.

Soil Variable	*Axis 1 38%*	*Axis 2 27%*	*Axis 3 13%*
Magnesium	0.87	-0.005	0.16
Cation exchange capacity (mmol of charge/100g soil)	0.85	0.09	0.23
pH in $CaCl_2$	0.85	-0.28	0.3
Nickel	0.77	0.3	-0.21
pH in water	0.73	-0.35	0.32
Cobalt	0.58	0.50	-0.13
Chromium	0.19	0.15	0.09
Zinc	0.12	0.29	0.03
Ca/Mg ratio	-0.75	-0.29	-0.02
Calcium	-0.57	0.05	0.61
Potassium	-0.35	0.01	0.68
Manganese	-0.17	0.79	0.22
Copper	-0.13	0.75	0.37
Iron	-0.12	0.88	-0.05
Sodium	-0.11	0.23	-0.24

5.4. Discussion

Reproductive character displacement can originate either as a by-product of evolutionary divergence (Macnair and Baker, 1994; Gardner and Macnair, 2001) or as a product of direct selection against maladaptive hybridization, i.e., reinforcement (Grant, 1966; Antonovics, 1968). Age, genetic distance, and ecology of the sympatric and allopatric populations are often not taken into consideration prior to attributing reproductive isolation to reinforcement (Coyne and Orr, 1989; Noor, 1999). In studies of heavy metal tolerance (McNeilly and Antonovics, 1968; Macnair *et al.,* 1989), adjacent metal-tolerant and -intolerant populations flower at different times providing a strong prezygotic barrier to reproduction. While this observation can be interpreted as evidence for reinforcement (McNeilly and Antonovics, 1968), an alternative explanation is that the flowering time difference represent differential adaptation to drought which is prevalent in these habitats (Macnair and Baker, 1994, Macnair and Gardner, 1998). In the discussion that follows, I examine evidence for reproductive character displacement in flowering time and pollen incompatibility and consider the possible role of ecological divergence and reinforcement in generating the observed patterns.

5.4.1. Premating isolation

Flowering time differences are a simple and effective means of isolating sympatric populations. While it may be tempting to invoke reinforcement in their origin, they illustrate the role that ecological selection can play in the evolution of reproductive isolation. At Jasper Ridge, peak flowering times between the two species differ by seven to ten days (Rajakaruna and Bohm, 1999) providing a potent barrier to reproduction.

This flowering time difference is maintained under greenhouse conditions, confirming that the trait is under genetic control. *Lasthenia gracilis* flowers early, produces significantly more flower heads, and dries out one-two weeks prior to *L. californica*. The study reported in Chapter Four reveals that the two species at Jasper Ridge are differentiated in their response to drought. *Lasthenia gracilis* is clearly the more drought-tolerant of the two species, as it is able to maintain flower production under high water stress, while *L. californica* suffers a sharp decline in flowering under such conditions. These observations suggest that *L. gracilis* employs a life history strategy known as phenological escape, hypothesized to result from selection for rapid completion of the life cycle, as a means of drought avoidance (Macnair and Baker, 1994; Macnair and Gardner, 1998). The ridge top soil where *L. gracilis* is found is much drier and has a lower water-holding capacity than the soils in the ridge bottom (Rajakaruna and Bohm, 1999). Early flowering gives *L. gracilis* the capacity to "avoid" drought and yet complete its life cycle. Further, due to chemical and physical stresses in the ridge bottom environment (Rajakaruna and Bohm, 1999), *L. californica* may grow more slowly, thereby reaching flowering at a later date. Both these scenarios suggest that reproductive isolation via flowering time differences could have evolved as a by-product of differing strategies (i.e., adaptations) for minimizing edaphic stress in ecologically distinct adjacent sites. Alternatively, as suggested for metal-tolerant races of several grasses by McNeilly and Antonovics (1968), flowering time differences at Jasper Ridge may have resulted from direct selection to reduce gene flow, i.e. reinforcement. Under this scenario, flowering time differences would have evolved in allopatry and later were reinforced at Jasper Ridge. While field observations document that the southern

populations (mostly *L. gracilis*) flower several weeks prior to northern populations (mostly *L. californica*), this difference was not clearly maintained in plants grown under uniform conditions. The results fail to show consistent patterns of flowering time differences for the two species, and therefore we have no support for this theory. Given the understanding of the distinct edaphic conditions at the Jasper Ridge site, I conclude that it is far more likely that isolation has evolved as a by-product of ecological divergence.

5.4.2. Postmating prezygotic isolation

The results provide clear evidence for the existence of reproductive character displacement in seed set; however, it is important to consider whether the pattern is likely to be attributable to reinforcement (i.e., selection for greater isolation), or whether it could be a byproduct of adaptation to greater ecological divergence in the parapatric location. Howard (1993) lists five additional criteria that can be used to support a hypothesis of reinforcement. First, heterospecific matings must be likely to occur in nature. Studies of allozyme variation (Figure 6 in Desrochers and Bohm, 1995) are consistent with a hypothesis of introgression between northern and southern clades. Two loci, *Nadhdh* and *6Pgd-1* are fixed for alternative alleles in northernmost and southernmost populations, but are polymorphic in the central portion of the range where the geographical clades are in contact. Populations of the southern clade have low frequencies of the northern alleles of both loci, and vice versa. At Jasper Ridge, although the two species differ in peak flowering times, some overlap in flowering exists. Despite the lack of apparent barriers to interspecific pollination among synchronously flowering

individuals, evidence for introgression is less obvious in allozyme data from Jasper Ridge. For example, *L. californica* individuals (race A) are fixed for a slow allele (b) at *Nadhdh*. This allele is present at low frequency in *L. gracilis* individuals. This pattern may represent one directional introgression of the *L. californica* allele or ancestral polymorphism in *L. gracilis* individuals; clearly data from a single locus are insufficient to characterize patterns of isolation at Jasper Ridge.

The second criterion for reinforcement is that there must be selection against hybridization. The data obtained here on seed germination provide the only indication of reduced hybrid fitness available to date. These data (Figure 5.2) indicate that hybrid seed from crosses between the species at Jasper Ridge are of decreased fitness relative to parental seed from this location. However, there is also reduced fitness in intraspecific crosses between populations in both *L. gracilis* and *L. californica*. While this pattern suggests that selection against interspecific hybridization is likely to occur at Jasper Ridge, it would seem that selection may act against both intra- and inter-specific gene flow in the remainder of the species ranges. Note, however, that germination tests were performed in benign environments and that ecological selection is known to be intense at Jasper Ridge. The upper reaches of the serpentine outcrop are ionically more benign, with moderate levels of ionic stress; however, this area is also subject to extreme drought conditions. The lower reaches tend to be wet throughout the growing season, but are ionically extreme. The boundary between the two species at this location has remained essentially unchanged in the more than 20 years since the discovery of the two species in parapatry. Rajakaruna and Bohm (1999) followed development of plants from both species in soil solutions from Ridge top and Ridge bottom soils. While *L. californica*

(race A) cypselae experienced similar rates of germination in both soil solutions, *L. gracilis* (race C) cypselae showed reduced germination in solutions from Ridge bottom soils. Further, a reciprocal transplant study in the greenhouse showed that *L. gracilis* is unable to reach reproductive maturity in Ridge bottom soils, whereas *L. californica* was able to grow equally well in both soils. It was suggested that while soil chemical features were responsible for keeping *L. gracilis* from inhabiting Ridge bottom soils, biotic factors may be responsible in keeping the slow-growing *L. californica* plants from colonizing the Ridge top soils. Further, differential response to water stress (Chapter Four) may play a role in keeping the drought-intolerant *L. californica* plants from colonizing the drier ridge-top soils. Physiological studies (Chapter Three) indicate that the two species also differ in adaptations related to ion uptake. Although no studies have examined the characteristics of hybrids under natural conditions, I hypothesize that the strong ecological selection noted for parental types is likely to impact the fitness of hybrids in one or the other parental environments.

Howard's third criterion for reinforcement relates to perception of interspecific differences and is not relevant in this case. The fourth criterion is that variation in the isolating trait must be heritable, which is particularly important to consider where behavioral traits are concerned. While I have no direct information on the heritability of pollen incompatibility in *Lasthenia*, there is no reason to assume that this trait is largely environmentally mediated.

The fifth and final criterion listed by Howard is that the pattern of character displacement must not be attributable to other selective forces, especially ecological selection. A few recent studies have attempted to control for the potentially confounding

effects of ecological divergence. For example, Rundle and Schluter (1998) carefully selected sympatric and allopatric sticklebacks of similar ecologies (in this case reflected in similar body sizes) for comparisons of levels of reproductive isolation. In the *Lasthenia* system, ecological differentiation has been well documented: soil characteristics are correlated with the pattern of distribution of the two edaphic races. While the races appear to have evolved in parallel in both species (Chapter Two), the crossing data presented here indicate that a significant portion of the variation in seed set among populations within species is attributable to racial identity, suggesting that ecological differentiation may also contribute to reproductive isolation. If one considers the pattern of edaphic differentiation among locations presented in Figure 5.4, it is evident that the Jasper Ridge populations represent highly ecologically divergent sites for both species. While the enhancement of pollen incompatibility reactions is unlikely to be subject to direct ecological selection (but see Searcy and Macnair, 1990), divergence in this trait may be a byproduct of divergence under ecological selection. Interestingly, the Mantel Test suggests there is a fairly high correlation ($r = 0.79$; $P = 0.077$) between ecological distance and the extent of reproductive isolation (via seed set) among four populations. By including data from several other populations, I will be able to better explore this relationship.

Comparisons of levels of reproductive isolation involving non-parapatric populations with similar levels of ecological divergence would also help clarify the role of ecological selection. In this study, only population $C3_A$-*L. californica* is likely to have experienced similar ecological selection as the Jasper Ridge populations (Figure 5.4). Levels of seed set between $C3_A$ and $G1_C$-*L. gracilis* (16) are substantially higher than

those found between the two species at Jasper Ridge (7.8). This comparison suggests that ecological divergence alone cannot account for patterns of reproductive isolation at Jasper Ridge. For *L. gracilis* (race C at Jasper Ridge), serpentine is an unusual habitat and the only known occurrence of race C on serpentine is at the Jasper Ridge site. Thus, I have no suitable comparison between populations of similar ecology for *L. gracilis*. Taken together, data presented here along with those from previous and ongoing studies suggest that reinforcement may have played a role in producing the pattern of reproductive character displacement in postmating prezygotic isolation in *Lasthenia*.

Reinforcement has only infrequently been implicated in the divergence of plant taxa, and Levin (1970) has argued that certain characteristics of plants, including increased opportunities for male gamete competition (a form of postmating, prezygotic isolation), may lessen selection against hybridization and make reinforcement less likely to play an important role in plants. Here I argue that such factors, rather than changing the likelihood of reinforcement, can be subject to reinforcement, as divergence in such traits decreases the frequency of maladaptive hybridization. As noted by Howard (1993), demonstrating that reinforcement has played a role in reproductive character displacement requires that several additional criteria be met, and I have examined evidence for these in the *Lasthenia* system. While the case for reinforcement in *Lasthenia* is far from complete, the data presented here, along with evidence from previous and ongoing studies suggest that this system provides an ideal setting in which both ecological selection and reinforcement may have contributed to reproductive character displacement.

CHAPTER SIX

Population differentiation and character evolution in the *L. californica* complex

6.1. Introduction

The study of the distribution of genetic variation within plant species has enhanced our understanding of population dynamics and divergence. Studies employing neutral to near-neutral markers have suggested that most widely distributed, outcrossing species have greater amounts of genetic variation within populations than among populations while inbreeding species are frequently characterized by a high degree of population differentiation and relative uniformity within populations (Hamrick and Godt, 1989). Greater genetic variation within populations of a species may enable short-term adaptation to environmental extremes and allow for long-term evolutionary change (Beadmore, 1983). The amount of gene flow is a major factor determining the structuring of genetic variation within a species' range. Gene flow depends on such factors as reproductive mode and breeding system, geographical distance, and selective forces (Loveless and Hamrick, 1984).

An understanding of factors governing the distribution of genetic variation is best sought in the study of species showing intermediate stages in the speciation process (Heywood and Levin, 1984). Edaphic factors are often considered potent agents of natural selection (Kruckerberg, 1986; Macnair and Gardner, 1998) and the study of edaphically differentiated species can shed light on the role of natural selection in shaping patterns of genetic diversity. However, very few studies have explored the genetic structure and relationships in species consisting of edaphically specialized

populations. Heywood and Levin (1984, 1985) examined patterns of allozyme variation for *Gaillardia pulchella* Foug. (Asteraceae), a widespread and edaphically diverse species, and found evidence for selection acting upon loci; the putative agent of selection was soil calcium content operating over a short distance. Similarly, Furnier and Adams (1986) found differences in genetic structure between serpentine and non-serpentine populations of *Pinus jeffreyi* Grev. & Balf. (Pinaceae). However, an allozyme study looking at the effect of serpentine soils on the population genetic structure of *Silene dioica* L. Clairv. (Caryophyllaceae) suggested that serpentine tolerant populations are genetically similar to those populations avoiding serpentine (Westerbergh and Saura, 1992, 1996). Similarly, a lack of association between edaphic features and genetic structure was reported for heavy metal tolerant species *S. vulgaris* (Verkleij *et al.*, 1985), *Minuartia verna* (Caryophyllaceae) (Verkleij *et al.*, 1989), and the salt tolerant species *S. maritima* (Baker *et al.*, 1975). The authors extend these findings to discuss multiple origins of edaphic specialization (i.e., tolerance may evolve with "relative ease") as well as suggest that unusual edaphic conditions may not exert strong selection acting upon allozyme loci or on those regions of the genome closely linked to allozyme loci.

The *Lasthenia californica* complex offers another opportunity to explore the genetic structure and relationships among geographically isolated populations that reflect varying levels of ecological divergence (Chapters Three and Four) and reproductive isolation (Chapter Five). Previous studies of allozyme variation in the complex (Desrochers and Bohm, 1995) suggested that the levels of variation found in the complex are comparable to those measured in outcrossing or widespread species (Hamrick and Godt, 1989, 1996). Based on their results, Desrochers and Bohm (1995) suggested that

the patterns of variation observed in the complex indicated the existence of two geographical races, a southern race consisting of predominantly race A plants and a northern race consisting primarily of race C plants. Genetic parameters indicating differentiation within and among populations suggested reduced gene flow among populations (G_{ST} = 0.36; Nm = 0.439) of the complex. The average genetic identity between populations of the two geographical races was 0.72, implying reduced gene flow between the northern and southern populations. Interestingly, reduced gene flow was also observed at Jasper Ridge, where the two races occur in parapatry on the serpentine ridge. The high G_{ST} (0.417) observed for the races at this site approached the mean value (G_{ST} = 0.51) estimated for selfing populations of species (Hamrick and Godt, 1989). Further, the calculated Nm value (0.35) was less than 1, which is commonly regarded as the point below which genetic drift can play a major role in determining the distribution of genetic variation among population subdivisions (Wright, 1951). Recent phylogenetic studies conducted by Desrochers and Dodge (in press) uphold the patterns found in the allozyme study. The two parapatric races at Jasper Ridge are genetically distinct, with race C plants clustering with populations from the southern clade (with mostly race A populations) while race A plants cluster with populations from the northern clade (with mostly race C populations). The reason behind this intriguing pattern was recently clarified (Chapter Two) when it was confirmed that the Jasper Ridge population was established by representatives of the two recently described species, *L. californica* subsp. *californica* and *L. gracilis* (Chan *et al.*, 2001, 2002). This discovery led to the testing of the hypotheses that flavonoid features (Chapter Two) and physiological traits (Chapter Three) differentiating the edaphic races have evolved in

parallel in both these species. The edaphic races (Rajakaruna and Bohm, 1999) were not described at the time the allozyme study was conducted, thus the genetic structure and relationships between and within the edaphic races have not been described.

The present study was conducted to clarify further genetic relationships among populations of the *L. californica* complex, specifically emphasizing relationships between and within the edaphic races. The objectives of the current study were to address the following questions: 1) What are the genetic relationships among populations of the *L. californica* complex and are the relationships comparable to those observed in previous studies?

2) What are the relationships between genetic distance and other tested parameters (i.e., ecological distance, geographical distance, and extent of reproductive isolation) for the complex, i.e., are populations in close proximity or with a greater capacity to interbreed genetically more similar to each other than populations further apart or having reduced crossability?

3) Can patterns of character evolution clarify whether one or both edaphic races have evolved in parallel in the complex ?

In order to address these objectives, I sampled genetic variation using Randomly Amplified Polymorphic DNA (RAPD) markers amplified using the Polymerase Chain Reaction (PCR) (Williams *et al.*, 1990). RAPD-PCR is a technique that is widely used for generating molecular fingerprints and for describing population genetic differences among closely related and recently diverged taxa (Chalmers *et al.*, 1992; Waycott, 1995; Weising *et al.*, 1995; Bussell, 1999). The technique relies upon the ability of short (10 bp) DNA primers of arbitrary sequence to bind to homologous sites in a target genome.

If primers adhere to complementary strands in the appropriate orientation (i.e., 3' ends toward each other on opposing strands) at a reasonable distance (100 bp to 4000 bp apart), the intervening fragment will be amplified via PCR. The set of fragments amplified in the presence of each primer can be separated by agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide.

In the present study, RAPD markers were used to describe genetic relationships among populations of *L. californica sensu* Ornduff and *L. macrantha* spanning the geographic and edaphic ranges of the species. Dendrograms based on levels of genetic similarity among individuals and populations were used to reconstruct the possible evolutionary history of the populations. The racial identities for each of these populations were mapped onto these trees in order to visualize correlations between genetic distances and racial identity and speculate on the patterns of race evolution.

6.2. Materials and Methods

6.2.1. Field collections and DNA extraction

Plant tissues for the study were collected from the field in April of 2000 and 2001. A total of 25 populations were collected from the range of the two races (from both *L. californica* and *L. gracilis*) spanning Southern Oregon to Southern California and Western Arizona. In addition, three populations of *L. macrantha* (recently revised as *L. californica* subsp. *macrantha*; Chan *et al.*, 2002) were included in the study. Locations, race, and specific status (where known) of the populations sampled are given in Table 6.1 and Figure 6.1

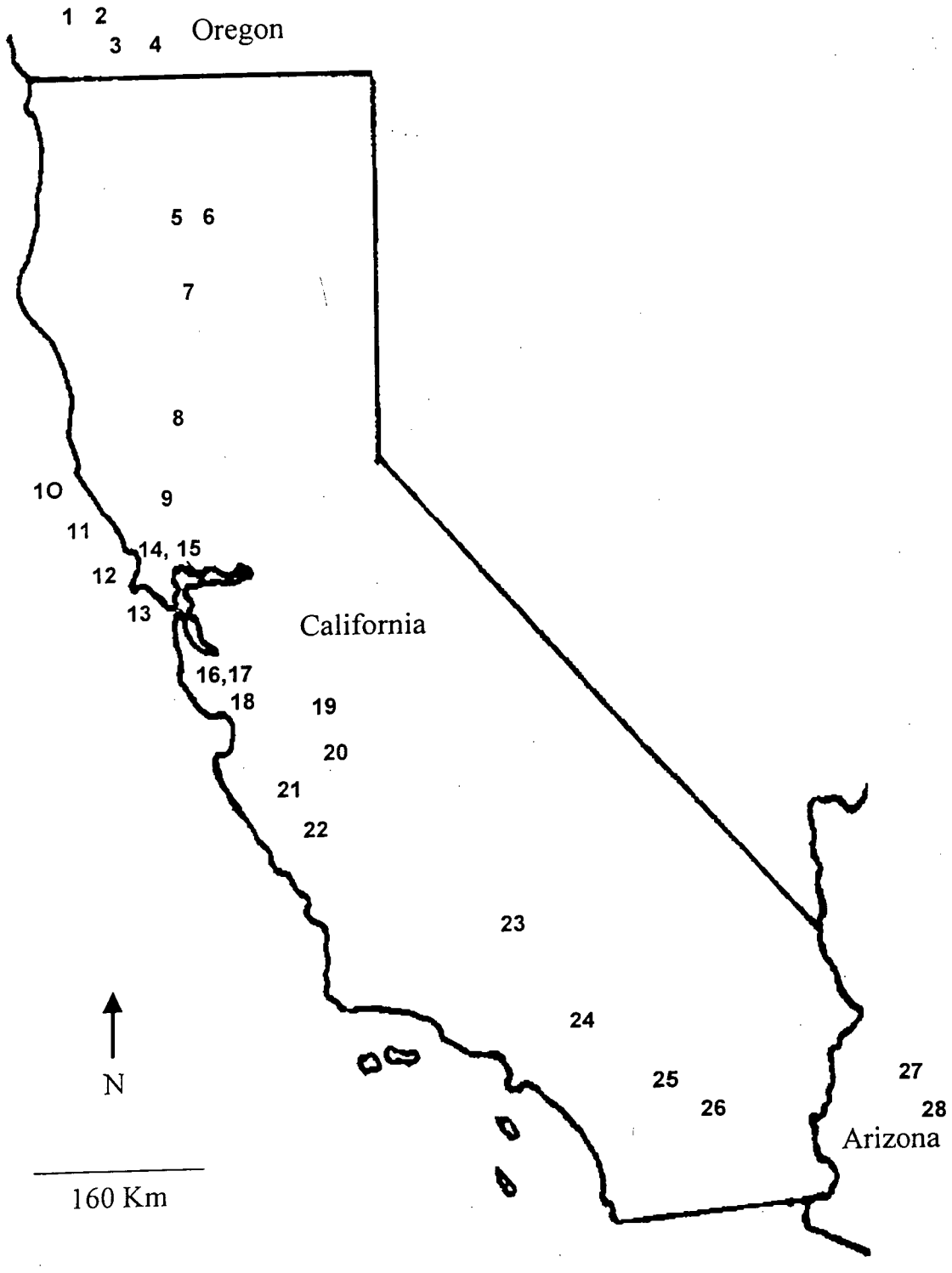


Figure 6.1. The distribution of all populations sampled for the RAPD study.

Table 6.1. Populations of *L. californica* complex sampled for the RAPD study. Populations listed from North-South. Specific status listed only for those populations confirmed by sequencing of the ITS region.

#	Population	Locality	Race	Species
1	OR1	On roadside pasture along Highway 234, 2.9 km W of intersection with Table Rock Road, near Gold Hill, Jackson Co., OR.	C	*L. californica*
2	OR2	0.5 km from intersection Kirtland Road and Table Rock Road. Roadside pasture in front of Water Treatment Plant, Jackson Co., OR.	C	*L. californica*
3	OR3	On gravelly pasture across road from population OR2. Jackson Co., OR.	C	*L. californica*
4	TR	On soils derived from andesite on summit of Upper Table Rock. Jackson Co., OR.	C	*L. californica*
5	44	Near Millville, 2 km from Old 44 Road/Route 44. On oak woodland at Route 44/A17, Shasta Co., CA.	C	
6	44A	Near Millville, 1 km from population 44 heading towards Redding, Shasta Co., CA.	C	
7	RB	Pasture along Route 36, 1.7 km from intersection with Highway 99. Near Red Bluff, Tehama Co., CA.	C	
8	R20	Roadside pasture along Route 20 between Colusa and Clear Lake, Colusa Co., CA.	C	
9	R29	Roadside pasture, 4 km N of Calistoga along Route 29. Napa Co., CA.	C	
10	SPS	On coastal bluff, Salt Point State Park, Sonoma Co., CA.	A	
11	CG	Off Highway 1 ca. 1 km N of Wright's Beach. Parking area at intersection Corlevaro Way and Grill Way. Sonoma Co., CA.		*L. macrantha*
12	DB	On coastal bluff growing aside *L. californica* on Dillon Beach, Marin Co., CA.		*L. macrantha*
13	PR	Along roadside on way to Light House, Point Reyes. 4 km from Drake Beach. Marin Co., CA.	A	
14	MT	On serpentine substrate at Mount Tamalpais State Park. Marin Co., CA.	A	
15	SRM	On rocky outcrop near Steep Ravine Campground, Highway 1, 3.5 km N Muir Wood National Monument, Marin Co., CA.		*L. macrantha*
16	JRC	On serpentine substrate, upper reaches of outcrop, Jasper Ridge Biological Preserve, Stanford University, San Mateo Co., CA.	C	*L. gracilis*
17	JRA	On serpentine substrate, bottom reaches of outcrop, Jasper Ridge Biological Preserve, Stanford University, San Mateo Co., CA.	A	*L. californica*
18	RS	On serpentine substrate, N-facing slope of Rattle Snake Rock, Jasp. Ridge Biol. Preserve, Stanford Univ., San Mateo Co., CA.	A	*L. californica*
19	KCN	On serpentine substrate, N-facing slope of Kerby Canyon, W of Coyote Ridge, Santa Clara Co., CA.	A	
20	25	Roadside pasture along Route 25, 8.5 km S of Hollister, San Benito Co., CA.	A	
21	PV	Pasture along Route 198, 4 km NW of County line of Monterey and Fresno Counties, Priest Valley, Monterey Co., CA.	A	
22	AS	Hillside N of 16-Mile Road on trail to stream, ca. 4 km from Arroyo Seco Campsite. Los Padres Nat. Forest, Monterey Co., CA.	A	
23	TW	Sandy roadside, along Tehachapi Willow Springs Road, approx. 3.3 km S of intersection with Highline Road. Kern Co., CA.	A	*L. gracilis*
24	Q	Roadside across from Holiday Inn on Avenue Q, Palmdale, Los Angeles Co., CA.	A	*L. gracilis*
25	CASR	Roadside drainage ditch. At intersection of California Ave. and Stowe Rd. W of Stetson & Warren Rd. Near Hemet, Riverside Co., CA.	A	
26	MR	On soils derived from granite. Motte Rimrock Reserve, Riverside Co., CA.	A	
27	AZ	Sandy soil along dry streambed. N of Apache Junction on Route 88, near Mile 203 marker, on left (W) side of road. Maricopa Co., AZ	A	
28	ARZ	Sandy ridge near a sidetrack ca. 1.5 miles along Dripping Springs Road, N of Mile 153 marker on AZ Route 77. Gila Co., AZ.	A	

(specific status was confirmed for nine populations in collaboration with Bruce Baldwin, UC Berkeley; Chapter Two). Ten to twenty individual plants in flower were collected from each population. Each plant was tightly packed into a 3 mL cryogenic collection vial in the field and stored in a liquid nitrogen-charged cryoshipping cannister (MVE Bio-Medical Systems, MN, USA). Vials were then transferred to a - 80° C freezer at the Department of Botany, University of British Columbia (UBC) and stored until further processing. A few whole representatives from each population were collected as vouchers to be deposited at the UBC Herbarium.

DNA was isolated from the whole plant (approx. 1 g frozen tissue) using the CTAB total DNA isolation protocol for fresh plant tissue (Doyle and Doyle, 1987). Unless otherwise noted, all steps were carried out at room temperature in a fume hood. Frozen tissue was first ground in liquid nitrogen using a mortar and pestle. Seven mL of 60° C 2xCTAB buffer (1M Tris-HCl, pH 8.0; 5M NaCl; 0.25M EDTA, pH 8.0; 2% (w/v) CTAB; 1% (w/v) PVP-40; 1% (w/v) sodium bisulfite, 0.2% (w/v) 2-β-mercaptoethanol) was added to frozen, finely ground tissue and mixed to make a slurry. The slurry was quickly transferred to a 15 mL Corex centrifuge tube and incubated in a water bath at 60° C for 30 minutes, inverting every five minutes to ensure mixing of contents and the even digestion and lysing of cells.

Following incubation, 2/3 volume of 24:1 chloroform:isoamyl alcohol was added to partition the organic fraction. Tubes were sealed and inverted twenty times to form an emulsion. The emulsified lysate was spun at 4000 rpm for 10 minutes in an IEC Clinical centrifuge (Thermo International Equipment Company, Neeham Heights, MA). After spinning, the aqueous top layer containing DNA was removed with a wide-bore pipette

tip and transferred to a clean 15 mL Corex centrifuge tube. Again, 2/3 volume of 24:1 chloroform: isoamyl alcohol was added, and the contents were emulsified and spun for 10 minutes. After the second spin, the aqueous layer was transferred with a wide-bore pipette tip to a clean 15 mL Corex centrifuge tube, to which one volume of ice-cold 100% isopropanol was added to precipitate DNA. The tube was sealed and inverted 10-20 times to mix contents thoroughly. The tubes were then stored in a - 20° C freezer overnight to precipitate DNA completely.

Following incubation, tubes were removed from the freezer, left at room temperature for five minutes, and again spun in the clinical centrifuge for 10 minutes to form a DNA pellet. The supernatant was discarded, and the pellet was soaked with 5 mL of 76% ethanol / 0.01M NH_4OAc for 10 minutes, after which the supernatant was again discarded. Samples were dried for 15-30 minutes at 40° C in a forced draft oven to evaporate any remaining ethanol. Finally, pellets were suspended in 400 μL of TE (10mM Tris-HCl , 1mM EDTA) and incubated at 37° C in a forced draft oven for 30-60 minutes until fully dissolved. Dissolved DNA was purified using the S & S Elu-Quik DNA Purification Kit (Schleicher & Schuell, Keene, NH) to remove residual contaminants such as polysaccharides and proteins.

DNA products were quantified using Hoefer DyNAQuant 200 fluorometer (Amersham Biosciences, Baie d'Urfé, PQ). Two μL of DNA was added to 2 mL of fluorometry solution (90 mL ddH_20; 10 μL commercially-prepared Hoechst dye H33258; 10 mL 10X TNE buffer), vortexed, and assayed in a crystal cuvette in the fluorometer. Fluorometry readings at or above 10 ng/μL are considered ideal to produce a clear, stable, repeatable PCR product. Since the majority of DNA samples gave readings above

the required concentration, 50 μL aliquots of standardized 10 ng/μL were prepared by diluting the appropriate volume of DNA with TE buffer.

6.2.2. RAPD-PCR and gel electrophoresis

The RAPD-PCR cocktail consisted of 10 ng template DNA, 30 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM of each dNTP (New England BioLabs, Mississauga, ON), 10 pmoles of primer (UBC), 5% acetamide, and 1.5 units Taq polymerase plus sterile, deionized distilled water to a 25 μL volume. Amplification was carried out in a 60-well PT-100 Thermal Cycler (MJ Research Inc., Waltham, MA), programmed for an initial denaturation step of 1 min at 94° C, followed by 45 cycles of 1 min at 94° C, 30 second annealing at 37° C, 90 second extension at 72° C, and a final extension step of 5 minutes at 72° C. Following amplification, samples were incubated at 4° C until electrophoresis.

RAPD-PCR products were electrophoresed on 1.5% agarose gels, run in 0.5X TBE buffer (pH 8.0) for about 2 hours at 180 V. Gels were dry loaded so that wells were filled with only the PCR product and no loading dye. Ladder lanes, containing loading dye for tracking the progress of the electrophoretic front, were loaded in the first and last wells of each gel. "Dry" gels were electrophoresed for 10-15 minutes, so that DNA products migrated into the gel, then were flooded with TBE buffer. Electrophoresed gels were stained with ethidium bromide solution for 10-15 minutes and floated in tap water for 15-30 minutes to remove residual stain. Gels were visualized and photographed under UV light using an AlphaImager 1200 Documentation & Analysis System and MultiImage Light Cabinet (Alpha Innotech Corporation, CA, USA).

Prior to running the actual samples, 5 individuals from 6 populations were used to screen RAPD primers for usability in subsequent analysis. Approximately 100 primers from the University of British Columbia NAPS unit were screened. Of these, 15 primers that produced consistent, bright, easily scorable, and highly polymorphic bands were selected for final analysis. The primers used in the study are UBC Primer numbers 6, 18, 23, 25, 28, 30, 34, 51, 54, 56, 67, 70, 101, 103, and 105.

Gels were scored manually, with presence of a band being scored as 1 and absence as 0. Bands were scored only if they were sharp, reproducible, and occurring in at least 2 individuals per population. On instances where the gels were not satisfactorily scorable, or when more than 25% of the individuals did not amplfy, the reactions were repeated and the gels were rerun. On such occasions, the gels were compared to confirm if any scorable bands were consistent. Overall, there was a high level of consistency and reproducibility, allowing greater confidence on the final selection of bands. To reduce bias attributed to the dominant nature of RAPD markers in subsequent analysis, band loci were retained as useful markers only if the observed frequency of a band was less than 1- 3/n, where n is the sample size of the population (Lynch and Milligan, 1994).

6.2.3. Data analysis

The binary data matrix resulting from RAPD profiles of 486 individuals from 28 populations was used to calculate descriptive summary statistics for the data set using TFPGA (Miller, 2000). Percent polymorphic loci, average heterozygosity (i.e., expected heterozygosity), and estimates of population differentiation (centroid F_{ST}) were calculated for each population. Genetic identities/distances were then estimated for all

populations using the method of Nei (1972, 1978). Using AMOVA-PREP (Miller, 1998) the data matrix was imported to WINAMOVA Version 1.55 (Excoffier *et al.,* 1992, Excoffier, 1993) to perform a hierarchical analysis of molecular variance (AMOVA).

The binary data matrix was converted to a NEXUS-format file using MacClade 3.07 (Maddison and Maddison, 1997). The file was then imported to PAUP* 4.0b8a (Swofford, 2002) to generate Neighbor-Joining trees based on genetic distances among all 486 individuals. To assess population-specific relationships the data matrix was imported to PHYLIP (Felsenstein, 1997) after converting the binary data matrix to a matrix of estimated allele frequencies for each population. Allele frequencies were estimated assuming that all markers were in Hardy-Weinberg equilibrium and then calculating the frequency of the null allele (no band) from the frequency of the null RAPD phenotype. The programs GENDIST and NEIGHBOR in PHYLIP were used to generate a Neighbor-Joining tree showing patterns of genetic relationships among the 28 populations. Using SEQBOOT in PHYLIP, 1000 bootstrap replicates were performed. The tree files generated from PHYLIP were then exported to TREEVIEW (Page, 1996) and MacClade 3.07 to reroot the Neighbor-Joining tree, add bootstrap support values, and trace patterns of character evolution.

Using the program Latitude/Longitude Position Finder from Juggling Information Services (http://www.juggling.org/bin/dc/map.find) locality coordinates for all 28 populations were estimated. Surface distances among all populations were then calculated using the site, http://www.wcrl.ars.usda.gov/cee/java/lat-long.htm.

Mantel Tests were conducted using TFPGA (Miller, 2000) to establish relationships between genetic distance and geographical distance, genetic distance and

ecological distance (Chapter Five), and genetic distance and the extent of reproductive isolation (Chapter Five).

6.3. Results

The 15 primers used in the study generated 235 RAPD bands. As is customary, each band was assumed to represent a distinct locus (Williams *et al.*, 1990). Loci were pruned based on criteria set forth by Lynch and Milligan (1994) and a final data matrix of 190 loci was obtained for the use in all subsequent analyses.

6.3.1. Population genetic parameters

6.3.1.1. Percent polymorphic loci and average heterozygosity

Descriptive statistics for all populations are reported in Table 6.2. Overall, percent polymorphic loci at the population level was lower than 50% with the highest recorded for a race A population (PV) from Monterey County (53.7%). SPS and MR, two race A populations, also had >50% polymorphic loci. At Jasper Ridge, race A plants had greater levels of genetic diversity (47.9% polymorphic loci; 0.1363 avg. heterozygosity) compared to JRC (40% polymorphic loci; 0.1034 avg. heterozygosity). This general trend contributed towards greater overall genetic diversity in race A plants: percent polymorphic loci (and average heterozygosity) was highest for race A plants 95.8 % (0.1517) compared to race C, 78.4 % (0.1461). A comparison of mean genetic diversity estimates between the five populations growing on serpentine (JRA, JRC, MT, KCN, RS)

Table 6.2. Percent polymorphic loci, average heterozygosity, and centroid FST values for the populations sampled in the RAPD study.

Population	*Polymorphic Loci (%)*	*Average Heterozygosity*	*Centroid F_{ST}*
OR1C	45.3	0.1551	0.160
OR2C	41.1	0.1413	0.155
OR3C	37.9	0.1284	0.133
TRC	43.7	0.1411	0.107
44AC	37.4	0.1088	0.116
44C	37.4	0.1104	0.081
RBC	42.1	0.1311	0.121
R20C	33.2	0.1056	0.100
R29C	42.6	0.1295	0.124
SPSA	50.0	0.1442	0.102
CGLM	43.2	0.1391	0.086
DBLM	43.2	0.1421	0.120
PRA	36.3	0.0944	0.075
MTA	37.4	0.1088	0.084
SRMLM	33.7	0.0964	0.046
JRC	40.0	0.1034	0.103
JRA	47.9	0.1363	0.130
RSA	45.3	0.1528	0.145
KCNA	44.7	0.1287	0.121
25A	37.9	0.1075	0.133
PVA	53.7	0.1325	0.093
ASA	36.3	0.1105	0.092
TWA	37.9	0.1376	0.228
QA	38.9	0.1304	0.166
CASRA	34.2	0.0936	0.097
MRA	50.0	0.1488	0.140
AZA	37.4	0.1489	0.221
ARZA	35.3	0.1293	0.208

and populations growing on other substrates did not show significant differences in diversity estimates (*t*-Test; $P > 0.05$). An examination of genetic diversity patterns among the 12 populations with confirmed specific status suggests greater genetic diversity for the six *L. californica* populations (% polymorphic loci: 77.3 %; avg. heterozygosity 0.1606) than for the three *L. gracilis* populations (% polymorphic loci: 63.7 %; avg. heterozygosity 0.1407). For the three *L. macrantha* populations, % polymorphic loci was 63.2 while avg. heterozygosity was 0.1406.

6.3.1.2. Estimates of differentiation

Centroid F_{ST} values were used to estimate the degree of differentiation of each population, race, and species from the pooled total of all genetic variation (Table 6.2). Wright (1978) suggests that F_{ST} values from 0-0.05 indicates negligible genetic differentiation, 0.05-0.15 moderate genetic differentiation, and 0.15-0.25 great genetic differentiaion. Based on these guidelines, it was evident that all populations show moderate to great genetic differentiation with race A populations TW (Kern Co.), AZ and AZR (both from Arizona) showing the greatest differentiation (0.23, 0.22, and 0.21, respectively). Populations from northern (OR1, OR2, OR3, TR, 44A, 44, RB, R20, R29 – all race C) and central (SPS, PR, MT, JRC, JRA, RS, KCN, 25, PV, AS – all race A except JRC) parts of the range showed significant differentiation from populations in the southern part of the range (TW, Q, CASR, MR, AZ, ARZ – all race A) (ANOVA; $P = 0.002$; Tukey Test; $P = 0.01$ between C and S populations, $P = 0.001$ between N and S). There was no significant difference in differentiation estimates between populations growing on serpentine and other substrates (*t*-Test; $P > 0.05$). Differentiation estimates at

the racial and species level indirectly suggests that the species identity contributes more to the estimate than the racial identity. Centroid F_{ST} for the two races is 0.0632 (moderate differentiation) compared to 0.1797 for *L. californica s.s.* and *L. gracilis* (great differentiation). Further, differentiation between *L. californica s.s.* and *L. macrantha* (0.0765) is lower than that between *L. gracilis* and *L. macrantha* (0.1537) confirming the previously established close genetic relationship between *L. californica s.s.* and *L. macrantha* populations (Ornduff, 1996; Chan *et al.*, 2001, 2002). As noted previously, *L. macrantha* is now described as *L. californica* subsp. *macrantha* based on results from a comprehensive phylogeny for the genus (Chan *et al.*, 2001, 2002). For Jasper Ridge, centroid F_{ST} between races A (*L. californica*) and C (*L. gracilis*) was 0.2122 suggesting great differentiation between the two populations. This level of differentiation is greater than that estimated for the two races (0.0632) and species (0.1797) from the entire range.

Table 6.3 lists results from the AMOVA. Overall, more genetic variation is found within groups (i.e., populations, species, races etc.) than among groups. However, among group differences still contributed significantly to the total variance, agreeing with the patterns of population differentiation observed in the above analyses. At the population level, 40% of the variation is due to differences among populations. Species differences again appear to be contributing more to variance than racial differences. Among species differences explain 24% of the variance compared to 15% of the variance explained due to racial differences. Differentiation among race A and C populations as well as among populations of the three species appears to be comparable. Between the two races, slightly more variance is due to differences among race A populations (40%) than race C populations (35%) while among the three species, slightly more variance is explained by

Table 6.3. Results from the Analysis of Molecular Variance (AMOVA) based on 190 RAPD loci. Partitioning of variance calculated among and within all populations, races, and species.

Source of Variation	d.f.	Variance Components	Total Variance %	*P*-value
Among all populations	13	9.65	39.55	< 0.001
Within all populations	442	14. 8	60.45	< 0.001
Between races	1	4	15.35	< 0.001
Within races	427	22.15	84.65	< 0.001
Among race A	14	9.9	39.9	< 0.001
Within race A	240	14.9	60.1	< 0.001
Among race C	9	7.5	34.4	< 0.001
Within race C	182	14.4	65.6	< 0.001
Among species	2	6.5	23.9	< 0.001
Within species	201	20.7	76.1	< 0.001
Among *L. californica*	5	7.1	31.3	< 0.001
Within *L. californica*	105	15.6	68.7	< 0.001
Among *L. gracilis*	2	8.8	39.2	< 0.001
Within *L. gracilis*	51	13.6	60.8	< 0.001
Among *L. macrantha*	2	5.7	26.4	< 0.001
Within *L. macrantha*	36	15.9	73.6	< 0.001

differences among *L. gracilis* populations (40%) than among *L californica* (31%) or *L. macrantha* (26%) populations.

Table 6.4 lists pairwise Genetic Identities (*I*) calculated using Nei's method (1972, 1978). The range of identities for all populations was 0.89-0.98. Overall, populations mostly had an *I* over 0.95 with the lowest *I* (0.89-0.9) recorded for populations that were most geographically remote (OR and AZ). At Jasper Ridge, the two races had an *I* of 0.959. Genetic Identities were estimated for populations representing the two races (A vs C: 0.9878) and the confirmed populations of the three species (*Lc* vs *Lg*: 0.9592; *Lc* vs *Lm*: 0.9833; *Lg* vs *Lm*: 0.9685). The *I* values further confirm the trends observed in the centroid F_{ST} estimates: races appear not to be as well differentiated as species, *L. californica* and *L. gracilis* have the greatest pairwise genetic distance among species' comparisons, and *L. californica* has a greater genetic similarity to *L. macrantha* than has *L. gracilis*. Similar to previous observations, the two races (and species) at Jasper Ridge were more distant (0.959) than the two races (0.9878) from the species' range. However, the level of identity between the two populations at Jasper Ridge was comparable to the level observed for populations of the two confirmed species (0.9592).

6.3.2. Relationships among populations

Figure 6.2 shows a dendrogram resulting from the genetic distance analysis conducted using PAUP* 4.0b8a for all individuals from all populations. Overall, the patterns confirm that intra-population genetic differences are low relative to inter-population

Table 6.4. Pairwise Genetic Distances as estimated by the method of Nei (1972/1978) for all populations sampled in the study.

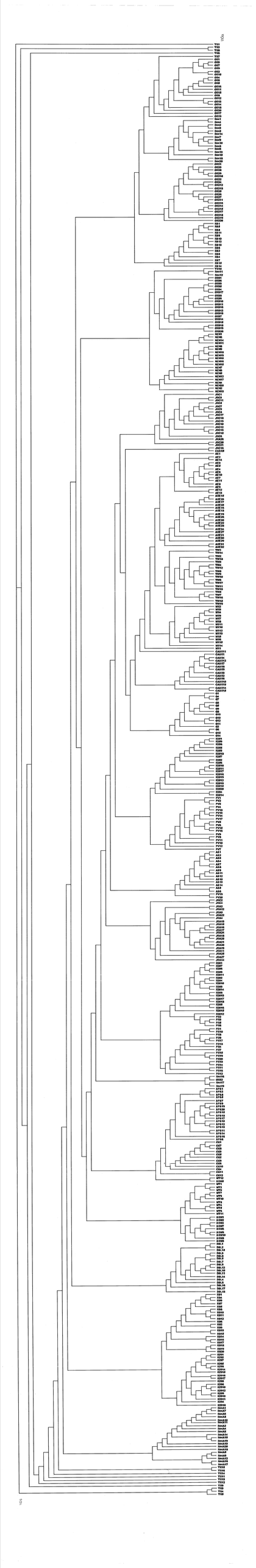
	TR	JRC	JRA	AZ	ARZ	44	25	SPS	R20	OR1	OR2	OR3	KCN	RB	CASR	44A	MT	SRM	PR	R29	TW	DB	Q	MR	AS	RS	PV	CG
TR	*****																											
JRC	0.9452	*****																										
JRA	0.9536	0.9590	*****																									
AZ	0.9029	0.9487	0.9112	*****																								
ARZ	0.9092	0.9473	0.9146	0.9755	*****																							
44	0.9717	0.9592	0.9633	0.9162	0.9228	*****																						
25	0.9400	0.9582	0.9418	0.9335	0.9339	0.9498	*****																					
SPS	0.9574	0.9519	0.9620	0.9170	0.9222	0.9636	0.9521	*****																				
R20	0.9584	0.9593	0.9508	0.9167	0.9244	0.9658	0.9525	0.9558	*****																			
OR1	0.9628	0.9347	0.9496	0.8858	0.8958	0.9641	0.9250	0.9469	0.9480	*****																		
OR2	0.9672	0.9379	0.9510	0.8883	0.8939	0.9629	0.9307	0.9452	0.9483	0.9707	*****																	
OR3	0.9710	0.9381	0.9545	0.8918	0.9002	0.9712	0.9314	0.9519	0.9484	0.9685	0.9697	*****																
KCN	0.9515	0.9466	0.9508	0.9089	0.9135	0.9658	0.9466	0.9660	0.9505	0.9522	0.9513	0.9626	*****															
RB	0.9714	0.9412	0.9562	0.8990	0.9068	0.9659	0.9436	0.9576	0.9601	0.9540	0.9587	0.9710	0.9549	*****														
CASR	0.9438	0.9724	0.9446	0.9548	0.9584	0.9566	0.9597	0.9517	0.9563	0.9309	0.9293	0.9373	0.9466	0.9403	*****													
44A	0.9704	0.9437	0.9526	0.9074	0.9166	0.9651	0.9410	0.9545	0.9577	0.9492	0.9583	0.9636	0.9532	0.9680	0.9443	*****												
MT	0.9564	0.9603	0.9528	0.9218	0.9294	0.9619	0.9510	0.9623	0.9604	0.9463	0.9446	0.9542	0.9650	0.9580	0.9583	0.9569	*****											
SRM	0.9647	0.9691	0.9596	0.9324	0.9390	0.9738	0.9571	0.9673	0.9715	0.9481	0.9514	0.9571	0.9638	0.9652	0.9676	0.9652	0.9820	*****										
PR	0.9689	0.9608	0.9516	0.9243	0.9310	0.9660	0.9617	0.9640	0.9748	0.9480	0.9497	0.9561	0.9584	0.9615	0.9613	0.9653	0.9698	0.9769	*****									
R29	0.9574	0.9444	0.9513	0.9086	0.9116	0.9589	0.9422	0.9616	0.9567	0.9436	0.9464	0.9504	0.9522	0.9619	0.9442	0.9645	0.9558	0.9633	0.9605	*****								
TW	0.9086	0.9457	0.9118	0.9442	0.9522	0.9178	0.9302	0.9128	0.9216	0.8891	0.8890	0.8970	0.9077	0.9039	0.9519	0.9094	0.9281	0.9364	0.9278	0.9094	*****							
DB	0.9611	0.9478	0.9458	0.9038	0.9115	0.9553	0.9474	0.9596	0.9525	0.9455	0.9484	0.9510	0.9564	0.9526	0.9454	0.9613	0.9638	0.9600	0.9645	0.9642	0.9113	*****						
Q	0.9243	0.9560	0.9245	0.9452	0.9480	0.9352	0.9414	0.9367	0.9395	0.9084	0.9094	0.9196	0.9280	0.9236	0.9668	0.9235	0.9443	0.9497	0.9488	0.9262	0.9483	0.9256	*****					
MR	0.9279	0.9644	0.9362	0.9544	0.9561	0.9429	0.9497	0.9403	0.9437	0.9158	0.9150	0.9206	0.9335	0.9229	0.9736	0.9348	0.9446	0.9540	0.9487	0.9320	0.9477	0.9343	0.9654	*****				
AS	0.9519	0.9596	0.9459	0.9410	0.9486	0.9636	0.9602	0.9547	0.9545	0.9382	0.9404	0.9452	0.9510	0.9460	0.9608	0.9523	0.9631	0.9693	0.9670	0.9541	0.9382	0.9520	0.9524	0.9546	*****			
RS	0.9553	0.9405	0.9565	0.8986	0.9056	0.9618	0.9370	0.9610	0.9449	0.9468	0.9475	0.9522	0.9532	0.9472	0.9377	0.9440	0.9595	0.9578	0.9506	0.9459	0.8959	0.9531	0.9151	0.9274	0.9459	*****		
PV	0.9482	0.9590	0.9433	0.9478	0.9506	0.9572	0.9695	0.9517	0.9607	0.9351	0.9366	0.9429	0.9507	0.9466	0.9673	0.9508	0.9570	0.9663	0.9708	0.9466	0.9495	0.9488	0.9577	0.9601	0.9711	0.9395	*****	
CG	0.9630	0.9535	0.9507	0.9141	0.9210	0.9635	0.9517	0.9709	0.9615	0.9530	0.9519	0.9606	0.9643	0.9643	0.9492	0.9595	0.9624	0.9744	0.9686	0.9579	0.9165	0.9692	0.9321	0.9400	0.9540	0.9587	0.9592	*****

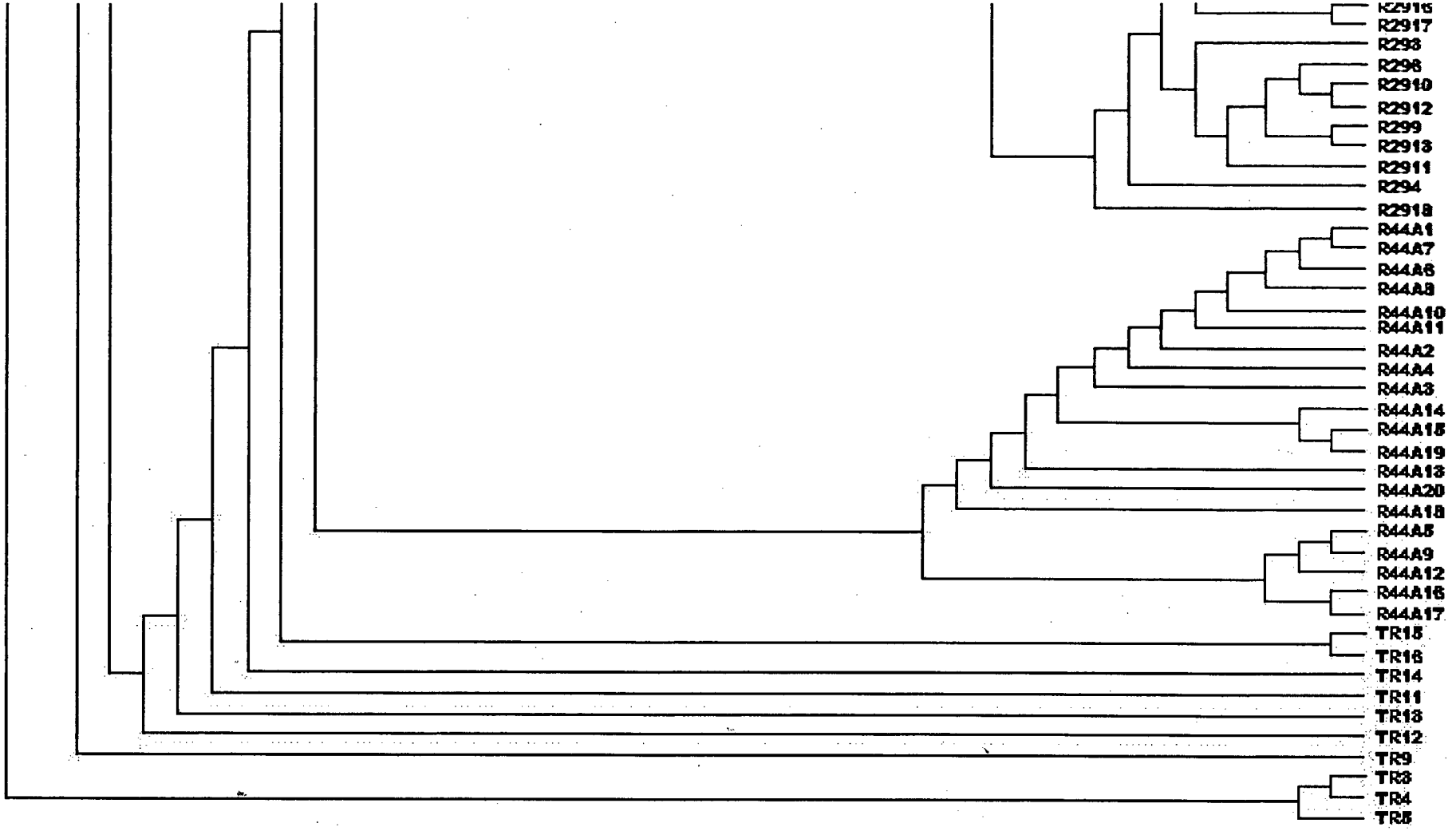
genetic differences, and that populations represent distinct demographic units. Out of 486 individuals 25 failed to group with their population, instead clustering with populations ollected from a nearby site and/or a population with which they share a higher genetic identity. Most of these individuals nest not within populations but as clusters lying outside populations. Notable exceptions are one individual from TR clustering within OR1 population and one JRA individual that clustered within the JRC population.

The Neighbor-Joining tree from PHYLIP is shown (Figure 6.3) with bootstrap values for the 14 well-resolved branches (bootstrap support > 50%). The tree is rooted to depict the known relationship of the two recently described phylogenetic species (Chan *et al.*, 2001, 2002), leading to the resolution of two well-supported clusters (91% bootstrap support; F_{ST} 0.1092), a northern cluster of populations representing plants collected from southern Oregon to Santa Clara Co., and a southern cluster of populations representing plants collected from San Benito Co. to Arizona. Races A and C from Jasper Ridge cluster with northern and southern populations, respectively. Many of the internal nodes within the southern cluster are strongly supported (> 70% bootstrap support) while within the northern cluster of populations, the four Oregon populations form a distinct unit (81% bootstrap support). The patterns depicted in the dendrogram are further supported by genetic distance (Table 6.4) and differentiation estimates (Table 6.2, 6.3) reported above. Figure 6.4 is similar to Figure 6.3, however, all branch lengths correspond to the extent of divergence among all populations (parallels trends observed from centroid F_{ST} estimates).

Figure 6.5 is a dendrogram generated by MacClade 3.07 to illustrate patterns of race evolution within the *L. californica* complex. The pattern reveals that it is more

Figure 6.2. Complete Neighbor-Joining distance tree from PAUP* based on RAPD marker fingerprints for 486 individuals collected from 28 populations. Genetic distances (Nei, 1978) were calculated from total character differences between pairs of individuals.




R2916
R2917
R293
R296
R2910
R2912
R299
R2913
R2911
R294
R2918
R44A1
R44A7
R44A6
R44A8
R44A10
R44A11
R44A2
R44A4
R44A3
R44A14
R44A15
R44A19
R44A13
R44A20
R44A18
R44A5
R44A9
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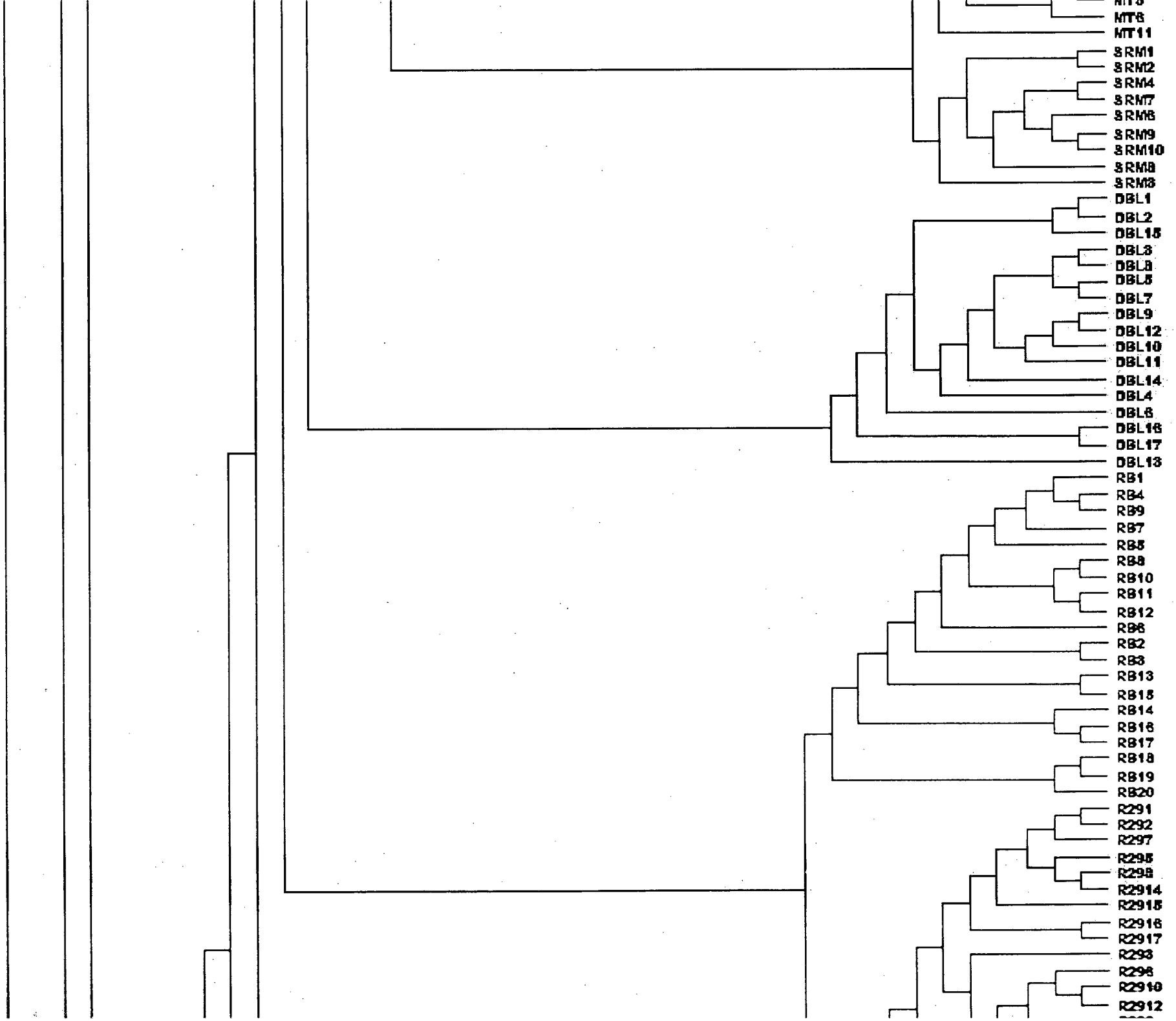
TR11
TR13
TR12
TR9
TR3
TR4
TR5


MT6
MT11
SRM1
SRM2
SRM4
SRM7
SRM6
SRM9
SRM10
SRM8
SRM3
DBL1
DBL2
DBL15
DBL3
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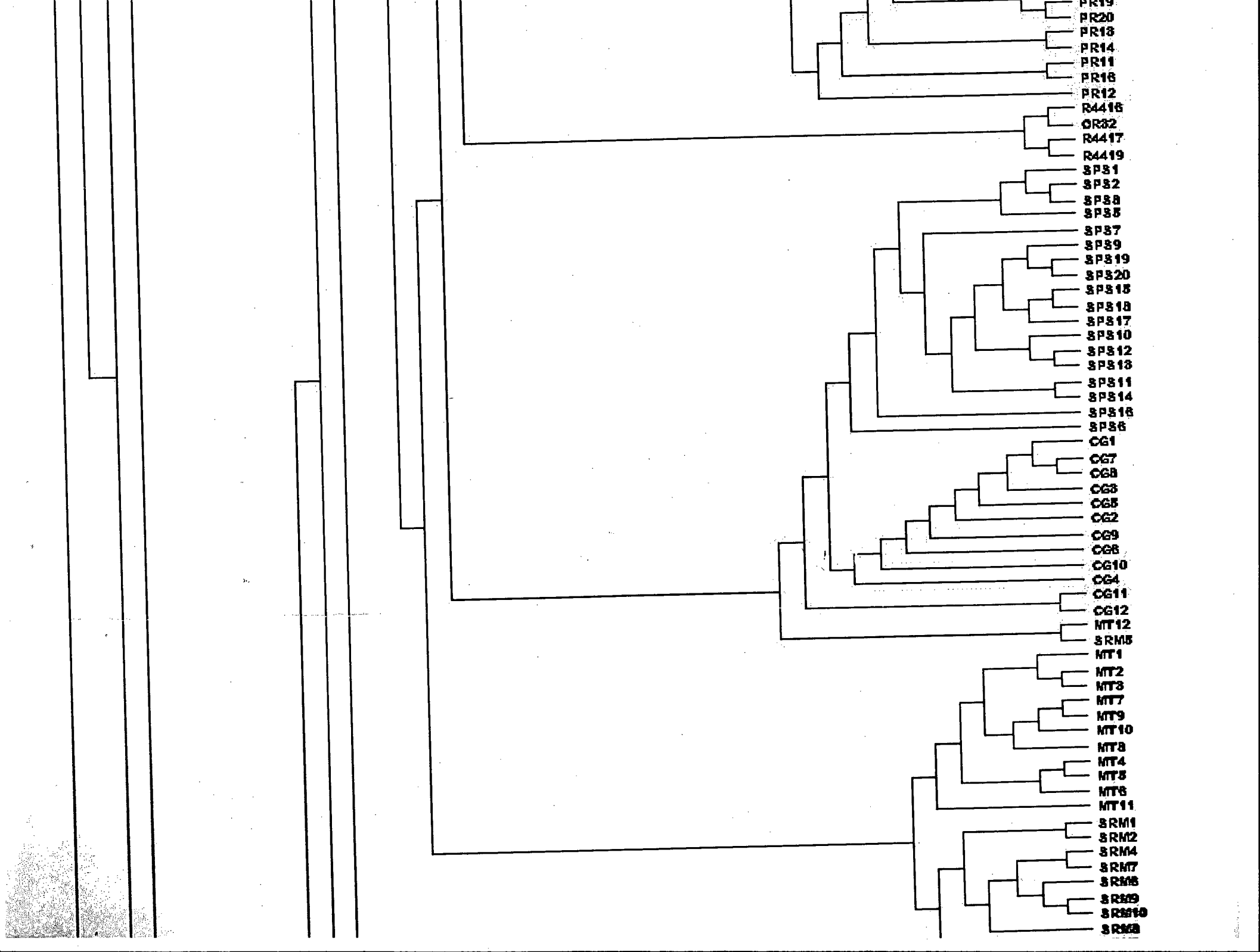
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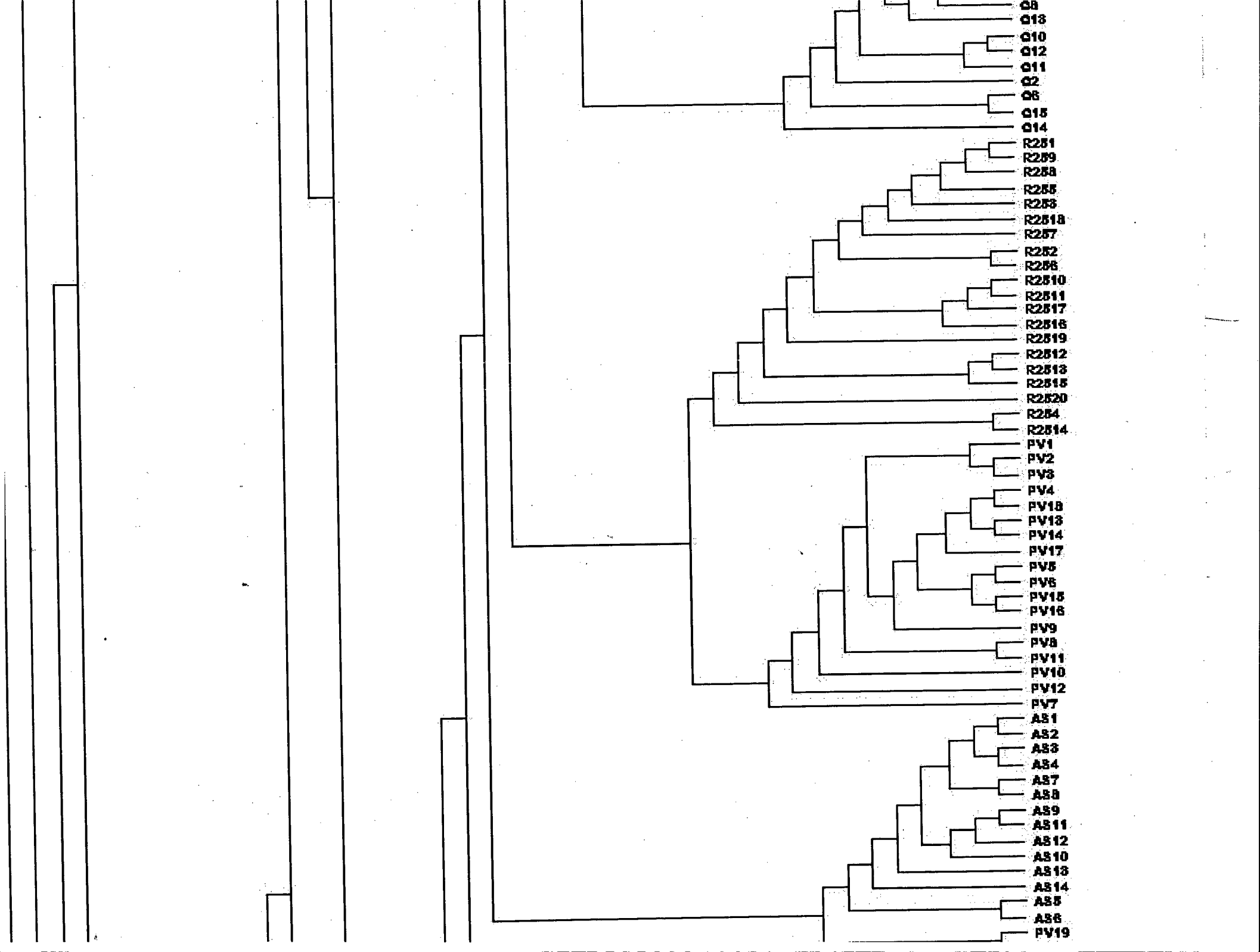
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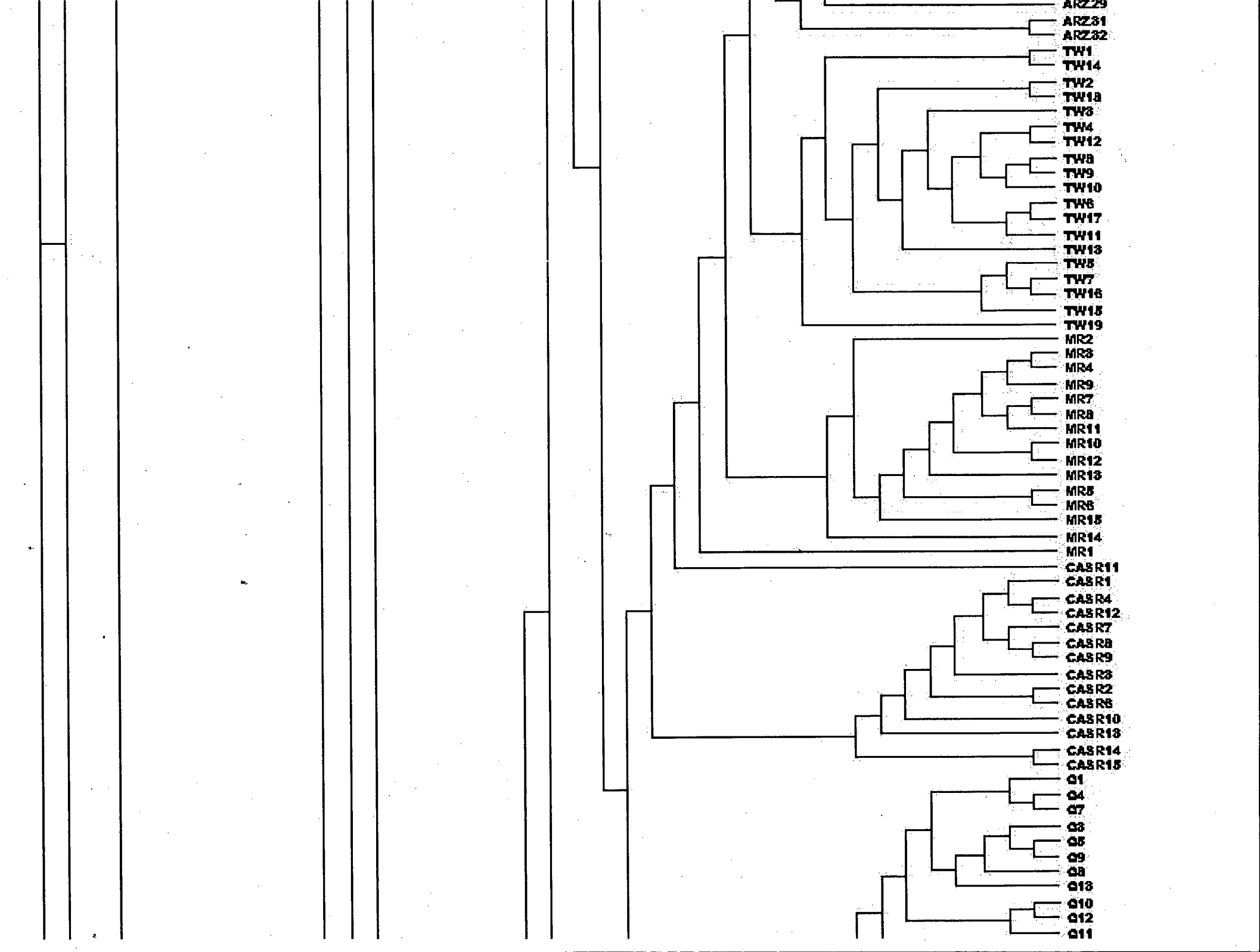
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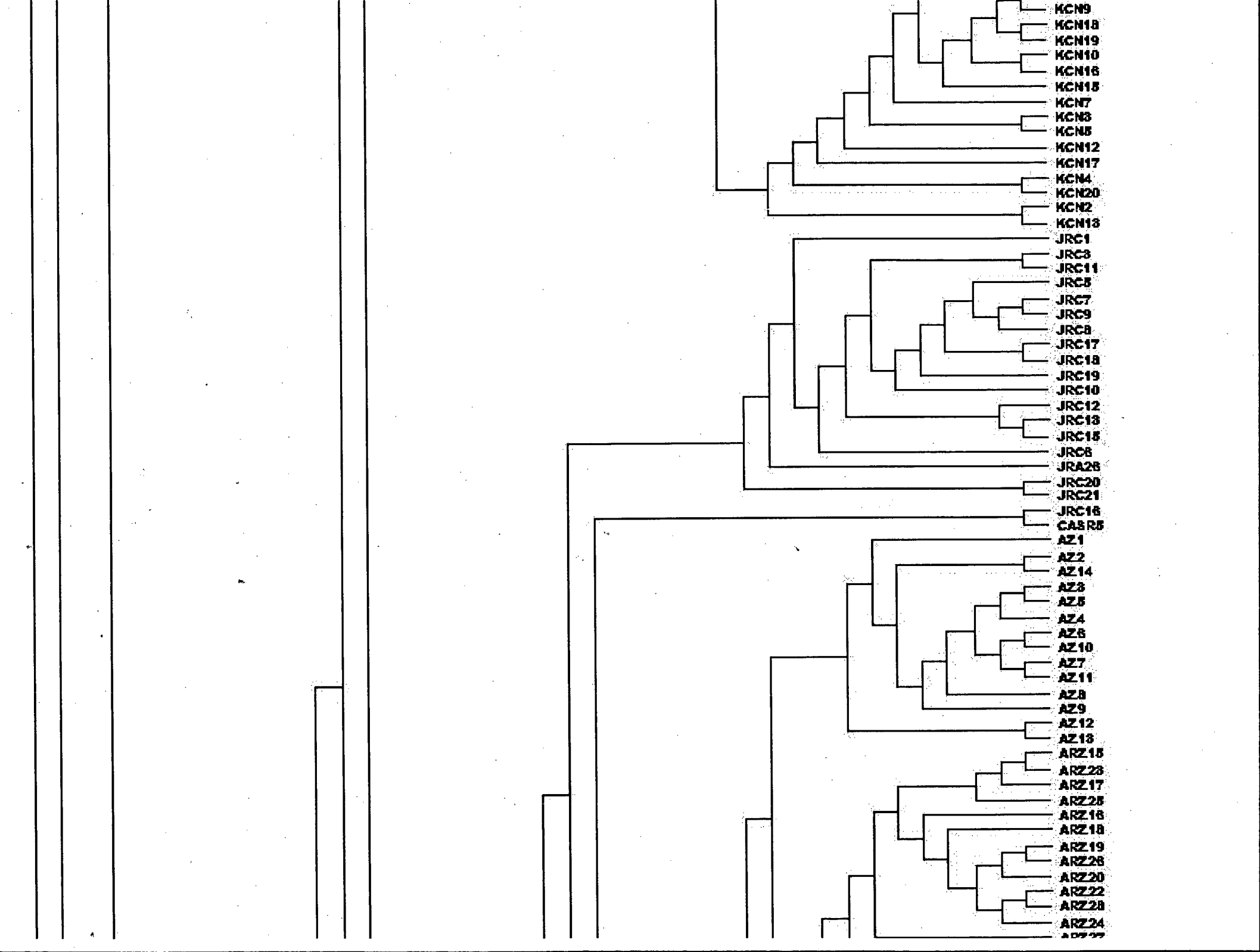
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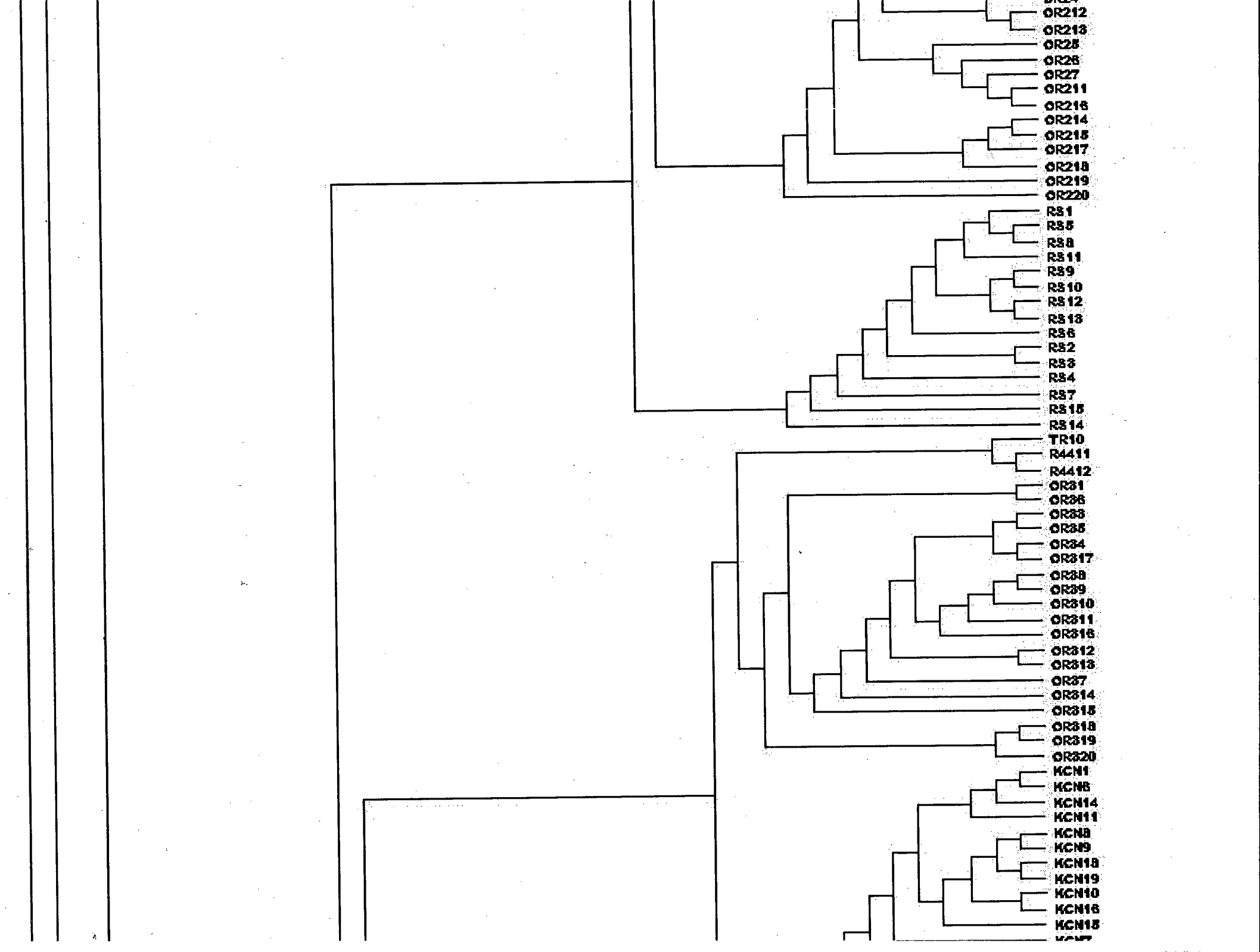
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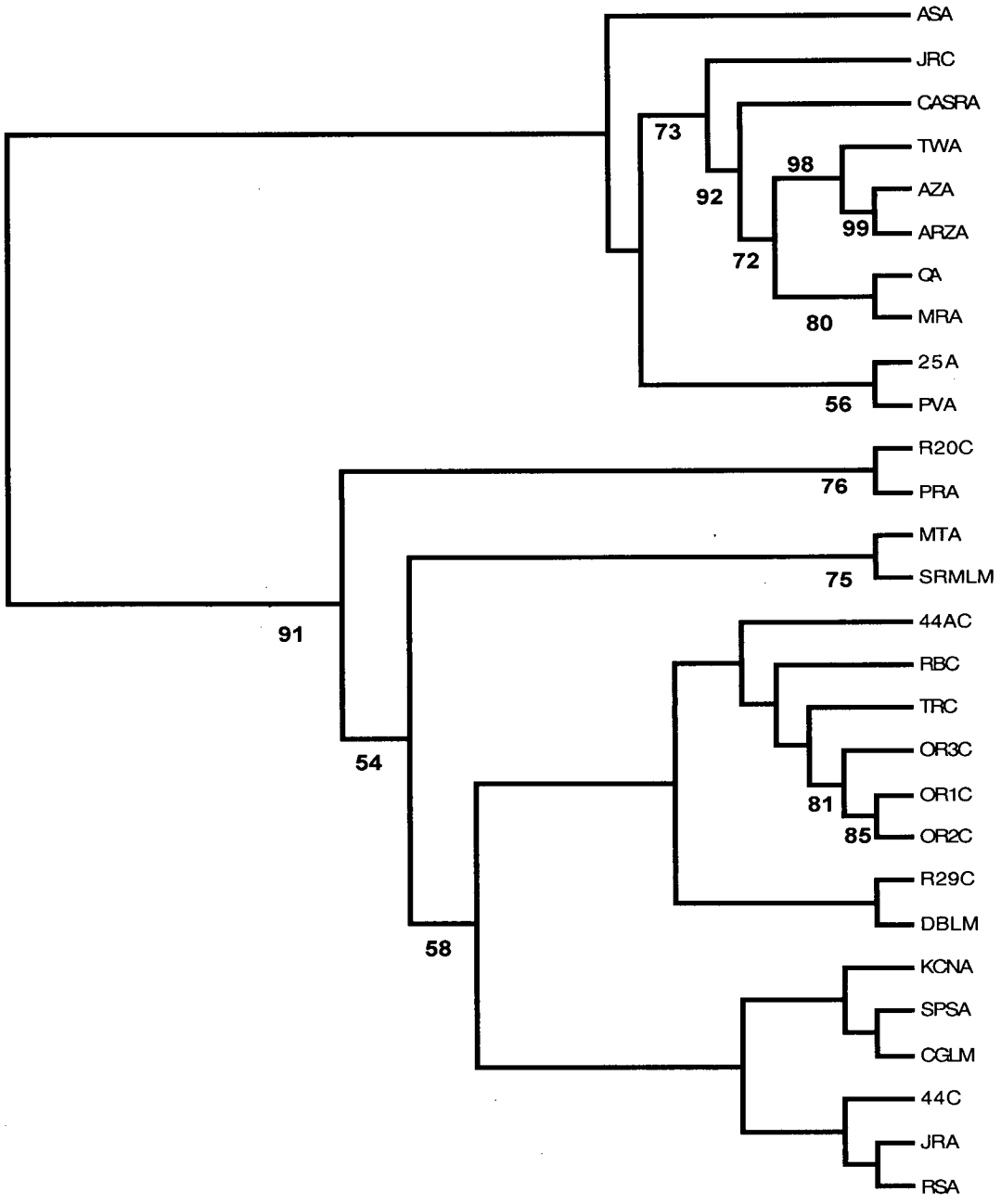

Figure 6.3. Neighbor-Joining distance tree from PHYLIP based on estimated allele frequencies for 190 RAPD loci. Rooting was done using the confirmed *L. gracilis* populations as the outgroup. Bootstrap support values over 50% are given. Race of each population is denoted by letters C and A.

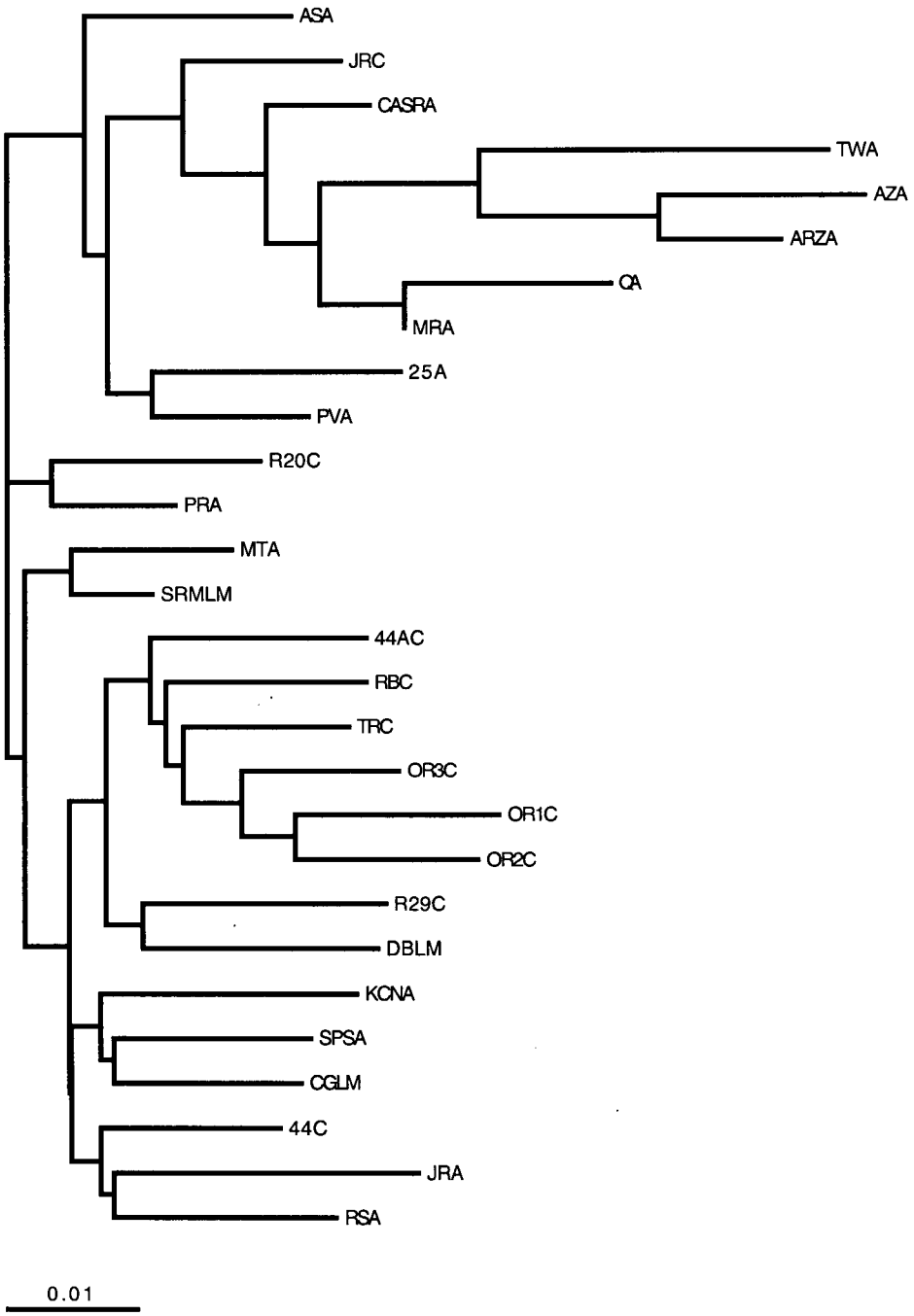

Figure 6.4. Neighbor-Joining distance tree from PHYLIP with branch lengths indicating the extent of genetic differentiation for each population. Populations TW, AZ, ARZ have the longest branch lengths (i.e., the most divergent). Trends seen in this dendrogram parallel the trends observed from F_{ST} values.

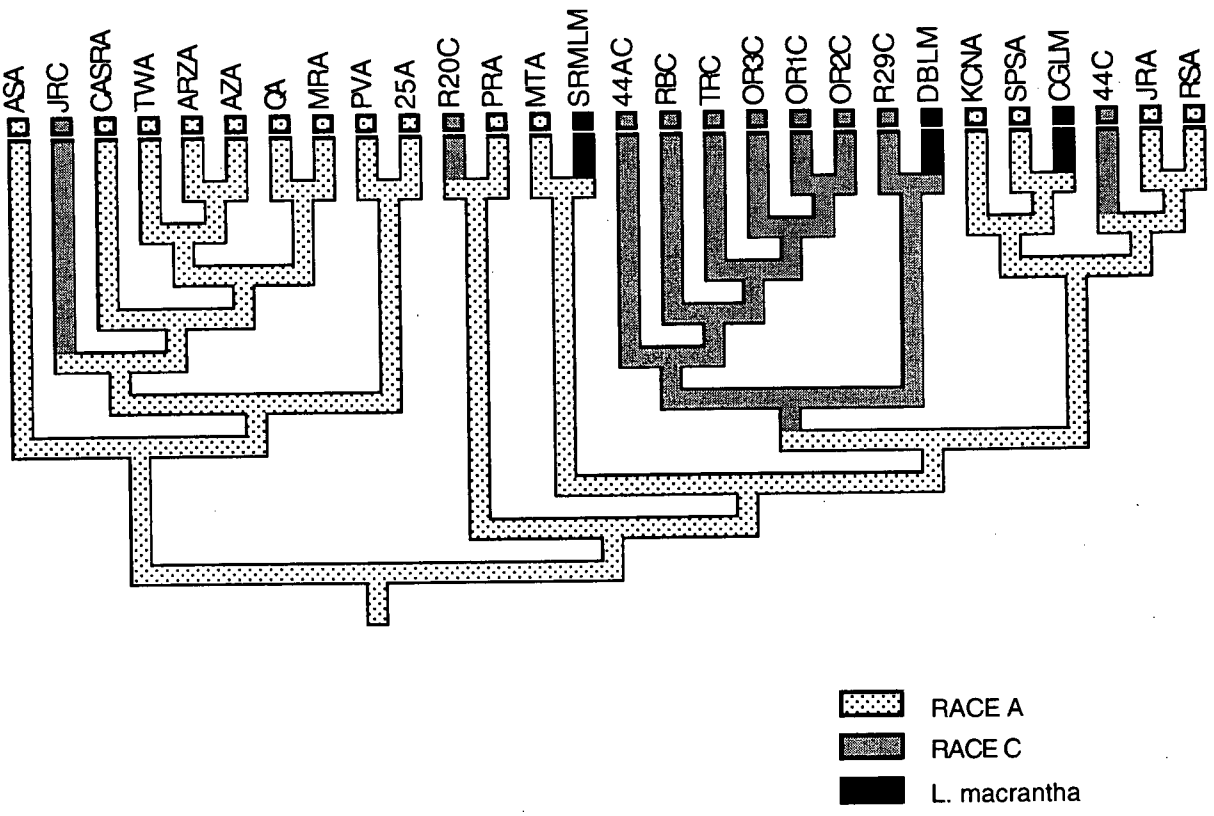

Figure 6.5. The Neighbor-Joining distance tree from PHYLIP redrawn using MacClade 3.07 to illustrate patterns of race evolution in the *L. californica* complex. It is more likely for the flavonoid trait for race A to be ancestral since race C appears to have evolved only four times. In comparison, race A would have evolved six times if the flavonoid trait for race C was ancestral.

parsimonious to assume that race A is ancestral within the complex (four steps account for distribution of race C versus six steps if race C is assumed ancestral). Manipulation of the position of the root did not alter this outcome.

6.3.3. Relationships between genetic distance and other matrices

Mantel Tests suggest that there is a low yet significant correlation ($r = 0.03$; $P = 0.001$) between genetic distance and geographical distance among all 28 populations. The correlation between genetic distance and ecological distance (Chapter Five) was insignificant ($r = 0.02$; $P > 0.05$) for 11 populations subjected to the analysis (JRC, JRA, RSA, OR1C, OR2C, OR3C, PVA, RBC, TWA, and TRC). Further, the correlation between genetic distance and seed set (i.e., an estimate of reproductive isolation; Chapter Five) was also insignificant ($r = - 0.2$; $P > 0.05$) for five populations included in the analysis (JRA, JRC, RSA, TRC, QA).

6.4. Discussion

The population genetic study strongly supports two distinct population clusters within the *L. californica* complex (91% bootstrap support; F_{ST} 0.1092). Regardless of the rooting, the patterns of clustering of certain populations are consistent with trends observed in previous studies conducted using allozymes (Desrochers and Bohm, 1995) and macromolecular markers (Chan *et al.*, 2001, 2002; Desrochers and Dodge, in press). Further, the relationship between the parapatric races at Jasper Ridge is similar to that observed in the allozyme study (Desrochers and Bohm, 1995) and the ITS phylogeny (Desrochers and Dodge, in press). This congruity is not unexpected given that

parameters describing genetic differentiation are also comparable qualitatively and rather similar quantitatively between the allozyme and RAPD analyses for the complex. Such similarity has been observed in some studies (Aagaard *et al.*, 1998; Buso et al., 1998; Bartish *et al.*, 1999; Bussell, 1999) while significant differences have been observed in others (Liu and Furnier, 1993; Lanner–Herrera *et al.*, 1996; Oiki *et al.*, 2001).

At the species level, *L. californica* subsp. *californica* showed the greatest levels of genetic diversity. Overall, the species complex showed greater genetic diversity than which has been estimated by allozyme analysis for congeneric species, *L. minor* (DC.) Ornduff and *L. maritima* (A.Gray) M. Vasey (Crawford *et al.*, 1985) and slightly higher levels of diversity than which has been estimated by allozyme analysis (Crawford and Ornduff, 1989) for the section *Ptilomeris* [*L. conjugens* Greene, *L. burkei* (Greene) Greene, *L. fremontii* (Torr. ex A.Gray) Greene]. Because the RAPD technique is known to generate greater numbers of detectable loci than allozyme studies (Aagaard *et al.*, 1998), it is not reasonable to compare levels of variation to those obtained with allozymes. The study pointed to a closer genetic relationship between *L. californica* subsp. *californica* and *L. macrantha* than between *L. californica* subsp. *californica* and *L. gracilis*, relationships strongly supported by the recent comprehensive phylogeny of Chan *et al.* (2001, 2002) and previous suggestions by Ornduff (1966, 1971). These close relationships have recently led to reclassifying *L. macrantha* as *L. californica* subsp. *macrantha* as well as the division between the cryptic species, *L. californica* subsp. *californica* and *L. gracilis* (Chan, 2001).

It is clear from all parameters calculated (F_{ST}, Genetic Identity, AMOVA) that species designation contributes more toward genetic differentiation than do racial

designations. However, the racial identities also play a significant role in the partitioning of molecular variance in the complex where 15% of the variance can still be attributed to variation between the two edaphic races. Genetic differentiation estimates suggest a close relationship with geographical distance for some populations, with three populations (TW, AZ, ARZ – all race A) from the southern part of the range showing the greatest levels of differentiation. Overall, the populations in the southern parts of the range (all race A) showed significant differentiation from populations found in the central (all but one race A) or northern parts of the range (all race C). The relationship between geographical distance and genetic distance was significant, however, only a very low r value was obtained for the correlation.

At the racial level, it is clear that race A populations show greater levels of genetic diversity than race C populations. Greater levels of overall diversity observed in the race that is edaphically specialized suggest an ancestral condition for the race (Gottlieb, 1973; Crawford, 1983). It is possible that greater levels of diversity in the race may have allowed tolerance to the variety of unusual edaphic habitats under which race A is found. However, it is possible that adaptations to unusual edaphic habitats may only depend on a few loci of major effect (Christie and Macnair, 1987; Bradshaw *et al.*, 1998). Further, local adaptations to serpentine and other such extreme conditions may only be based on selection on specific combinations of loci rather than depending on extensive genetic differentiation. The limited number of populations subjected to the correlation analysis between genetic distance and reproductive isolation suggested an insignificant relationship between the two variables. It is interesting to note, however, that a high correlation ($r = 0.78$; $P = 0.07$) was obtained for the relationship between

ecological distance and reproductive isolation (Chapter Five), suggesting that ecological features may be a better predictor of reproductive isolation than genetic distance among populations.

All estimates show significant differentiation between the two races at Jasper Ridge agreeing with previous studies of allozyme variation (Desrochers and Bohm 1995), ITS phylogenies (Desrochers and Dodge, in press; Chapter Two), physiological differences (Chapter Three, Four), and reproductive isolation (Chapter Five). It is now clear that the Jasper Ridge site was established by representatives of the southern *L. gracilis* and the northern *L. californica* subsp. *californica*. Racial differences between these two colonizers may have resulted in the habitation of different parts of the outcrop, with race A plants (*L. californica*) occupying the harsh ridge bottom while race C plants (*L. gracilis*) became restricted to the ionically-benign ridge top. It is unclear whether specific traits such as early flowering in race C plants at this site resulted from local adaptation to the drier ridge top or whether they reflect traits common to populations in the southern cluster that gave rise to the Jasper Ridge colonizers. It is also clear from the discussion in Chapter Five that reinforcement or ecological selection may have played a role in greater levels of divergence of the two races at Jasper Ridge. It is interesting to note that the estimate for genetic differentiation for the two races at Jasper Ridge is much greater than the estimate obtained for differentiation between the two races or the two species from the entire range. A preliminary examination of RAPD loci from the transition zone between races A and C at Jasper Ridge, however, shows indications of introgression of certain RAPD markers between the two races (results not included) and that the Jasper Ridge site may in fact represent a hybrid zone. Although JRC and JRA

cluster with populations from south and north, respectively, genetic identity estimates for the two races at this site show a close relationship between the two populations. JRA population has JRC as the fourth most similar population while for JRC, JRA is the eighth most similar population. While it is difficult to put much emphasis on specific values that are found within a narrow range of identities (0.89-0.98) for the complex, this pattern is still worthy of speculation. The breeding study (Chapter Five) suggested that JRA is a better parent than JRC. Levels of seed set between JRA and all other populations were much greater than levels observed between JRC and other populations. Thus, it is reasonable to hypothesize that there may be asymmetrical gene flow between the races at this site with more gene flow occurring from JRC to JRA than vice versa. Further, the early flowering of the more numerous race C plants at this site may lead to more inter-specific pollen being available for pollination of the late flowering race A plants. This is especially true if pollen longevity is high so that when race A reaches receptivity, race C pollen is still abundant in the environment. However, when race A plants reach anthesis, most race C plants have already been pollinated by the freely available race C pollen and have reached the seed maturation stage. Thus, flowering time differences could potentially lead to an asymmetry in gene flow between the two races. Whether this is the cause of the asymmetry in genetic identity at this site will be clarified by detailed analysis of gene flow at this site.

An examination of trait evolution (race) in the complex corroborates evidence from genetic diversity estimates: race A appears to be ancestral to race C. The number of steps required for race C to have evolved from race A is fewer than the number of steps required for race A to have evolved from race C. This observation is of interest in light

of the known trends in flavonoid evolution (Harborne, 1977; Gornall and Bohm, 1978). The primary difference in flavonoid pigments between the two races is in the sulfated flavonoids that are found in race A but not in race C. Overall, the flavonoid profile in race A is more complex than that of race C (Bohm *et al.*, 1989; Desrochers and Bohm, 1995); however, structural complexity does not necessarily imply derived states. It is overwhelmingly clear that, in flowering plants, the dominant evolutionary trend in flavonoids is one of reduction in both structural complexity and number of flavonoids produced (see review by Gornall and Bohm, 1978). Hence, it is reasonable speculate that the race C flavonoid profile may have been derived from the more complex profile found in race A plants.

The notion that edaphic specialization is ancestral is also contrary to common belief; many examples to date suggest that edaphic specialists evolve from non-specialized ancestors and that evolution to extreme edaphic conditions can occur independently in populations exposed to similar selective pressures (Westerbergh and Saura, 1992; Mayer *et al.*, 1994a,b; Westerbergh, 1996; Schat *et al.*, 1996). In the genus *Streptanthus* (Brassicaceae), tolerance to serpentine appears to have evolved multiple times (Kruckeberg, 1954; Mayer *et al.*, 1994a,b). Rapid evolution of serpentine tolerance was also inferred for populations of *Prunella vulgaris* L. (Lamiaceae) and *Rumex acetosella* L. (Polygonaceae), two species with populations that occupy serpentine substrate exposed just 100 years ago by receding glaciers (Kruckeberg, 1967). The heavy metal-tolerant literature is also full of examples of rapid and recurrent evolution of metal tolerance (reviewed in Antonovics *et al.*, 1971; Lefebvre and Vernet, 1990; Levin, 2001).

Although there is abundant speculation that edaphic specialization evolves with relative ease, it is difficult to exclude the possibility that edaphic specialization is an ancestral condition in some species, that is eventually lost by descendent populations on ionically-benign substrates. This appears to be the case within the *L. californica* complex.

There is considerable evidence that tolerance to extreme edaphic conditions involves a cost (Macnair and Watkins, 1983; Harper *et al.*, 1997a,b). Classic studies of heavy metal tolerance in populations adapted to mine tailings (McNeilly, 1968; Cook *et al.*, 1972; Hickey and McNeilly, 1975) or serpentine outcrops (Kruckeberg, 1951, 1954, 1967) demonstrate that physiological differentiation can be maintained because genotypes that are edaphically specialized compete poorly on uncontaminated soils. Reciprocal growth studies conducted on the two races from Jasper Ridge clearly suggested that race A plants are able to grow in race C soil (Rajakaruna, 1998; Rajakaruna and Bohm, 1999), whereas race C could not reach reproductive maturity in race A soil. However, in many years of field collections (1981-present), only the occasional race A individual has been located in soils where race C is found. This suggests that this pattern results from biotic control (i.e., competition). Race C germinates earlier in greater percentages and reaches flowering 7-10 days prior to race A plants. Faster growth to reproductive maturity may simply out-compete the generally slow-growing race A plants on race C soils. This explanation is comparable to the one given for metal and serpentine tolerant genotypes in previously mentioned studies, suggesting that the cost of tolerance could provide the selective push for reversion to non-tolerance. It also would not be surprising if distribution of tolerance among populations of edaphically diverse species such as *Silene*

and *Streptanthus* actually reflects a complex history of multiple derivations and/or losses of various traits. Such multiple gains and reversions of numerous chromosomal, chemical, and morphological features have become increasingly apparent through phylogenetic analyses (Soltis and Soltis, 1995).

Fourteen out of the 18 species in *Lasthenia* are tolerant of ionically extreme edaphic habitats. It is possible that tolerance to ionic stress reflects a condition that is ancestral for the genus. Tolerance may have been retained in species that radiated into extreme habitats while it was lost in those few that occupy benign habitats. Within the *L. californica* complex a similar trend could have occurred, with features that correspond to race A (flavonoid sulfates, Na^+ and Mg^{2+} tolerance – Chapters Two, Three) being retained in populations that were exposed to edaphic stresses while lost in those populations that came to occupy benign inland habitats (see Chapter Seven for a detailed discussion of trait evolution in *Lasthenia*). For example, ion localization mechanisms such as those required to pump Na^+ into vacuoles require energy (Apse *et al.*, 1999) and it is reasonable to speculate that there would have been a selective drive to lose such energy consuming traits in populations not exposed to ionic stresses. Regardless, it is clear that both edaphic races occur in both geographical clusters of populations (Figure 6.3) that appear to correspond to the two cryptic phylogenetic species recently described by Chan *et al.* (2001, 2002). Additional ITS sequencing of populations used in the RAPD study or flavonoid/physiological profiling of populations subjected to the ITS study (Chan *et al.*, 2002) will further our understanding of the origins and maintenance of racial features within the complex.

Further, a closer examination of the RAPD profiles between JR populations and populations of the two races (species) from other locations will inform us of possible patterns of introgression within the complex. If hybrids/introgressants can be identified via RAPD profiles, it will be possible to examine post-zygotic isolation (via hybrid inviability, hybrid sterility, reduced hybrid fitness) between races. This information will add to our knowledge on the extent of isolation (Chapter Five) between the races and further our understanding of the role of ecological divergence/reinforcement in diversification (Chapter Five). Most importantly, such information will be critical to further the hypothesis of edaphic differentiation in the *L. californica* complex.

CHAPTER SEVEN

Lasthenia—A model genus for studies in evolutionary ecology: Conclusions and future directions

"*They grow in wet places, and appear to be uninteresting weeds.*"

John Lindley and Thomas Moore, 1876

They do "*grow in wet places*," but studies of *Lasthenia* over the last several decades have shown the genus to consist of anything but "*uninteresting weeds*." Commonly known as goldfields, species of *Lasthenia* occupy large areas of the Californian landscape, casting spectacular carpets of brightly-colored golden yellow flowers in early spring. A recent phylogenetic study recognized 21 species and subspecies belonging to seven sections (Chan *et al.*, 2001), with all but one species endemic to the Californian Floristic Province. *Lasthenia kunthii* (Less.) Hook & Arn., the only member of the genus found outside western North America, is endemic to vernal pools and marshes in central Chile (Ornduff, 1966). Members of the genus have wide edaphic tolerance; species are found in habitats such as coastal bluffs, guano deposits, vernal pools, salt and alkaline flats, serpentine outcrops, deserts, grasslands, and open woodlands (Ornduff, 1966, 1993). Members of *Lasthenia californica sensu* Ornduff have the widest edaphic tolerance within the genus, with populations occurring in all but guano habitats. Keck (1959) stated that *L. californica sensu* Ornduff [then *Baeria chrysostoma* (Fischer & C. Meyer) E. Greene] was the most abundant composite in California. Other *Lasthenia* taxa have rather restricted distributions, with seven taxa now listed in California Native Plant Society Inventory of Rare and Endangered Vascular Plants (Tibor, 2001).

Following Ornduff's extensive monograph (1966), *Lasthenia* has received considerable attention. Early studies examined inter- and intra-specific variation in flavonoid features (Saleh and Bohm, 1971; Ornduff *et al.*, 1973, 1974; Bohm *et al.*, 1989; Desrochers and Bohm, 1993) to assess biosystematic relationships and determine trends of biochemical evolution in the genus. Electrophoretic work followed to establish evolutionary relationships among closely related taxa within the genus (Crawford *et al.*, 1985; Crawford and Ornduff, 1989) as well as within the highly variable *L. californica* complex (Desrochers and Bohm, 1993). More recently, comprehensive phylogenetic studies (Chan *et al.*, 2001, 2002; Desrochers and Dodge, in press) have contributed to our understanding of patterns of divergence in *Lasthenia*. The study by Chan *et al.* (2001) agrees with observations made earlier (Ornduff, 1966, 1976) that divergence patterns in the genus conform to expectations of catastrophic selection and saltational diversification (Lewis, 1962).

Existing knowledge, along with the current findings on the *L. californica* complex, suggests that *Lasthenia* can serve as a model genus for studies in evolutionary ecology, specifically, in understanding the role of edaphic factors in differentiation. Species of *Lasthenia* have successfully colonized diverse habitats within California, including those that exclude the vast majority of species (Ornduff, 1966, 1993; Kingsbury *et al.*, 1976; Vasey, 1985; Rajakaruna and Bohm, 1999; Noe and Zedler, 2000; Parsons and Whelchel, 2000). Further, in some sections (for example, sect. *Hologymne* and sect. *Ornduffia*) all species occupy the same edaphic habitat yet generally do not grow intermixed (Ornduff, 1966), while in others (eg., sect. *Ptilomeris*), species occupy contrasting edaphic habitats (Vasey, 1985; Crawford *et al.*, 1985). Thus it

is likely that edaphic factors have played an important role in the diversification of the genus. In this final chapter, I summarize my findings on *L. californica,* suggest possible avenues for future research, and discuss several other taxa in the genus with emphasis on the possible role of edaphic factors in their diversification.

The detailed ecological survey of *L. californica sensu* Ornduff (Rajakaruna, 1998; Rajakaruna and Bohm, 1999) documented that the edaphic races occur in distinct sets of habitats that can be classified on the basis of ionic stresses and water availability. Race A plants predominate in habitats subject to ionic stress. Although the soils in these environments are ionically harsh, the percent clay content is generally high, increasing the water holding capacity of the soil. Plants are often restricted to moist or even saturated soils in such environments. In contrast, race C populations are found in ionically "benign" inland environments. The soils are often sandy, rocky, and shallow, drying out early in the growing season. The conditions at Jasper Ridge, where the races occur in parapatry, mirror the trends seen across the range of the species, with the two races occupying distinct microhabitats: Race A occupying the wet, yet ionically harsh soils at the bottom of the ridge, while race C occupies the fast-drying, yet ionically less stressful upper reaches.

The studies described in this thesis clearly show that the two races are physiologically differentiated to deal with their distinct edaphic habitats. Race A is better adapted to deal with ionic stresses, specifically with Na^+ and Mg^{2+}, that characterize their edaphic habitat. Results from the hydroponic studies (Chapter Three) confirm that the racial differences in ion accumulation, first observed under field conditions (Rajakaruna,

1998; Rajakaruna and Bohm, 1999), are not exclusively the outcome of soil ionic composition, since they are sustained even under identical growth conditions in the laboratory. This strongly suggests a genetic basis for the observed patterns of ion accumulation. Measures of germination, survivorship, and root length also showed that race A plants from Jasper Ridge are better adapted to grow in Na^+ - and Mg^{2+} -rich habitats. The tolerance was not based upon ionic exclusion, because root absorption and shoot accumulation of these ions by race A plants were substantially higher than by race C plants, suggesting internal mechanisms of tolerance. The significant Genotype x Treatment interactions observed in all three measures of tolerance further confirm that the two races are genetically differentiated in their tolerance responses to these ions.

The study reported in Chapter Four clearly suggests that race C plants are better adapted to drought, a feature that characterizes their edaphic environment. Race C is better able to avoid as well as tolerate drought. The faster growth to reproductive maturity observed under the low-water treatment in the study is consistent with the observations made at Jasper Ridge. Race C appears to be avoiding extreme water stress by completing its life cycle faster, using a strategy often referred to as phenological escape. The experiment also revealed that race C allocates relatively more biomass to reproduction under water stress, which is again consistent with a strategy employed by drought-tolerant herbaceous species. The significant Genotype x Treatment interaction observed for the number of flower heads, the measure of reproductive fitness, clearly indicates that the races are genetically differentiated in their tolerance response to water stress.

The physiological studies I have conducted are based on environmental variables that appear to be associated with differences in racial distribution (Rajakaruna, 1998; Rajakaruna and Bohm, 1999). It is now clear that the races are physiologically differentiated and that they appear to achieve higher fitness under those environmental conditions that best match their natural environment. Thus it is reasonable to hypothesize that the unique distribution pattern of the edaphic races may have been achieved through differential adaptation (Wade and Kalisz, 1990; Sultan, 2001).

Since the studies on ionic (Chapter Three) and water (Chapter Four) stress were conducted separately, it is unclear how the findings relate specifically to conditions experienced in the field. It appears that in these edaphic races, physiological stress results from an interaction of water availability and chemical features of the soils. An important aspect of eco-physiological studies that is currently under design is the ability of the races to withstand low osmotic potentials (i.e., to test the combined effects of water and ionic stress). A large-scale experiment using natural soils will soon be conducted to clarify further the relationship between ionic strength, water availability and fitness within environments and further explore factors that affect the distribution of these races at Jasper Ridge and across the species' range. The *L. californica* complex provides an ideal system with which to conduct detailed analysis of selection gradients (Wade and Kalisz, 1990; Sultan, 1995; Dudley, 1996a, b; Pigliucci and Schlichting, 1996) to infer the role of natural selection in achieving fitness differences under field conditions.

The study reported in Chapter Five suggests that the edaphically differentiated races are reproductively isolated. Most intriguing, the Mantel Test suggests that there is a strong relationship between ecological distance and reproductive isolation. Clearly more

data are needed to confirm this almost significant (P=0.077) relationship; however, the trend observed lends another line of support to the hypothesis of adaptive differentiation. Another fascinating observation requires further study. The races are strongly isolated at Jasper Ridge, suggesting the possibility of reinforcement of reproductive isolation in the parapatric location. However, it is possible that ecological selection may also have contributed to isolation, as revealed by flowering time differences in response to water stress (Chapter Four). Thus, reproductive isolation may have been enhanced as a bi-product of ecological selection. More studies are clearly needed to clarify the roles of ecological selection versus reinforcement on patterns of reproductive isolation seen within the complex.

An understanding of the relationship between traits for adaptation and reproductive isolation is critical to further the hypothesis of edaphic differentiation in the *L. californica* complex. Studies suggest that reproductive isolation can be achieved as a bi-product of a physiological adaptation to unusual soil conditions (Chapter One). The work of Macnair and colleagues (Macnair and Gardner, 1998) is the only available literature that suggests that reproductive isolation can be achieved as a bi-product of a physiological adaptation to unusual soil conditions. They have shown that the linkage block associated with copper tolerance in an ecotype of *Mimulus guttatus* also produces hybrid inviability; however, it is unclear whether inviability is achieved via pleiotropy or hitchhiking. My research on *L. californica* clearly identifies several traits that are directly related to measures of fitness (Chapter Three and Four) as well as traits that are responsible for reproductive isolation between the races (Chapter Five). Whether relationships, such as those observed for *M. guttatus*, exist between edaphic tolerance

and observed reproductive isolation in *L. californica sensu* Ornduff is worthy of investigation.

An intriguing pattern emerging from the current research is that the edaphic races have evolved in parallel in two closely related species. Such patterns of recurrent origins of plant taxa (intraspecific and specific) is becoming ever more apparent in the literature (reviewed in Levin, 2001), suggesting that genetic similarity of progenitor populations combined with parallel selective pressures can lead to multiple independent origins of taxa. Thus, studies of parallel evolution can be powerful for demonstrating the role of ecological selection in speciation (Schluter, 2001). Schluter and Nagel (1995) and Levin (2001) suggest four criteria to be satisfied to demonstrate parallel evolution of taxa. First, separate populations that occur in similar environments must be phylogenetically distinct. Second, the shared characteristics must be the products of natural selection. Third, separate descendent populations that are found in similar environments must be reproductively isolated from the ancestral populations. Finally, the separate descendent populations must not be reproductively isolated from one another. All these criteria have rarely been satisfied in current cases of parallel evolution (Schluter and Nagel, 1995) and Levin (2001) indicates that there are almost no unassailable examples of parallel origins in the plant literature. It is clear from my studies that the edaphic races in the complex appear to satisfy the criteria important to suggest parallel evolution. Although, the work is far from complete, the *Lasthenia* system can provide a model for studies in multifaceted parallelisms (Levin, 2001) in traits responsible for adaptation (i.e., salt tolerance, flavonoid biosynthesis, drought tolerance) and reproductive isolation and serve as an unparalleled model for the study of parallel evolution in plants.

The majority of *Lasthenia* species (14 of 18) are tolerant of unusual edaphic conditions. Although the *L. californica* complex represents the edaphically most-diverse group of taxa in the genus, several other species show fascinating examples of ecological radiations that are also worthy of discussion.

Lasthenia minor (DC.) Ornduff occurs in a variety of habitats, including alkali flats, coastal bluffs, sand dunes, pond margins, and disturbed sites while *L. maritima* (A. Gray) M. Vasey is restricted almost exclusively to islands and offshore rocks harboring seabird nesting and roosting sites (Ornduff, 1965, 1966; Vasey, 1985). The soils on these sites are high in nitrogen, low in pH, and highly disturbed resulting from the activities of the birds as well as due to constant wind and salt spray (Vasey, 1985). The physiological basis for guano tolerance in *L. maritima* is not known. Limited work suggests that *L. maritima* accumulates high concentrations of nitrates in its foliage (Ornduff, 1965) and Vasey (1985) suggests that high nitrate content in the cells may have allowed plants to develop tolerance to these osmotically-challenging sites. Preliminary studies by Vasey (1985) also show that *L. minor* is not tolerant of guano-modified soils. All lines of evidence to date suggest that the self-compatible *L. maritima* is a recent descendent from the self-incompatible *L. minor* (Ornduff, 1966; Crawford *et al.*, 1985; Chan *et al.*, 2001). Crawford *et al.* (1985) suggest that speciation probably involved a switch to self-compatibility, development of autogamy, and subsequent divergence driven by edaphic factors. Given that variation for tolerance is first required to colonize the extreme guano habitats, it is likely that self-compatibility arose post-colonization. An examination of parapatric populations of the two species has failed to reveal any indication of natural interspecific hybridization (Vasey, 1985). It is possible that strong ecological selection is

responsible for limiting introgression. Whether traits responsible for reproductive isolation (i.e., self-compatibility, hybrid inviability or reduced hybrid fitness) arose as a bi-product of an adaptation to guano or is directly linked to a gene/s conferring adaptation is not known.

Lasthenia chrysantha, L.glabrata, and *L. ferrisiae* (sect. *Hologymne*) occupy saline habitats and form vigorous, moderate to highly fertile artificial hybrids (Ornduff, 1966). An interesting observation made by Ornduff (1966) is that these three closely related species, with edaphic requirements that appear to be identical, are never sympatric. A pure population of one of these species may exist as near as a few meters to a pure population of another. Ornduff (1966) attributes this pattern to occupation of a site on a "first come, first served" basis, yet admits that a detailed ecological study probably would reveal factors that are different among such sites. It is likely that the three taxa differ in their tolerance regime to edaphic features associated with salinity.

Lasthenia fremontii, L. conjugens, and *L. burkei* (sect. *Ornduffia*) are freely inter-crossable (Ornduff, 1966, 1969). Although all three species colonize vernal pools, their distributional pattern within a pool seems to be related to water level, soil moisture, and salinity (Ornduff, 1966). Ornduff (1966) claimed a similar situation to that described for the three halophytes in sect. *Hologymne*, where the species are rarely sympatric although they may occupy sites only a few meters apart. Again, specific edaphic tolerances may exist in each of these vernal pool taxa, and the sites they are restricted to may, in fact, show micro-scale differences in edaphic features. Only a close examination of these sites, such as my detailed study of the serpentine outcrop at Jasper Ridge (Rajakaruna, 1998; Rajakaruna and Bohm, 1999), will reveal patterns of soil heterogeneity in these

apparently uniform edaphic habitats. In a study conducted in an artificial vernal pool created at Berkeley, Ornduff (1966) demonstrated that *L. conjugens* was always restricted to the soil immediately above and below the water level while *L. fremontii* occurred from water level to the upper limit of soil moisture (Ornduff, 1966). *Lasthenia conjugens* appeared to be the more water-dependent of the two species and may be occupying deeper vernal pools that dry out later in the growing season. Though physiological differences obviously exist among the three species, the range of tolerance to salinity and osmotic effects is unknown. Characterization of the ecological amplitude of these species and their artificial hybrids may shed light on importance of edaphic features in the diversification of this group.

From the detailed studies of the *L. californica* complex, as well as from observations reported above for several other species, it is reasonable to suggest that edaphic features have played an important role in the divergence within this relatively small genus. Even within an apparently uniform edaphic habitat, such as a salt flat or vernal pool, the species are rarely sympatric, suggesting that micro-scale differences in edaphic features are likely responsible for their distribution. Although species discussed above are obviously adapted to deal with different specific ions (e.g., heavy metals, magnesium under serpentine, sodium and magnesium under saline, nitrate under guano), an important factor that is common to all these edaphic habitats is the low osmotic potential of the soil solution. From the information currently available for *Lasthenia*, it appears that ion accumulation and sequestration is a common strategy used to counter this stress. However, there are inter- and intra-specific differences in mechanisms of

tolerance to osmotic stress and these differences may have set the stage for the fascinating ecological divergence seen in the genus.

Many *Lasthenia* species occur in geologically rather recently available land, since a large proportion of their present range was covered by sea water during Miocene and Pliocene times (Howard, 1951; Axelrod, 1956). The inland sea retreated at the close of the Pliocene, although saltwater lakes of varying extent existed in the Central Valley during Pleistocene time (Flint, 1947). Some of the lakes and marshes persisted into the 19th century (Mason, 1957) and it is reasonable to speculate that the ancestor of *Lasthenia* was tolerant of salinity (specific ion effects) and osmotic effects. Salinity and osmotic tolerance may in fact represent ancestral traits that have been retained in the vast majority of species while it has been lost in others. Alternately, tolerance to osmotic and salinity effects may have independently evolved in the various species in response to their radiation into unique habitats such as vernal pools, alkali flats, serpentine outcrops, and guano deposits. A closer look at recent phylogenies for Asteraceae (Baldwin and Wessa, 2000; Baldwin *et al.*, 2002) lends more support to the first hypothesis. Many of the genera found in the sub-tribe *Baeriinae* Benth. & Hook., of which *Lasthenia* is a member, have taxa that are drought-, salt- and serpentine-tolerant. *Amblyopappus pusillus* Hook. & Arn., a monotypic genus, is the closest relative of *Lasthenia* and is restricted to salt-rich habitats such as coastal bluffs, dunes, and beaches (Hickman, 1993). The other close relatives are *Monolopia* DC., *Baeropsis* J. Howell., and *Eriophyllum* Lagasca., three genera having several species tolerant of drought, coastal, and serpentine conditions (Kruckeberg, 1984; Hickman, 1993). An examination of the consensus tree for *Lasthenia* (Chan *et al.*, 2001), however, does not conclusively support

the notion that tolerance to edaphic extremes is ancestral since the loss as well as the gain of tolerance within the genus can be explained by an equal number of steps. However, given that the closest relative to *Lasthenia* is restricted to salt-rich habitats and that the majority of species within the genus occupy edaphically harsh environments, it seems more likely that salt-tolerance is an ancestral condition for the genus. In this regard, it is interesting to note that the population genetic study for the *L. californica* complex (Chapter Six) suggests that the salt-tolerant race A is ancestral to the salt-intolerant race C, with several lines of evidence (e.g., trends in flavonoid evolution, edaphic specialization) supporting the notion that race C evolved from a race A ancestor. Testing of the hypothesis of an adaptive role for sulfated flavonoids (Chapter Two) will add further credence to the trends speculated for the evolution of edaphic races (Chapter Six).

Edaphically restricted species provide fascinating examples for the study of plant speciation. The study of the processes leading to the evolution of such species can shed light on the relationship between adaptation and speciation. The genus *Lasthenia* provides numerous opportunities to examine the link between adaptation to substrate and speciation. Many closely related species in *Lasthenia* generally avoid sympatry and appear to be wholly distinct in areas of contact, suggesting that they are ecologically divergent and reproductively isolated. In *Lasthenia*, the extent of isolation as well as the extent of ecological differentiation among closely related species has not been well documented except in the case of the *L. californica* complex (Chapter Five). Traits that are associated with ecological specialization in closely related species can be identified and their adaptive significance can be demonstrated. If the ecologically divergent species

are reproductively isolated, it will be possible to examine any links between adaptation to substrate and reproductive isolation and, thus, identify potential "speciation genes."

The available phylogenetic information (Chan *et al.*, 2001, 2002, Desrochers and Bohm, 1995, Desrochers and Dodge, in press), along with the relatively small size of the genus, small base chromosome number (n=8), generally annual habit and obligately outcrossing nature, and the ease with which the species can be grown for experimental studies make *Lasthenia* an ideal model for studies in evolutionary ecology.

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