Bohm & Rajakaruna. 2006. Published in *Natural Product Communications*. 11:1013-1022.

The *Lasthenia californica* Story: It Started with Flavonoids

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Dedicated to Professor E. Wollenweber on the occasion of his 65th birthday.

Our laboratory's study of *Lasthenia* (Asteraceae) began with an examination of flavonoid profiles of all species of the genus. The finding of two distinct flavonoid races within the *L. californica* complex led us to investigate environmental factors that might have been responsible for selection of these particular forms. Data were gathered on soil chemistry, allozyme variation, breeding biology, the effect of water stress on plant growth, and ion uptake physiology. In conjunction with workers at other institutions, DNA studies were undertaken to determine evolutionary relationships between the two races. These studies led to the recognition that racial differences had arisen in parallel in two phylogenetically independent lineages. These results are discussed in terms of the evolution of edaphic (soil type) races, and the possible usefulness of the system as a model for future studies of parallel speciation in plants.

Keywords: *Lasthenia*, edaphic races, serpentine soils, speciation, speciation model.

Lasthenia is a small genus in Asteraceae that consists of as many as 21 species and subspecies, depending on taxonomic opinion, with a mainly western North American distribution but with a single species in Chile. *L. californica*, the main topic of this review, is the most widespread of the North American species with populations in southern Oregon, throughout California, in central Arizona, and northern Baja California (Mexico). Work on members of the genus began in the senior author's laboratory because of a combined interest in anthochlor chemistry—several species of *Lasthenia* make aurones and chalcones—and comparative phytochemical studies in the California flora in general.

Lasthenia offers a wide variety of interesting and challenging problems for the comparative phytochemist and evolutionary biologist that would require the special talents of both. Species within the group occupy sites that vary from comparatively benign prairie grasslands to seabird islands characterized by highly disturbed and acidic substrates. Several species grow in and around saline pools in the Central Valley of California, while others occur on serpentine-derived soils. Edaphic specialization seems to be one of the major features of the genus and the Chilean species represent one of several California-Chile disjunct species pairs suggesting long distance dispersal by birds. As the reader will see, the genus had other surprises awaiting us.

According to Robert Ornduff [1], the most recent monographer of the genus, *Lasthenia* consists of 16 species (20 taxa counting subspecies) assorted into six sections based upon morphological and cytological characteristics. A subsequent study of *L. minor* subsp. *minor* and subsp. *maritima* supported elevation of the latter to species rank, i.e. *L. maritima* [2, 3]. Other taxonomic changes resulting from recent DNA-based phylogenetic analyses will be commented on below. Until that point, Ornduff's treatment of the genus will be used.

The first mention of flavonoids in the genus (as *Baeria*) came from the work of Gertz [4] who applied

a simple color test for anthochlors to dried material of several species. The positive tests (anthochlors turn from pale yellow to dark orange or purple with alkali) observed were substantiated by a detailed examination of *L. chrysostoma* (now recognized as *L. californica*) by Shimokoriyama and Geissman [5], who reported the chalcones okanin-4'-*O*-glucoside and butein-4'-*O*-glucoside and the aurones maritimetin-6-*O*-glucoside and sulfuretin-6-*O*-glucoside. Other compounds identified were caffeic acid, chlorogenic acid, quercetin, and a quercetin glycoside. Preliminary studies from our laboratory revealed the presence of a mixture of quercetin and patuletin (6-methoxyquercetin) glycosides in *L. conjugens* and *L. fremontii* [6]. An investigation of the flavonoids of artificial interspecific hybrids in *Lasthenia* revealed that patterns of anthochlors, kaempferol glycosides, and some patuletin glycosides were additive, whereas inheritance of luteolin and quercetin glycosides was not always so [7]. In some progenies, quercetin glycosides were produced that were not observed in the parents, which may have simply been a concentration effect.

An analysis of flavonoids for the entire genus [8] revealed a moderately complex array of compounds based on aurones, chalcones, the flavonols kaempferol, quercetin, and patuletin, and a single flavone, luteolin. The pattern of occurrence of this array of compounds paralleled the sectional taxonomy of the genus as defined by Ornduff [1]. Thus, aurone and chalcone derivatives were identified from both species of section *Baeria* (*L. californica* and all three subspecies of *L. macrantha*), all three species of section *Burrielia* (*L. debilis*, *L. leptalea*, and *L. microglossa*), and in two members of section *Hologymne* (*L. chrysantha* and *L. ferrisiae*, but not in either subspecies of *L. glabrata*). Sections *Baeria* and *Burrielia* were distinguished from the rest of the members of the genus by the presence of luteolin glycosides (glucosides and glucuronides). Differences were seen within section *Burrielia*, however, with only *L. leptalea* exhibiting luteolin-7-*O*-glucoside. A rich assortment of quercetin mono- and diglycosides was observed with a good deal of variation in the occurrence of individual compounds; no pattern was evident however. A kaempferol diglycoside was observed in a few individuals of *L. macrantha*. Patuletin glycosides and related derivatives were observed in the only member of section *Platycarpha* (*L. platycarpha*), and in all three members of section *Ptilomeris* (*L. burkei*, *L. fremontii*, and *L. conjugens*). The related compounds were identified as patuletin -7-potassium bisulfate and patuletin-3-*O*-glucoside-7-potassium bisulfate. The distribution of flavonoids within the genus appears to be associated with the loss of the capacity to accumulate aurones and chalcones in more advanced members. Ornduff [1] concluded that members of sections *Baeria* and *Burrielia* are the most primitive members of the genus based on morphological and cytological data. It is interesting to note, as well, that patuletin derivatives were not observed in the two putatively primitive genera, but were present in some members of section *Ptilomeris*, thought by Ornduff [1] to consist of more advanced species. Patuletin can be thought of as representing an advanced (or derived; apomorphic in cladistic terminology) flavonoid character in that it is biosynthetically more complex than quercetin. It must be borne in mind in considering statements of this sort, however, that the flavonoid feature does not stand alone, but only takes on any meaning when considered in combination with other characters, morphological features and chromosome number in this case.

One other element from this study deserves mention. The only non-North American species of *Lasthenia*, *L. kunthii*, is a Chilean endemic that shares a suite of morphological and cytological features with *L. glaberrima*; together these two taxa constitute section *Lasthenia*. This species pair represents a fairly common phenomenon involving closely related species native to California and Chile, thought to have been brought about by long distance transport by migrating birds. The members of these pairs occupy very similar habitats in their respective countries. For example, *L. kunthii* occupies sites roughly in the range 33°- 38° S, well within the corresponding range of its North American cousin's (sibling's?) range of 36°- 45°30' N latitude. Although *Lasthenia* is referred to as an essentially Californian genus with an outlier in Chile, it might be more appropriate to identify it as a Chilean genus with several outliers in North America. This reversal of tradition would reflect the fact that the type species for *Lasthenia* is, in fact, the Chilean taxon, which was named after Karl Sigismund Kunth (1788-1850). Incidentally, the type specimen for the species was destroyed during the bombing of the great herbarium in Berlin during the latter stages of World War II.

During the course of most of the surveys of *Lasthenia* species summarized above, we observed differences in pigment profiles within several of them suggesting

that within-population as well as between-population variation might be factors that should be examined in a more systematic fashion [9]. For the initial study we chose to examine pigment patterns in the northern Californian taxon *L. burkei* [10]. There were four reasons for selecting this species: (1) it existed in comparatively small, geographically discrete populations; (2) it has been postulated as possibly of hybrid origin between. *L conjugens* and *L. fremontii*; (3) since it is/was the rarest species in the genus, it was/is at the greatest risk of extinction, particularly because of significant commercial development in its small natural range; and (4) the earlier studies had revealed the existence of two different flavonoid profiles suggesting the existence of pigment races. Since only a small number of dried specimens had been examined in the earlier study, we felt it necessary to expand the study using material freshly collected from the field. One hundred and eight individuals were collected from four populations, two in Sonoma County and two in adjacent Lake County in northern California (populations in Mendocino County could not be relocated, presumably owing to construction activities). One of the populations was bisected by California Route 29. The greatest distance separating any two populations was ca. 40 km. The flavonoid profile of the species was based upon quercetin and patuletin glucosides, glucuronides, xylosides, diglucosides, and glucosyl-xylosides. A trace of a patuletin triglycoside was also observed, but was not further studied. Confirming the earlier findings, two races were observed based on the occurrence of xylose-based glycosides, quercetin and patuletin diglycosides, and the distribution of glucuronides of both flavonols. Distinctions between races were not absolutely likely owing to quantitative variation, but the overall differences in the various types of derivatives was marked. Relationships between populations and pigment patterns were not evident. Cause and effect relationships of such differences are extremely difficult to establish without extensive experimental studies. Such work will be addressed in detail below in our study of *L. californica*.

In 1978 the late Professor John Thomas, of Stanford University, invited the senior author to visit the extensive population of *L. californica* growing on serpentine-derived substrate in the Jasper Ridge Biological Preserve of Stanford University. Analysis of approximately 100 individuals, collected at intervals along a kilometer long transect paralleling the long dimension of the ridge revealed the existence of three different pigment patterns. The composition of the three patterns was subsequently determined. In line with earlier work on this species, all plants exhibited a suite of aurone and chalcone glycosides, as well as a set of highly polar flavonol glucuronides. The three patterns were distinguished from one another on the basis of compounds present, in addition to the base array. Thus, type **C** consisted solely of the base array; type **B** had luteolin-7-*O*-glucoside in addition to the base array; type **A** was characterized by the base array plus the flavanone eriodictyol-7-*O*-glucoside and flavonol-3,7-diglycoside bisulfates. Subsequent analyses revealed that recognition of type **B** was unnecessary owing to its very infrequent occurrence; the few **B**-type plants seen were thereafter included in type **C** tallies. In 1982 we initiated a program of collection along the original transect and expanded collections at several shorter transects established at right angles to the original. Subsequently, collection along the ridge line was discontinued in order to avoid interfering with other researchers' plots in the area. Some details on the research area are relevant.

Jasper Ridge Biological Preserve of Stanford University is located west of Palo Alto, California, San Mateo County, in the Santa Cruz Mountains. Approximate coordinates of the Preserve are 37°25' N, 122°2.5' W; the collection area lies at ca. 180 m elevation. One of the most prominent features of the research preserve is an extensive serpentine outcrop that runs in a west-northwesterly to east-southeasterly direction. The serpentine outcrop forms the spine of the Preserve and is the feature from which the name arises. [Jasper is a silicate mineral, described as a granular, cryptocrystalline quartz, often colored by hematite. It is a semiprecious stone from which decorative items including jewelry is made]. The serpentine outcrop is characterized by a flora consisting of species that are endemic to, or can grow facultatively on ultramafic soils. One of the prominent species found on Jasper Ridge is *L. californica*, which often blankets the entire outcrop in a mantle of golden-yellow. At the boundary of the serpentine ridge, where the mineral rich substrate slips beneath the sandstone-based substrate, the vegetation abruptly changes to that of typical Californian oak-grassland. The research area encompassing the several shorter transects runs from the Preserve's Fire Road down-slope to the boundary with the grassland; a 7% drop exists over a distance of approximately 65 m from the Fire Road to the grassland boundary.

In 1982 sampling along the shorter transects was done by selecting individual plants at one meter intervals from the Fire Road to the grassland junction. Chromatographic analysis revealed the presence of two flavonoid types, type **A** and type **C** (a single type **B** plant was included in the **C** tally). The noteworthy observation was the abrupt change from one pigment type to the other. From meter 0 at the Fire Road to meter 44-45 plants exhibited type **C** flavonoid pattern with an occasional type **B**; from that point to the oak-grassland boundary plants having type **A** profiles were the predominant type observed. Sampling was continued for the next several seasons (1983-1987 inclusive) in order to determine whether this sharp geographical separation between the two pigment types was a chance observation or whether it represented the result of some intrinsic factor in the plant's environment. With only slight deviation, the pattern of type **C** plants above the "transition point" and type **A** plants below was observed in each of the subsequent seasons. The deviations involved the occasional type **A** plant in the upper portion of the transect and the occasional type **C** plant in the lower portion. One of the strategies followed in the 1985 season involved collecting one plant every 10 cm from the 40- to the 50 meter mark along the transect in order to see how abruptly the pigment pattern changes. The results are shown in the following sequence of plant pigment types starting at the 44 meter mark: …C[44]CCCCC<u>CCACB</u>[45]<u>AAAAA</u>AAAAA[46]AAAAC AAAAC[47]ACAAAAAAAA[48]AAA…The transitional zone is underlined. Two additional type **C** plants were observed beyond meter 48; the remainder was type **A** [11].

With the establishment of a distinct pattern in the distribution of flavonoids in the Jasper Ridge population, it was of interest to determine if other populations of this species exhibited similar patterns. To this end, material of *L. californica* was collected from three other populations that grew on or near serpentine, two north of the Jasper Ridge site, and one to the south. The population collected from a serpentine outcrop near Kirk Creek in southern Monterey County exhibited only type **C** plants. A population growing on non-serpentine soil at Rock Creek in Mount Tamalpais Park (Marin County) exhibited all three flavonoid profiles (types **A**, **B**, and **C**), while a population in the park growing on a serpentine outcrop exhibited only type **A** plants. Other populations will be encountered below. The possibility occurred to us early in this research that the presence of sulfated flavonoid glycosides, i.e., type **A**, was in some way a reaction to an extreme soil type, an idea discussed by J. B. Harborne [12]. There appears to be no direct relationship between soil type and flavonoid profile type, at least as judged from analysis of field-collected specimens. This is in agreement with earlier studies in which several *Lasthenia* species, including *L. californica*, were grown under controlled conditions in a glasshouse using standard potting soil. Regardless of the origin, e.g., saline pools, grassland, serpentine, coastal bluffs, flavonoid chemistry of the plants grown from seed under common garden conditions was identical to profiles observed in wild-collected plants.

With the apparent elimination of soil type as the primary driving force in explaining differences in flavonoid chemistry (but more on this below), the study was expanded to include other possible factors. In addition to being the most widely distributed species in the genus, with populations in southern Oregon, throughout California, extreme northern Baja California, and central Arizona, *L. californica* is also highly variable morphologically. It exhibits a high degree of ecological tolerance with populations known from oak-grassland, coastal bluffs, serpentine-derived soils, and sand. The species is also chromosomally diverse with diploid (n = 8), tetraploid (n = 16), and possibly higher numbered populations (there has also been a report of a population with n = 24). Several questions were asked: (1) Are there populations other than Jasper Ridge that exhibit mixed flavonoid types? (2) Is chromosome number a factor? (3) Are there any other features by which the two flavonoid types can be distinguished, either morphological or biochemical? In order to address these issues, *L. californica* was recollected from populations representing its entire range.

The existence of flavonoid, or other secondary metabolite, races in Nature is a well-known phenomenon, much of the literature of which has been reviewed by the senior author [13, 14]. The persistence of geographic patterns of the sort observed in the studies of *L. californica* represented, at least at the time of its first reporting, a unique observation. The only boundaries as abrupt as this of which we were aware came from the mine-tailings studies of heavy metal tolerance in plants, which have been reviewed by Antonovics and coworkers [15], and Jain and Bradshaw [16]. These represent clear-cut examples of selection in the presence of

potentially toxic heavy metals. Relationships between flavonoid patterns in *Lasthenia* and soil chemistry, should they exist, remained cryptic.

With the establishment of time-stable local geographic patterning in the population on Jasper Ridge, it was of interest to see if similar patterns of flavonoid variation existed in other populations of *L. californica*. Thirty-three populations—two from Oregon, two from northern Baja California, three from Arizona, and the rest from California—were sampled for flavonoids [17]. The two Oregon populations, the two northernmost Californian populations, and one population from the coast north of Santa Barbara were characterized by type-**C** flavonoid patterns. Two populations north of the San Francisco Bay area exhibited highly unusual pigment patterns and were not further examined. The remaining populations consisted either exclusively or primarily of plants with type-**A** flavonoid profiles. An examination of allelic variation within and among populations from the entire range of the species revealed frequency differences in two systems that also varied with geographic origin of the populations [18, 19]. Thus, the *b* allele of *Nadhdh* (nicotinamide adenine dinucleotide dehydrogenase) is the major or sole allele in populations from Oregon and in the northern half of California roughly as far south as Monterey County, with the *a* allele present as either the predominant or only allele in populations from the southern half of California, Arizona, and Baja California. A very similar distribution of alleles of *6Pgd-1* (6-phosphogluconate dehydrogenase) was observed with a strong north-south differentiation: alleles *a*, *b*, and *c* were the primary alleles in northern populations, while alleles *d*, *e*, and *f* occurred in southern populations.

Local differences in allele distribution of these two enzymes were observed at the population level as well. Specimens taken at regular intervals along the main transect at Jasper Ridge were analyzed for electrophoretic variation employing the same enzymes as were used in the range-wide study. The two systems that showed differentiation in the range-wide study were also distributed along the transect in strong concordance with the array of flavonoid types. Thus, flavonoid type-**A** plants were characterized by *Nadhdh-b* and the faster-moving allozymes coded for by *6Pgd-1*. Plants with flavonoid type-**C** (**B** plus **C** in that paper) were characterized by *Nadhdh-a* and the slower-moving allozymes of *6Pgd-1*. The genetic identity among populations representing the two flavonoid clusters was 0.72, implying reduced gene flow between northern and southern populations. Genetic parameters indicating differentiation within and among populations also suggested a reduction in gene flow among populations of the complex (G_{ST} = 0.36; Nm = 0.439). Reduced gene flow was also observed between the two races of plants at Jasper 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 species [20]. Further, the calculated value for Nm (0.35) was less than unity, 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 [21].

Among the several morphological features that exhibit variation in this species is the absence, or when present, the shape of the pappus [1, 18]. Four character states for pappus were coded in the morphometric study: linear, subulate, lanceolate, or pappus absent. In the range-wide study, plants with primarily linear pappus were observed in populations from Oregon and northern California; none with this feature were seen in populations south of the San Francisco Bay area. Distribution of the other two pappus types varied to some degree throughout the range, but populations characterized by subulate pappus occurred primarily south of the San Francisco Bay area. Similarly, plants lacking a pappus were observed mostly in southern populations. A strong correlation emerged when pappus structure was examined for plants along the Jasper Ridge transects. Thus, plants with type-**A** flavonoids, the *Nadhdh-b* allele and the faster-moving allozymes coded for by *6Pgd-1*, consistently exhibited a linear pappus; while plants with type-**C** flavonoids were characterized by *Nadhdh-a* and the slower-moving allozymes of *6Pgd-1*.

In spite of consistent differences in flavonoid chemistry over time, variation in pappus structure, electrophoretic differences, different flowering times, and reduced capacity to cross [1, 18], there is no clear-cut justification for recognizing different taxonomic entities, a conclusion reached by Ornduff [1]. The existence of distinct races of *L. californica*, however, cannot be disputed. It is obvious that different populations have responded to different environmental influences in a variety of ways. The more or less clear-cut differentiation of the population at Jasper Ridge could be explained by colonization of the site by propagules from different

races, each characterized by different suits of flavonoid-pappus-allozyme characteristics. Without further study of specific environmental features, however, little more could be said. Additional studies were undertaken to address these issues.

Despite the range of habitats occupied by *L. californica*, a detailed ecological survey of 23 populations [22], including Jasper Ridge, suggested that the two flavonoid races previously described correspond to edaphic races. Race **A** plants are often found to occur on coastal bluffs, serpentine outcrops, and salt and alkaline flats, all habitats that are subject to ionic stress. The soils of these habitats are generally ionically extreme with high concentrations of sodium and magnesium ions. As well, the percent of clay in these soils is generally high, which increases their capacity to retain water. Plants are often localized in moist to saturated soils in such environments. In contrast, race **C** plants are found in more ionically benign inland environments such as oak woodlands and pastures where the soils are often sandy, rocky, and shallow, drying out fairly early in the growing season. Ionic strengths of soils in these two types of environment are often quite different, 2.23-111.7 mM for race **A** soils and 1.4-26.8 mM for race **C** soils. Electrical conductivities also differ: race **A** plants can occur in soils with values reaching 7.49 $mScm^{-1}$, while race **C** plants generally occur in soils with conductivities less than 1.79 $mScm^{-1}$. Electrical conductivity above about 4.0 $mScm^{-1}$ is considered toxic to most plants [23]. Differences in sodium and magnesium ion concentrations are the most likely contributors to differences in ionic strength, and hence conductivity. Thus, race **A** plants occur in soils averaging 60.8 ppm sodium and 1147 ppm magnesium, whereas race **C** plants occur in soils that average 19.9 ppm and 280.6 ppm of these elements, respectively. Soil conditions at Jasper Ridge, where the two races occur in parapatry, mirror the trends seen across the range of the species, with the two races occupying distinct microhabitats characterized by specific edaphic conditions: race **A** occupies the moist, yet ionically-rich, soils at the bottom of the serpentine outcrop, while race **C** occupies the fast-drying, ionically moderate upper parts of the outcrop.

Analyses of plant tissue concentrations of various elements in the two races suggest a number of differences in the patterns of ion accumulation between the two races [22]. A key difference lies in the capacity to deal with sodium. Race **A** plants are capable of accumulating sodium to concentrations three to four-fold greater than race **C** plants. At some extreme sites, sodium accounts for 5.2% of dry weight of race **A** plants, a value that approaches levels found in halophytic plants [24]. Given that race **A** plants often occur in ionically harsh environments, it seems reasonable to speculate that accumulation of high levels of sodium ions serves to maintain a favorable water potential gradient between plant and soil. Race **A** plants also show strong and significant correlations between soil and tissue sodium ($r = 0.87$; $P<0.001$), magnesium ($r = 0.81$; $P<0.05$), and calcium ($r = 0.78$; $P<0.05$) ion concentrations. The correlations for race **C** plants for these three ions are not significant ($P>0.05$). These observations clearly indicate that the two races are physiologically distinct, at least with regard to uptake of and tolerance to these normally abundant cations.

A greenhouse experiment, using field-collected soils, revealed that the two races from Jasper Ridge respond differently with regard to germination and growth responses to the different soils [22]. Race **A** cypselae germinate, grow to maturity, flower, and set seed similarly in the two soil types. Although race **C** cypselae germinated in both soil types, growth was significantly poorer and the plants never attained reproductive maturity in soils from the lower part of the outcrop. Combination of these observations and the differential accumulation of and tolerance to various cations suggests that these are key factors in determining the occurrence of the two races throughout the range of *L. californica*.

A study of the response of the two races to water stress revealed differences in life history strategies adopted to dealing with soil moisture [25]. Race **C** plants avoid severe drought conditions by reaching reproductive maturity faster—essentially setting seed before their environment dries out entirely. This has been referred to as phenological escape [26]. In addition to early maturation, race **C** plants also allocate more biomass to reproduction and maintain flower head development despite limitation of soil moisture. In contrast, race **A** plants, which grow under water-stress conditions, have adopted a slow-growth strategy which consists of a reduced flower head production and higher allocation of biomass to root production relative to shoot production. These strategies appear to be widespread in species growing under nutrient and/or water stress [27].

A comprehensive phylogenetic analysis of *Lasthenia* using ITS/ETS/cpDNA markers revealed the

existence of two geographically-based, non-sister clades [28-31]. The authors of that work recognized the two clades as cryptic taxa: *L. californica* subsp. *californica*, corresponding to a northern clade, and *L. gracilis* representing a southern clade. Interestingly, the existence of these geographically-based clusters of populations within *L. californica* (in the broad sense) was previously suggested by the genetic distance-based dendrogram generated from the earlier allozyme study of Desrochers and Bohm [19], a point evidently overlooked by Chan et al. The molecular phylogeny provided a model with which relationships of the edaphic races to the newly described taxa could be studied. If edaphic selection has played a role in the origin of flavonoid races, as appears to be the case, then similar edaphic tolerances may have evolved in parallel within the two phylogenetic taxa. A suggestion that something of this sort may have occurred came from the allozyme data where there was a northern cluster of populations, which were mainly of the **C** type, and a southern cluster of populations, which were mainly of the **A** type, but in each area a few of the other type were present.

In order to examine this situation in greater detail, the edaphic race and phylogenetic affinities of 33 populations from throughout the range of the *L. californica* complex were determined. Flavonoid profiles were used to assign the Chan et al. [28-30] populations to edaphic races, and nuclear ribosomal ITS regions of several representatives from the edaphic races were used to determine phylogenetic affinities [32]. The flavonoid and sequence data revealed that edaphic races are not in agreement with the newly defined taxa, *L. californica* subsp. *californica* and *L. gracilis*. Thus, of the 16 populations of *L. gracilis* examined, 13 were race **A** and 3 were race **C**. Of the 17 populations of *L. californica* subsp. *californica* tested, 11 were race **C** and 6 race **A**. These observations lead to an intriguing conclusion: both flavonoid races occur in parallel in both phylogenetic lineages. Careful analysis of the allozyme study results [19], and a more recent study of the phylogeny based on nuclear ribosomal ITS markers by Desrochers and Dodge [33], confirm the parallel formation of edaphic races. Interestingly, the two races on the serpentine outcrop at Jasper Ridge belong to the two phylogenetic species (32) suggesting that racial divergence did not occur *in situ* but that the two races colonized the site independently and have been maintained in their respective microhabitats within the site.

A distance-based analysis of RAPD (randomly amplified polymorphic DNA) variation rooted using the information from phylogenetic markers indicated that race **A** is more likely ancestral in the complex [34]. This led to the inference that race **C** evolved in parallel in the two clades. This is contrary to many examples of evolution of edaphic races found in the literature where it is generally considered that stress-tolerant races evolve from the more "normal" populations [15, 35]. However, given the climatic and edaphic histories of the Central Valley region of California where *Lasthenia* is thought to have evolved [1], it is possible that salt tolerance is an ancestral trait, and that it has been lost as the climate became drier and soils became less saline owing to the retreat of inland seas [36, 37]. Thus, a transition from wet, saline to dry, non-saline conditions could have led to relaxation of selection for traits conferring salt tolerance, while selecting for traits that conferred drought tolerance, leading to the evolution of race **C** populations from ancestral race **A** [38].

Independent origin of race **C** in the two phylogenetic species would have entailed loss of sulfated flavonoid derivatives, compounds that characterize race **A**. The coincident loss of both sulfated flavonoids and sodium ion tolerance is of interest owing to the possible linkage of the two phenomena [39]. Sulfated flavonoids have often been reported from plants growing under saline conditions [12]. It has also been shown that over 50% of radioactive sulfur fed to the halophyte *Zostera* (seagrass) becomes concentrated in the flavonoid fraction [40]. An ecologically-based adaptive mechanism linking sulfated flavonoids and salt tolerance has been discussed by Rajakaruna and coworkers [41]. Of interest in this regard is the recent study of the salt-tolerant hybrid species *Helianthus paradoxus* (Asteraceae) by Karrenberg and coworkers [42] showing that sulfur concentration in this species was four to five-fold greater than in its non-salt tolerant ancestral species. Whether the sulfur was associated with the flavonoid fraction was not determined. Changes in salt tolerance and water stress physiology in these plants would follow the climatic and edaphic shifts known to have taken place in California [43]. The occurrence of different edaphic races in two phylogenetic clades on Jasper Ridge provided an excellent opportunity to study the nature of local adaptation. Hydroponic studies were conducted to examine the physiological differences with respect to sodium ion uptake [41]. Mean sodium ion uptake rates and tissue accumulation of the ion

were estimated. Results indicated that race **A** plants from both lineages are more tolerant: sodium ion uptake rates of race **A** plants were 20-fold higher than those of race **C** plants. Further, race **A** plants translocated ca. 50% of absorbed sodium ion to the shoot compared to ca. 30% in race **C** plants. When combined with higher uptake rates, race **A** plants were shown to be able to accumulate about 5-fold greater concentrations of sodium ion in above-ground tissues than race **C** plants. Regardless of phylogenetic lineage, race **C** plants restricted translocation of sodium ion to the shoots, a mechanism often seen in plants sensitive to toxic elements [44]. In such plants sodium ions are sequestered in vacuoles [45] by means of Na^+/H^+ antiporters [46, 47]. A future study of interest would be an examination of the expression of the Na^+/H^+ antiporter gene in the two races in the *L. californica* complex, which might provide useful information on the underlying biochemical/genetic basis for salt tolerance in these plants.

Studies of germination, root growth, and plant survivorship were also performed [41] results from which also indicated greater tolerance to sodium ions of race **A** plants. Significant genotype x treatment interactions were observed for all tolerance measures, which suggested that these races are genetically divergent in their tolerance responses to sodium ions. It is not yet clear, however, how loss of these traits to produce race **C** is adaptive, unless they are reflected in a relative fitness cost under the conditions in which race **C** plants occur. With the establishment of the phylogenetic relationships between the two edaphic races, and the physiological basis of the edaphic differences, attention was turned to the nature of the reproductive isolation in the system. Early greenhouse studies, as well as field observations, suggested strong prezygotic isolation between the two races at Jasper Ridge. Flowering time differs by 7-10 days, with race **C** plants finished flowering in many cases before race **A** plants had begun to flower [19, 22]. The question remains, however, as to whether this difference is the result of direct selection for flowering time [48, 49], or is the by-product of selection for some other feature [50]. What is abundantly clear, however, is that flowering time differences provide an effective strategy for reproductive isolation.

If pollen flow among members of different races does occur, however, how effective is it? Based upon a limited number of crosses, Desrochers [18] observed generally low crossability of the Jasper Ridge races. Recall that the allozyme study revealed reduced gene flow between them (G_{ST} = 0.417; Nm = 0.35). Ornduff [1] had also noted generally low crossability among plants originating in separate populations, which he took to suggest that reduced gene flow may be population-specific rather than controlled by either race or clade. The absence of any definitive data on this key part of this system's biology represented a significant gap in understanding reproductive and evolutionary activities within the complex. Examining the extent and nature of reproductive isolation between races of the complex was critical to testing the hypothesis of adaptive evolution of edaphic races. More importantly, the parallel occurrence of physiologically distinct races in divergent lineages would also provide an opportunity to test for the parallel origin of reproductive isolation between ecologically divergent populations. If the races are reproductively isolated within each clade, and between clades, it would be possible to infer parallel and independent origins of the edaphic races. Although parallel evolution of taxa has been documented [51, 52], parallel evolution of traits conferring reproductive isolation—parallel speciation—has only rarely been considered in the plant literature [52]. Independent and recurrent evolution of functional traits could best be explained by the action of natural selection [51], and a link between adaptive traits and traits that cause reproduction isolation could lead to multiple, independently-derived populations. The physiologically distinct races in the two divergent lineages within the *L. californica* complex provided an opportunity to examine the possible link between adaptation and reproductive isolation.

A test of reproductive isolation between races was performed using seven populations [34, 38]: three populations of *L. californica* subsp. *californica*, two race **A** populations and a single race **C** population; and four populations of *L. gracilis*, three of which were race **A** populations with a single race **C** population. Seed set per cross was estimated by the number of ripe seeds (based on usual criteria) and then by averaging the numbers from two heads used in reciprocal crosses. The results indicated that reproductive isolation appeared to be strongest (reduced pollen flow) in inter-racial crosses both within and between clades [38]. That is, **A** x **C** (and the reciprocal **C** x **A**) crosses yielded fewer viable seeds than did crosses between like races (**A** x **A** and **C** x **C**). This is in agreement with the idea that reproductive isolation is evolving as a direct

consequence (or by-product) of edaphic specialization. A separate experiment, that involved measuring pollen tube growth, revealed that pollination success rate of **C** x **A** crosses from within the same clade was about 5%, whereas the success rate for **C** x **C** crosses from different origins was ca. 40% (34). Although based on a preliminary crossing study—more work is always indicated—the results meet the criteria that are essential to demonstrate parallel speciation [51]: descendent populations must be reproductively isolated from ancestral populations, and separate descent populations (arising from different clades) must not be reproductively isolated.

In following the history of our, and others', studies in the genus *Lasthenia*, from the initial chromatographic analysis of flavonoid pigments to the application of modern macromolecular techniques, we have demonstrated the importance of applying all of the array of tools available to the evolutionary systematist. Moreover, the story moves from the straightforward analysis of simple chemical structures to a point—far removed from the beginning—of recognizing the *Lasthenia californica-L. gracilis* system as a potential model system for continued studies of speciation, the fundamental process in biology [53]. There are those who would have one believe that only the most sophisticated techniques are necessary to understand the evolution of an organism. We would hope that readers appreciate the shortsightedness of that philosophy.

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