# Two California Lineages of *Oxalis oregana*: Genetic Evidence for a Pleistocene Separation into Northern and Southern Glacial Refugia[1]

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## Abstract

In the Pacific Northwest, there are discontinuities in the lineages of several plant and animal species in the northern California/Oregon region that are thought to have their origins in the separation of populations into refugia during the Pleistocene glacial periods. Redwood sorrel (*Oxalis oregana* Nutt.), a common understory species of the California redwood forests and other Pacific Northwest temperate rainforests, was found to have two distinct genetic lineages in California based on sequence analysis of two chloroplast intergenic loci (*psbJ-petA* and *trnQ-5'rps16*) and the nuclear rDNA internal transcribed spacer (ITS) region. A "southern" lineage was detected in five populations from Big Sur to southern Humboldt County, and a "northern" lineage was dominant in two populations in northern Humboldt County and Del Norte County. The southern individuals had mixed sequence chloroplast haplotypes (presumably due to locus duplication and divergence or from chimeric tissue) while the vast majority of northern individuals had single sequence haplotypes. The northern and southern ITS variants were markedly divergent from each other, indicating a long period of separation between the lineages. Hybridization is occurring, as evidenced by an individual in a northern population that possesses a hybrid ITS genotype. The data suggest that these two groups were derived from an ancestral form that separated into two glacial refugia: a northern refugium within, or north of, the Klamath-Siskiyou ecoregion and a southern refugium in the California coastal forests.


Keywords: chloroplast DNA, glacial refugia, internal transcribed spacer, Klamath-Siskiyou ecoregion, *Oxalis oregana*, phylogeography, redwood sorrel

## Introduction

Redwood sorrel (*Oxalis oregana* Nutt.) is a perennial herb that ranges from northern California to southern British Columbia in coastal temperate forests. In California it is strongly associated with *Sequoia sempervirens* (D. Don) Endl. and is often a dominant understory species of the redwood forest. Like the coast redwood, redwood sorrel is capable of both sexual and vegetative reproduction. The requirement for moist and shaded habitat makes redwood sorrel especially susceptible to the predicted climatic changes that will occur in much of the redwood region this century (Hayhoe et al. 2004). Therefore, it is important to document the genetic diversity and population structure of redwood sorrel in California as a climatic "indicator species" of the redwood forest.

The ranges of species with widespread Pacific Northwest coastal distributions were likely altered significantly by fluctuating climate during the glacial/interglacial periods of the Pleistocene epoch (2.58 to 0.012 mya). Genetic discontinuities have been observed in the north-south distributions of many Cascade and Sierran species, including Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.). (Furnier and Adams 1986), western sword fern (*Polystichum munitum* (Kaufl.) Presl ) and red alder (*Alnus rubra* Bong.) (Strenge 1994), tailed frog (*Ascaphus truei*) (Nielson et al. 2001), dusky shrew (*Sorex monticolus)* (Demboski and Cook 2001) and Pacific giant salamander (*Dicamptodon tenebrosus*) (Steele and Storfer 2006). This phylogeographic pattern has been attributed to the contraction of ancestral populations into glacial refugia followed by southern and/or northern post-glacial recolonization (Brunsfeld et al. 2001, Soltis et al. 1997). Such studies yield insights into past

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responses to climate change and might also help predict how species will respond to the current warming trend.

In this study, seven populations of *O. oregana* from Big Sur to the Oregon border were analyzed at two chloroplast loci and one nuclear locus for DNA sequence variation. The discovery of two geographically distinct genetic lineages of redwood sorrel in California not only expands our knowledge of the genetics of redwood forest flora, but also provides strong evidence for a Pleistocene glacial refugium in coastal California for the southern lineage.

# Methods

## Sampling and DNA Extraction

Sampling was conducted between May 25 to June 2, 2012 in seven redwood state parks and reserves in California, ranging from Monterey County to Del Norte County (fig. 1). In each population single leaves from 10 individuals spaced a minimum of 50 m apart were collected and dried on silica gel desiccant. Genomic DNA was extracted from dried tissue using the method of Xin et al. (2003) and the Plant Genomic DNA Mini-prep Kit (Bay Gene, Burlingame, CA).

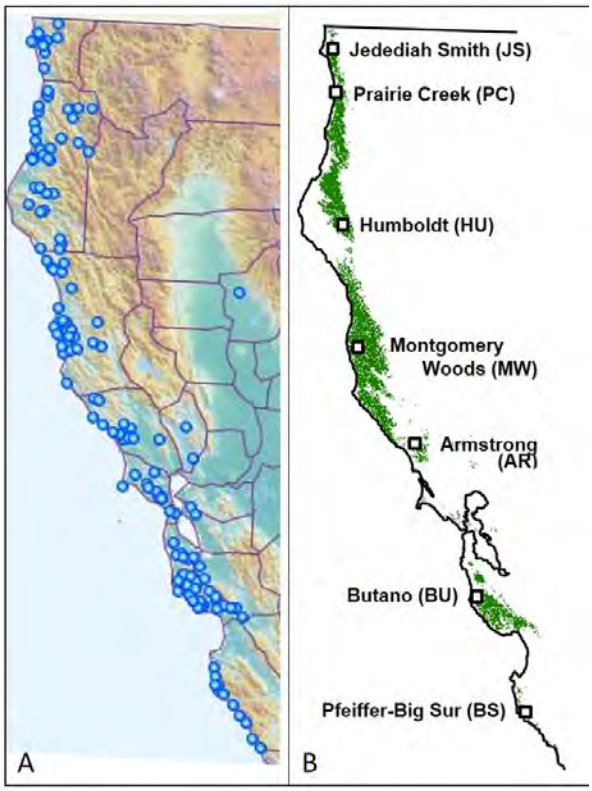


Figure 1—A. *Oxalis oregana* distribution in California (Calflora 2016). B. Coast redwood forest distribution with sites of state parks and reserves where *O. oregana* populations were sampled.

## Amplification Conditions

The chloroplast intergenic sequences *psbJ-petA* and *trnQ-5'rps16* were amplified using the primers of Shaw et al. (2007). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (including ITS-1, 5.8S rDNA and ITS-2) was amplified using the ITSA and ITSB primers of Blattner (1999). All PCR reactions (20 µL) contained 1X PCR Gold Buffer (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 0.1 percent BSA, 0.2 mM each dNTP, 0.5 µM each forward and reverse primer, 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems) and 1 µL DNA extract.

Amplification of the *psbJ-petA* locus was performed with an 8 min polymerase activation at 94 °C followed by 30 cycles of 94 °C (30 sec), 56 °C (30 sec) and 72 °C (1 min), then a final extension at 72 °C (6 min). The *trnQ-5'rps16* locus PCR conditions were the same except 35 cycles were used. For the ITS locus, denaturation was at 94 °C (45 sec) and primer annealing was at 55 °C (1 min), otherwise conditions were the same as with the *psbJ-petA* amplification. PCR products were checked by electrophoresis in 2 percent agarose gels.

## DNA Sequence Analysis

PCR products were purified for sequencing using PCR Clean-up Kit spin columns (Bay Gene) or DNA Clean and Concentrator-5 spin columns (Zymo Research, Orange, CA). Sequencing was performed by the University of Maine DNA Sequencing Facility (Orono, ME) using the psbJ, $trnQ^{(UUG)}$ and ITSA PCR primers. Sequences were aligned using ClustalX v.2.0 (Larkin et al. 2007). Areas of duplicate sequences in the "mixed se quence" haplotypes were analyzed manually and confirmed with Mixed Sequence Reader (Chang et al. 2012). Heterozygous genotypes at the ITS locus were identified by double peaks at variable sites in the sequencing electropherograms. Bootstrapped neighbor-joining trees of ITS sequence variants were constructed in MEGA5 (Tamura et al. 2011) using IUPAC ambiguity codes for the heterozygous genotypes.

# Results

## Chloroplast DNA Haplotypes

### psbJ-petA Locus

PCR amplification of the *psbJ-petA* locus resulted in a product of approximately 700 bp. The alignment length was 616 bp of which 548 to 557 bp comprised the intergenic sequence (the length variation due to an indel). Three variable sites (two substitutions and a 9 bp indel) were detected in six haplotypes (table 1).

**Table 1—Haplotypes of the chloroplast *psbJ-petA* locus in *O. oregana***

| | Variable site[b] | | |
|---|---|---|---|
| Haplotype[a] | 168 | 275 | 276-284 |
| A | G | T | ATCGAAACT |
| B | T | T | ATCGAAACT |
| C | T | T | ACCGAAACT |
| D i | G | C | deletion |
| ii | T | T | deletion |
| E i | G | T | ATCGAAACT |
| ii | T | T | deletion |
| F i | T | T | ATCGAAACT |
| ii | T | T | deletion |

[a] Haplotypes D-F are "mixed sequence" haplotypes, each with two sequences indicated by i and ii.
[b] Haplotype A Genbank accession number is KX906973. Haplotype B-F polymorphisms are annotated as "variations" in the Haplotype A Genbank feature table.

Haplotypes A-C were single sequences as expected from haploid chloroplast loci. However, individuals with the other three haplotypes (D-F) had "mixed sequences" due to the presence of two closely related templates (indicated by i and ii in the table) in their PCR products. There were double

electropherogram peaks at site 168 (in Haplotypes D and E) and site 275 (in Haplotype D). A sudden double sequence began at site 276 in the electropherograms of Haplotypes E and F and continued to the end, the cause due to the deletion of the nine bases at positions 276 to 284 in one of the two templates.

Changes in PCR annealing temperatures, the use of touchdown PCR protocols, the addition of PCR enhancers (DMSO and betaine), and the resequencing of all samples amplified from reisolated DNA gave the same results. In addition to the reproducible nature of the single and mixed sequence haplotypes, there was also a distinct geographic pattern to their distribution (see next section).

It should be noted that the Haplotype A sequence is the same as the Haplotype Ei sequence, and the Haplotype B sequence is the same as the Haplotype Fi sequence. Also, the second sequences (ii) of all three of the mixed haplotypes are the same.

### trnQ-5'rps16 Locus

For the *trnQ-5'rps16* locus, PCR amplification resulted in a product of approximately 850 bp. The alignment length was 798 bp of which the intergenic sequence was 758 bp. Four *trnQ-5'rps16* haplotypes were detected due to base substitutions at three variable sites (table 2).

**Table 2—Haplotypes of the chloroplast *trnQ-5'rps16* locus in *O. oregana***

| | Variable site[b] | | |
|---|---|---|---|
| Haplotype[a] | 195 | 250 | 264 |
| 1 | T | T | A |
| 2 i | T | T | A |
| ii | T | T | T |
| 3 i | T | G | A |
| ii | T | T | T |
| 4 i | G | T | T |
| ii | T | T | T |

[a] Haplotypes 2-4 are "mixed sequence" haplotypes, each with two sequences indicated by i and ii.
[b] Haplotype 1 Genbank accession number is KX906974. Haplotype 2-4 polymorphisms are annotated as "variations" in the Haplotype 1 Genbank feature table.

Only Haplotype 1 had a single sequence. The others (Haplotypes 2-4) were mixed sequence haplotypes, each with two closely related templates (i and ii) in their PCR products. Haplotype 1 is the same as the Haplotype 2i sequence, and the second sequences (ii) of all three of the mixed haplotypes are the same.

Results were reproducible regardless of PCR conditions or DNA preparation as with the *psbJ-petA* locus. Similarly, there was a distinct geographic distribution to the single vs. mixed sequence haplotypes. All individuals that had a single sequence haplotype at one chloroplast locus also had a single sequence haplotype at the other locus. The same was true for individuals with mixed sequence haplotypes.

## Chloroplast DNA Haplotype Distributions

The distributions of chloroplast haplotypes for each locus are shown in fig. 2. In the pie charts, mixed sequence haplotypes are color-coded in orange and red shades while the single sequence haplotypes are displayed in blue shades.

Both the *psbJ-petA* (fig. 2A) and *trnQ-5'rps16* (fig. 2B) loci clearly show a sharp demarcation between populations having the single vs. mixed sequence haplotypes. All populations south of, and including, Humboldt Redwoods State Park (HU) in southern Humboldt County have mixed sequence

haplotypes. For the *psbJ-petA* locus, Haplotypes E and F comprise 54 percent and 44 percent of the haplotypes in these populations; Haplotype D (2 percent) was found in only one HU individual. At the *trnQ-5'rps16* locus, Haplotype 2 was found in 90 percent of individuals in the five southern populations, with Haplotypes 3 and 4 contributing 8 percent and 2 percent, respectively.

Ninety percent of individuals in the two northernmost populations (PC and JS) had single sequence haplotypes at both loci. Haplotype A was dominant at the *psbJ-petA* locus (80 percent) and Haplotype 1 was dominant at the *trnQ-5'rsp16* locus (90 percent). Two individuals in Jedediah Smith Redwoods State Park (JS) had mixed sequence haplotypes at both loci (Haplotypes E and 2).

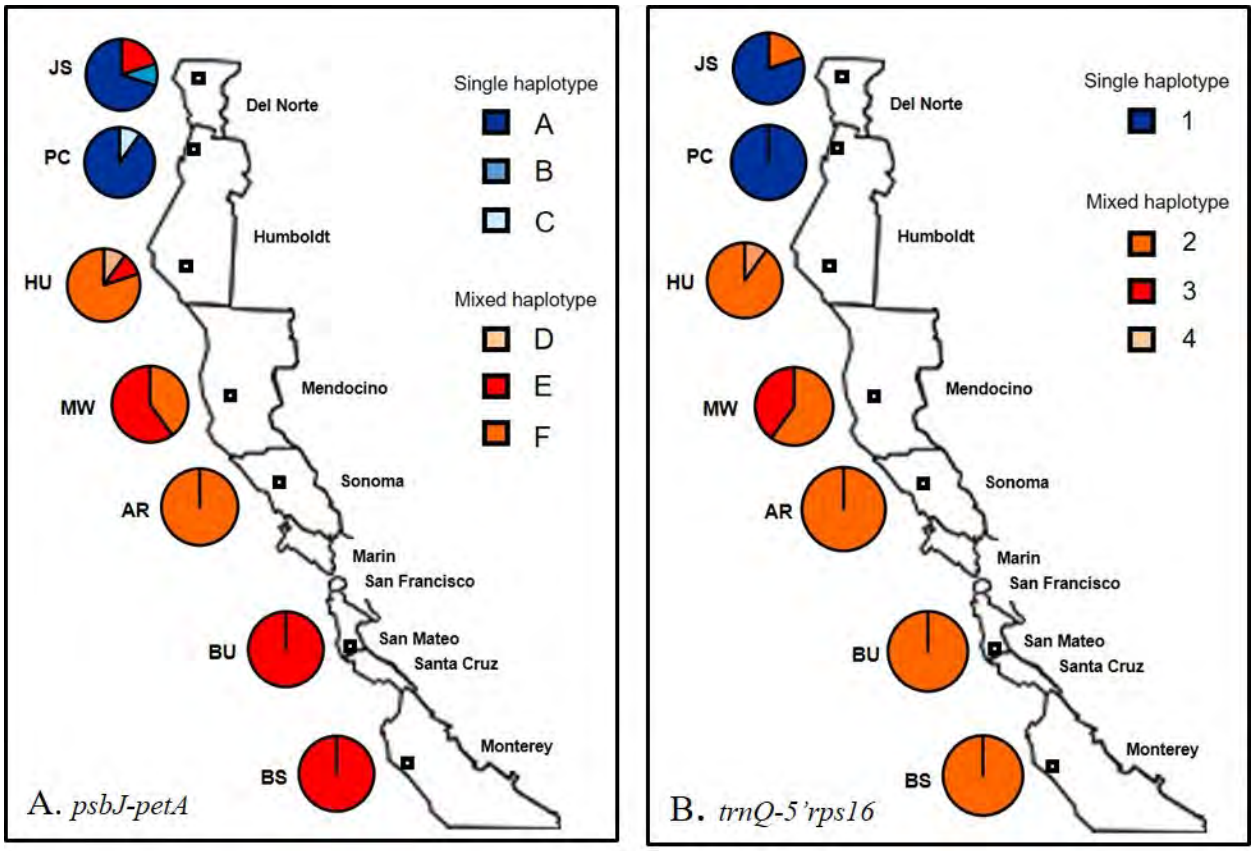


Figure 2—Distribution of chloroplast DNA haplotypes from loci *psbJ-petA* (A) and *trnQ-5'rps16* (B) in seven populations of *Oxalis oregana*. Two-letter abbreviations of sampled populations are at left. County names are at right. Haplotype descriptions are provided in tables 1 and 2.

## ITS Sequence Variant Distributions

PCR amplification of the nuclear rDNA ITS region resulted in a product of approximately 850 bp. The total alignment length was 665 bp. The ITS-1 and ITS-2 alignment lengths were 223 bp and 221 bp, respectively.

There were 10 variable regions in the ITS-1 region and six in the ITS-2 region (table 3). Six closely related variants ($S_1$-$S_6$) were found almost exclusively in the five southernmost populations (fig. 3). A more divergent variant (N) was only found in the two northernmost populations. Four variants were homozygotes with only one allele ($S_1$, $S_2$, $S_4$ and N) while the other three ($S_3$, $S_5$ and $S_6$) were heterozygous, each having alleles that differed at only one variable site (as indicated by the G/A, C/T and C/T designations at sites 136, 469 and 591, respectively). A single individual in the JS population had a heterozygous N/$S_1$ genotype (not shown in table 3, but marked by an asterisk in fig. 3).

**Table 3—Internal transcribed spacer (ITS) sequences of the southern (S) and northern (N) *O. oregana* variants**

| ITS variant | Variable site[a] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ITS-1 | | | | | | | | | | ITS-2 | | | | | |
| | 49 | 77 | 93 | 99 | 114 | 126 | 136 | 168 | 223 | 226 | 394 | 422 | 469 | 572 | 591 | 594 |
| $S_1$ | G | C | G | G | T | – | G | G | T | A | T | C | C | A | C | T |
| $S_2$ | A | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| $S_3$ | · | · | · | · | · | · | G/A | · | · | · | · | · | · | · | · | · |
| $S_4$ | · | · | · | · | · | · | · | · | · | · | · | · | T | · | · | · |
| $S_5$ | · | · | · | · | · | · | · | · | · | · | · | · | C/T | · | · | · |
| $S_6$ | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C/T | · |
| N | · | T | – | T | G | A | · | T | G | C | C | T | · | G | · | C |

[a] Dots indicate the same base as the $S_1$ variant. The N variant Genbank accession number is KX906971. The $S_1$ Genbank accession number is KX906972. The $S_2$-$S_6$ polymorphisms are annotated as "variations" of the $S_1$ sequence in the Genbank feature table.

## ITS Phylogeny

A neighbor-joining tree was constructed (fig. 4) using the southern and northern *O. oregana* ITS variants from table 3 (using IUPAC ambiguity codes for the three southern heterozygous variants) and three Genbank *Oxalis* ITS sequences: an *O. oregana* accession from southwestern Washington (JN836782), *O. acetosella* (JN836783) and *O. rosea* (JN836784), the latter serving as an outgroup.

The northern *O. oregana* variant (N) is 100 percent homologous with the Genbank *O. oregana* accession from Washington. Along with *O. acetosella* (common wood sorrel), these two form a cluster that is a sister clade to the southern *O. oregana* variants.

# Discussion

Genetic analysis of one nuclear locus (ITS) and two chloroplast loci (*psbJ-petA* and *trnQ-5'rps16*) show conclusively that there is an abrupt genetic break in *O. oregana* in Humboldt County. Only mixed sequence chloroplast haplotypes and the southern ITS variants were found from Humboldt Redwoods State Park (HU) in southern Humboldt County to Pfeiffer-Big Sur Redwoods State Park (BS) in Monterey County. The single sequence chloroplast haplotypes and the northern ITS variant were found exclusively in Prairie Creek (PC) and Jedediah Smith (JS) Redwoods State Parks in northern Humboldt County and Del Norte County. The presence of two individuals with mixed sequence haplotypes in the JS population suggests a northward migration of the southern group, and the detection of a north/south ITS hybrid genotype in one of those individuals indicates that the two lineages are capable of mating.

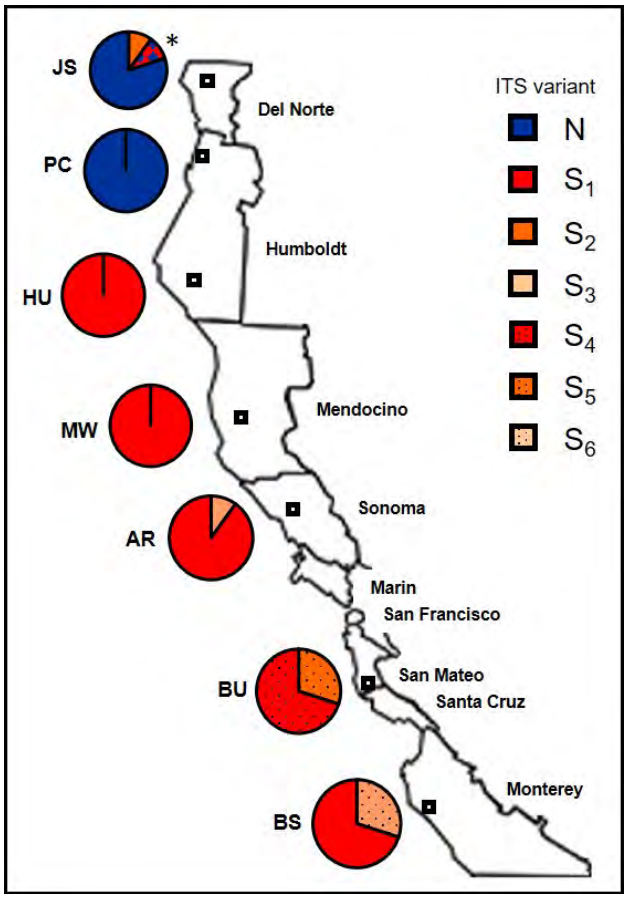


Figure 3—Distribution of nuclear ITS genotypes in seven populations of *Oxalis oregana*. The asterisk marks one individual in the JS population that had a north/south hybrid genotype (N/$S_1$). Two-letter abbreviations of sampled populations are at left. County names are at right.

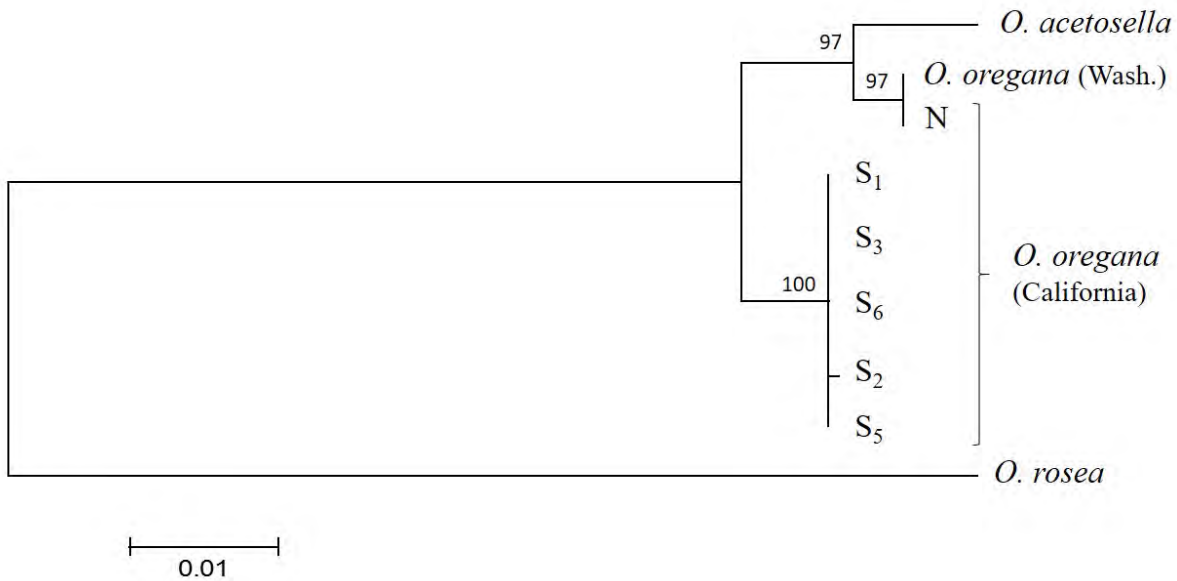


Figure 4—Neighbor-joining tree of northern and southern *Oxalis oregana* ITS variants with related *Oxalis* species. Numbers at nodes are bootstrap percentages.

The most obvious physical barrier separating these two genetically distinct groups is the northwest to southeast oriented mountain range that forms the southwestern border of the Klamath-Siskiyou ecoregion and divides the Redwood Creek watershed from the Klamath River watershed (fig. 5). Of the two northern *O. oregana* populations, JS is well within the Klamath-Siskiyou ecoregion while the PC collection site is just within the Redwood Creek watershed but only 8 km (5 mi) from the Klamath River at a coastal location where the mountains dividing the two watersheds are at their lowest elevation of less than 300 m (1000 ft). This area could be a migration pathway between watersheds for *O. oregana*.

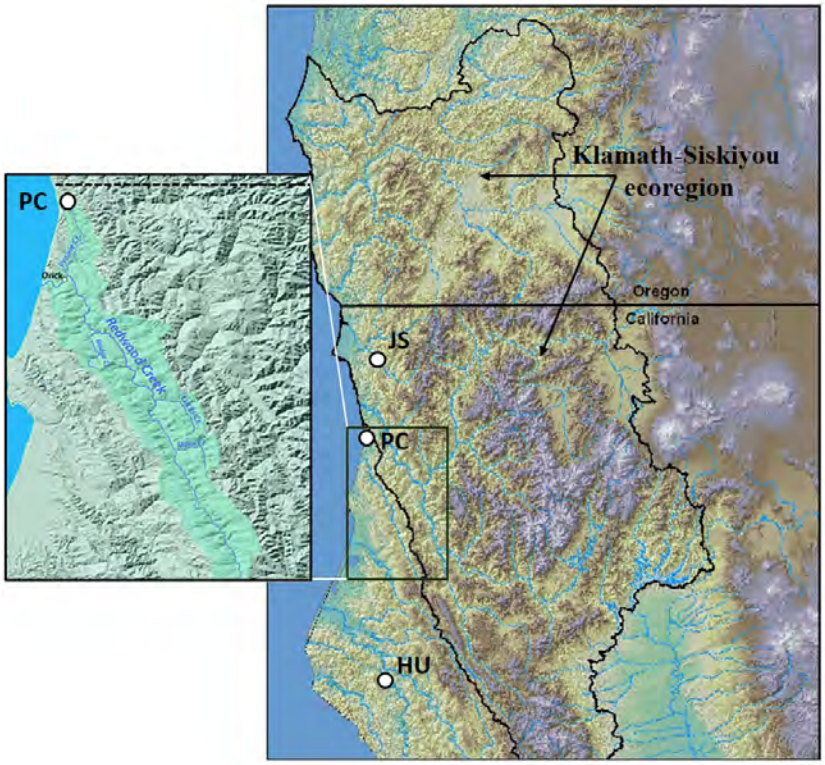


**Figure 5**—*Oxalis oregana* collection sites HU, PC and JS relative to the Klamath-Siskiyou ecoregion (Klamath-Siskiyou Wildlands Center 2016). Inset: Redwood Creek watershed (light green) and the PC collection site. Dashed line is the border between Humboldt and Del Norte counties.

The Klamath-Siskiyou ecoregion has been suggested as one of many possible Pacific Northwest glacial refugia (Roberts and Hamann 2015, Smith and Sawyer 1988, Whitaker 1961). Other potential refugia include Vancouver Island and the Olympic Peninsula as well as other coastal sites (Brunsfeld et al. 2001, Soltis et al. 1997).

In a review of chloroplast DNA-based phylogeographies of six Pacific Northwest plant species, Soltis et al. (1997) identified “northern” clade populations ranging from Alaska to central/southern Oregon and “southern” clade populations ranging from central Oregon to northern California. The authors offered two explanations for the observed data: the “north-south recolonization” hypothesis and the “leading edge” hypothesis. The first hypothesis requires that populations are separated into two distinct northern and southern refugia which diverge during their isolation. Post-glacial expansion might reunite these populations, but there will still be a marked north-south genetic discontinuity. The leading edge hypothesis allows for only one southern refugium. Rapid post-glacial northern dispersal and survival of limited genotypes (through drift and bottlenecks) at the leading edge of migration will ultimately result in less diverse, or even fixed, populations in the northern regions. In their analysis of several plant and animal studies, Brunsfeld et al. (2001) found the data to be more consistent with the north-south recolonization hypothesis.

The very different genetic compositions of the two groups of California redwood sorrel identified in this study support the conclusion that the lineages are derived from separate northern and southern glacial refugia which have met in northern California after post-glacial recolonization. The sharp geographic transition from one lineage to the other and the absence of northern cpDNA haplotypes and ITS genotypes in the southern group are strong arguments against the leading edge hypothesis as an explanation for the observed phylogeographic distribution.

It is possible that the southern redwood sorrel lineage was isolated in coastal California in the Pleistocene coast redwood ecosystem that at times extended as far south as Santa Barbara (Sawyer et al. 2001). The greater genetic variation found in the southern lineage suggests that its refugial population was larger than that of the northern lineage. The location of the northern lineage's refugium is much less certain. The northern ITS variant (N) from California and an accession from Washington have identical sequences (as do their *psbJ-petA* loci). This is consistent with a rapid post-glacial migration from a bottlenecked northern lineage and suggests two possible migration scenarios: 1) the northern lineage's refugium was in the Klamath-Siskiyou ecoregion with a subsequent migration northward during the Holocene, or 2) the refugium was in a more northern location followed by southward migration to its current southern limit in Humboldt County. A third possibility, though less likely from geographic considerations, is that the "northern" lineage was derived from a small, bottlenecked population at some other well isolated refugium in California, i.e., a second "southern" group that migrated northward through the Klamath-Siskiyou region.

The fact that the ITS region sequence of the northern variant is more closely related to common wood sorrel (*O. acetosella*) than to the southern variants indicates a very long separation between the two lineages. Löve (1968) once proposed that *O. oregana* be reclassified as a subspecies of *O. acetosella*, but results from morphological and hybridization studies countered her argument (see Packham 1978). However, the ITS data show the close evolutionary relationship between these two species, confirming the work of Gardner et al. (2012).

The mixed sequence haplotypes detected in the chloroplast loci of the southern lineage have two possible origins, one being that each locus underwent duplication and divergence. These types of mutations have been reported in many plant species (Xiong et al. 2009) and could result in mixed sequence haplotypes as long as the PCR priming sites are conserved. For this to occur in two widely separated loci such as (*psbJ-petA* and *trnQ-5'rps16*) is improbable. However, I have evidence (data not shown) of mixed haplotypes occurring only in the southern populations at two other chloroplast loci, as well. These combined four loci span the entire Large Single Copy (LSC) region of the chloroplast genome. There are no documented examples of duplication and divergence of the entire LSC region.

The other explanation for mixed sequence haplotypes is chimerism – the existence of two different variants of chloroplasts in different leaf cells of the same individual. This could originate by the mutation of chloroplast DNA in a leaf meristematic cell. Division of that cell (and of wild-type meristematic cells) would result in chimeric leaf tissue and lead to the amplification of diverged loci from two different chloroplast sources in the same individual. Chimerism, in the form of leaf variegation, is common in *Oxalis* species and occurs to a small degree in some natural populations of *O. oregana*. For entire populations to become chimeric would require vegetative reproduction since chimerism cannot be propagated sexually. Vegetative propagation would be an advantage in the more southern extent of the *O. oregana* range where summer temperatures are higher and precipitation is far less than the northern part of the range. In *S. sempervirens*, the diversity of a chloroplast microsatellite locus is significantly reduced in the species' southern populations (Brinegar 2011) which could be an indication of greater rates of vegetative reproduction. A more detailed genetic analysis will be required to determine whether the southern lineage of *O. oregana* is indeed chimeric.

Over the past century coastal fog has decreased approximately 33 percent in northern California (Johnstone and Dawson 2010) and summer temperatures are predicted to rise which will put more stress on the flora of the southern redwood forests and cause a northward recession as coastal

woodlands and savanna expand (Hayhoe et al. 2004). If, in fact, the southern lineage of *O. oregana* is chimeric and propagates vegetatively, it might be somewhat buffered against these changes.

It is perhaps premature to consider the northern and southern lineages of *O. oregana* as subspecies, especially without conclusive evidence of phenotypic differences to support such a reclassification (as argued by Patten 2015). However, this study has shown that these lineages fit the other requirements of subspecies status: geographic and genetic distinctiveness, and the ability of the two groups to hybridize. Further research is needed to determine whether such a taxonomic change is warranted.

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