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Genotyping a second growth coast redwood forest : a high throughput methodology

Charles Steven Glavas
San Jose State University

GENOTYPING A SECOND GROWTH COAST REDWOOD FOREST: A HIGH THROUGHPUT METHODOLOGY

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By
Charles Steven Glavas
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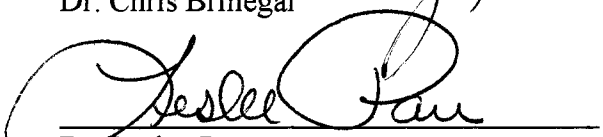
Dr. Chris Brinegar



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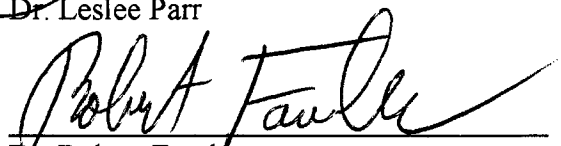

Dr. Robert Fowler

APPROVED FOR THE UNIVERSITY

ABSTRACT

GENOTYPING A SECOND GROWTH COAST REDWOOD FOREST:
A HIGH THROUGHPUT METHODOLOGY

by Charles S. Glavas

Five newly developed microsatellite markers have been used in this study to thoroughly visualize the genetic structure of a second growth coast redwood (*Sequoia sempervirens*) stand. The study area was six square miles. A total of 510 trees were genotyped at ten different sampling locations. Obvious clones were specifically avoided. A high throughput methodology was developed to minimize the time required for such a large study. Data acquisition was performed via a 96-tip robotic platform and a 96-capillary genetic analyzer. The trial population studied showed limited variation from site to site. One nuclear locus, 37G5, showed a strong gradient in amplification across sites. This homogeneity is not characteristic of other distant old growth population studies, but correlates well with nearby second growth studies. In addition, the potential autoallopolyploidism (AABBBB) of coast redwood is supported by these data. A method more suited to the cost restrictions of conservation genetics is suggested.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

INTRODUCTION

Sequoia sempervirens, also known as the California coast redwood, is an ancient conifer occurring along the foggy coast of northern and central California. It is naturally occurring from the southern Oregon border to Monterey County in a somewhat continuous 30-mile wide coastal strip. The total area covered is 2500-3000 square miles (Olson et al., 1990) which is the remainder of the former northern hemisphere distribution. Further, natural barriers, such as geological formations or climate, and human disruptions, such as logging and land development, have created today's fragmented forest.

Currently, approximately 95% of the redwood forest is second and third growth. The high degree of logging in the late 1800s has left few surviving old growth groves. *S. sempervirens* can reach ages of up to 2200 years (Fritz, 1957). It is uncertain exactly when a given grove will begin showing old growth characteristics, but general consensus is over 400 years. Typically, an old growth tree has a broad and complex canopy providing roosts for endangered species, such as the marbeled murrelet and spotted owl. Alternatively, the second growth characteristics are more uniform conical canopy shapes with a less diverse speciation in the surrounding area.

Today's redwood forests, like the old growth forests at the turn of the 19th century, are a valuable timber resource. Given their sheer size, the total stem biomass of an old growth stand can reach up to 8072 cubic meters per hectare. Equally impressive, second growth stands have been projected to reach 3600 cubic meters per hectare within

70 years (Olson et al., 1990). With current prices of second growth redwood logs of $1000/MBF (thousand board feet) representing a 50% price increase from two years ago, a properly managed redwood plantation can provide good returns while minimizing the impact on non-farmed redwoods.

Characteristics

There are three identified sections of the redwood range. These can be broken down into the northern (southern Oregon to Humboldt County), central (Humboldt County to San Francisco), and southern (Alameda County to Monterey County) redwood forests.

The northern forest terrain is marked by rounded ridges; precipitation exceeds 2500 mm annually including snow in late winter; the summers are generally cooler with respect to the rest of the range. It is often compared to the temperate rain forests of Oregon and Washington rather than the central and southern redwood counterparts (Olive et al., 1982).

Central redwood stands have a higher degree of speciation among tree types including Douglas-fir, tanoak, and other hardwoods (Zinke, 1988). The weather pattern of this section has similar precipitation as its northern counterpart, but only sometimes surpasses the 2500 mm level. Snow is uncommon. Summers are typically warmer, and foggy days by and large are restricted to the coast.

Finally, southern forests, the focus of this study, are characterized by their relatively high degree of interspeciation. They are made up of redwood, Douglas-fir,

California bay, coast live oak, and tanoak (Zinke, 1988). Southern stands are considered compositionally, ecologically, and even genetically different than their counterparts to the north. The forests are marked by stark boundaries between the redwood forest and conifer, hardwood, chaparral, and scrub. Precipitation here is limited to 1000 mm annually with snow being extremely rare. Summers are cooler than their central counterpart and are also foggy.

Redwood reproduction and genetics

Redwoods have three methods of reproduction: sexually via seeds and pollen, asexually via stump sprouting, and asexually via cuttings or fallen stem material.

In order for seeds to germinate, the best bed is a warm, moist mineral soil. Seeds have been shown to germinate in duff, on logs, under other plants, or basically anywhere adequate water over a three- to six-week period is available. Seedlings are able to survive sustained surface temperatures of 60°C if a continued water supply is available during the hot summer months. However, other obstacles to seedling survival include the banana slug, rabbits, nematodes (Snyder, 1992), and leaf litter (Olson et al., 1990). These selective pressures, particularly the lower precipitation in the southern section, limit the number of successful sites for seedling establishment.

Redwood stump sprouting – which is unusual for a conifer – is efficient after felling. Approximately 100 sprouts typically surround the remaining stump. Although these sprouts use the existing stump root system, they quickly form their own (Cole, 1983; Olson et al., 1990). The lignotuber, or burl, is a normal part of the redwood

anatomy and has been shown to develop on 75% of seedlings by their sixth year (Simmons, 1973).

Yet another method for the redwood to propagate is through cuttings or fallen stem material. No special treatment is needed for the root system to develop and a 90% success rate has been shown when taken from young trees (Olson et al., 1990). This can be very useful in a farm setting where a single cutting can produce in excess of one million clones in a three-year period (Libby and McCutchan, 1978). This capability of the redwood can also be harnessed in an explant tissue culture method that produces successful redwood individuals (Olson et al., 1990).

These three different types of reproduction can present a challenge to genetic research on *S. sempervirens.* Adding to the issue is the fact that redwoods are unusual in conifers by having a hexaploid genome made up of 66 chromosomes (Saylor and Simons, 1970). Current genetic studies of the coast redwood are limited; however, some evidence is suggests the existence of different founders for the southern versus the central and northern populations. From terpene analysis, Hall and Langenheim (1987) speculated that the southern population may have arisen from one ancestral population, whereas the northern and central populations were founded by two similar, yet incompletely mixed, populations. Enough time has passed between the founding of the different populations that each has adapted to its local environment (Anekonda et al., 1994). Common garden studies suggest that redwoods from the southern population will be less successful in the northern section and vice versa (Millar et al., 1985; Anekonda., 1992). This does not mean a transplanted redwood will not grow in a non-

native environment; rather it is suggestive of the need for care during restoration efforts. Because it will survive, the genes of the foreign tree will be passed on to the extant population potentially polluting the local genetic structure.

Molecular genetics of redwoods

Recently, molecular studies of coast redwoods have contributed to conservation efforts of this species. One of the first molecular studies was the restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) inheritance by Neale et al. (1989) The authors of this study reported that the coast redwood is one of the few eukaryotic organisms with paternal inheritance of mtDNA. This demonstrated that mtDNA and chloroplast DNA (cpDNA) can be used in paternally tracking pollen flow in a given population. A decade later, Rogers began characterizing population differences via allozymes (Rogers, 1997; 1999; 2000). She (2000) found clones in the coast redwood forest propagate in a linear, circular, or disjunct arrangement, thus laying the groundwork for understanding the coast redwood dimensional sexual and asexual reproduction, as well as clone size and diversity. This was based on previous studies (Rogers, 1997; 1999) in which she was able to determine pedigree and uniquely identify individuals via allozymes. Subsequent work by Douhovnikoff et al. (2004) further supported the spatial clonal reproduction patterns in a second growth stand via amplified fragment length polymorphisms (AFLP).

Concurrently, an effort by Bruno (2002) to characterize the first viable microsatellite loci for the coast redwood was completed. Using these new markers, a

small southern population was then characterized (Bruno and Brinegar, 2004) to determine the practical range of the given loci. Investigation by Brinegar et al. (2006) began looking at a small sampling of populations throughout the coast redwood range. Some of the data in this thesis was included in that study. With the development of a PCR-based method, the door for inexpensive, accurate, and a high throughput compatible genotyping method for coast redwoods has been opened.

Study objectives

The main objective of this study was to develop a high throughput method of microsatellite DNA analysis compatible with capillary electrophoresis and amenable to the study of redwood population genetics. As a trial, the methodology was used to ascertain the genetic structure in a second growth redwood population and to determine whether watershed topology plays a role in directing gene flow.

MATERIALS AND METHODS

Collection area and collection technique

Collection of material was with permission of the California Department of Parks and Recreation. The collection area was a six square mile watershed environment located in Big Basin Redwoods State Park in the Santa Cruz Mountains (Fig. 1). This area is a protected second growth coast redwood forest that was heavily logged up to the late 1800s. At the beginning of the 1900s, the remaining old growth stand and some second growth were protected. Additional regions of interest were acquired and added to the existing Big Basin Redwoods State Park jurisdiction over time.

Large elevation changes and a network of ridges that channel the West Waddell Creek and its tributaries to the Pacific Ocean mark the watershed terrain. The ten collection sites (Table 1, Fig. 2) were both upstream of the confluence or at the confluence of various tributaries. Access to the sample sites was generally by footpath. Ten samples were collected on each bank approximately 100 m up and downstream of the collection site coordinates with approximately 10 m separating each tree.

Depending on the number of tributaries present at the collection site, a range of 40 to 90 samples were collected per site. As seen in Fig. 2, site 5 had only 40 samples whereas site 7 had 60 samples. Site 8 was unique in that two confluences were separated by approximately 100 m, thus allowing for 90 samples given this configuration. Two branchlets per tree were collected by hand at ground level. New growth branchlets, marked by their lighter color, were preferentially chosen over the

previous season's growth when possible. Obvious clonal trees were avoided. Branchlets for each tree were placed in a #1 coin envelope, air dried, then desiccated at ambient temperatures. Samples were named by site location, bank, then tree number. Bank is defined as the approximate map bearing (eg, N for north) and bank side (R for right, L for left) when facing upstream. The tree number begins with 1 at the confluence, ending with 10 at the farthest point from the confluence. Site 8 had an additional notation of "C" for center section where numbering began at the farthest upstream point.

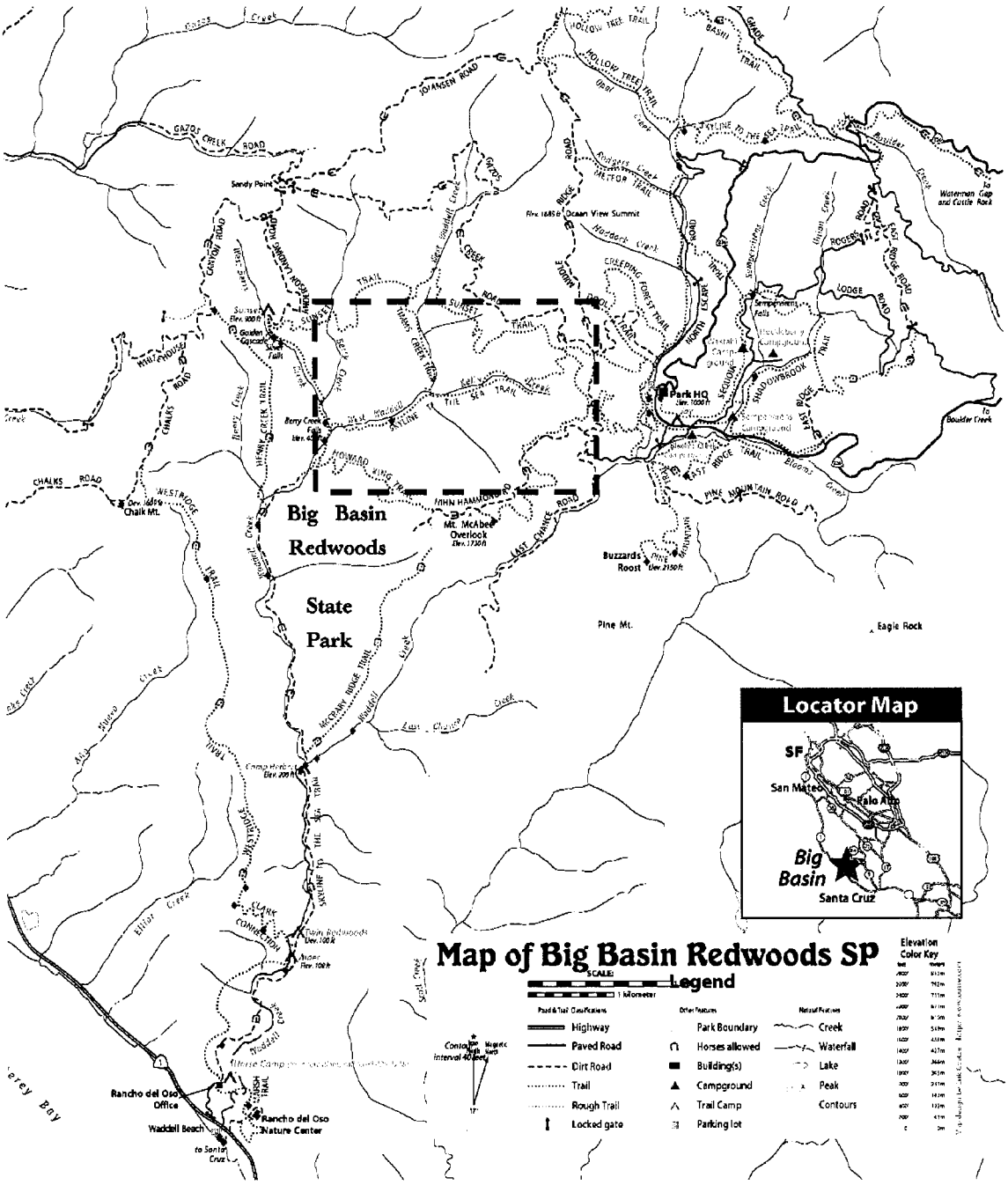

Figure 1. Sample site within Big Basin Redwoods State Park. The sample site is defined with a dashed line. Inset is the approximate location of the park in California.

Table 1. GPS coordinates of and number of samples (n) at each collection site.

Site	n	GPS Coordinates
1	40	N 37.18185, W 122.26384
2	40	N 37.17898, W 122.27259
3	60	N 37.17130, W 122.26547
4	80	N 37.16805, W 122.26331
5	40	N 37.18492, W 122.25009
6	40	N 37.17981, W 122.24571
7	60	N 37.17773, W 122.25238
8	90	*Confluence 1:* N 37.17247, W 122. 25078 *Confluence 2:* N 37.17228, W 122.25251
9	40	N 37.16639, W 122.24421
10	40	N 37.17208, W 122.23678

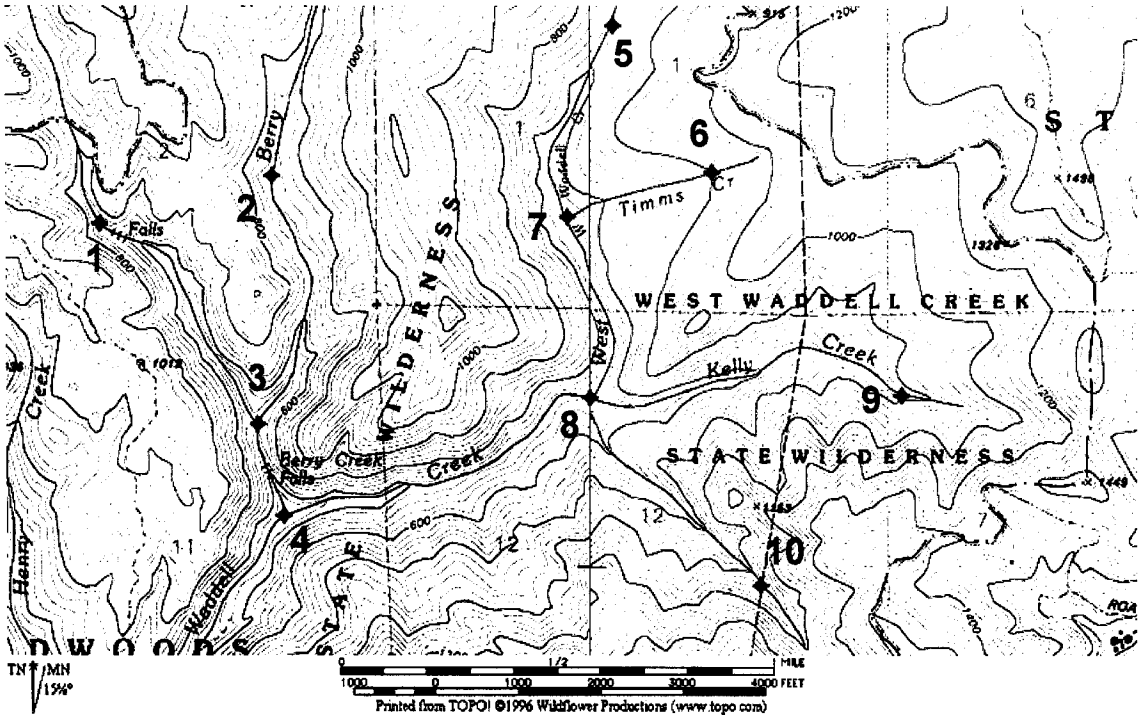

Figure 2. Map of the collection area in the Big Basin Redwoods State Park. Approximate position of collection sites is noted (numbered 1-10).

DNA extraction

Genomic DNA (gDNA) was extracted according to Xin et al. (2003). In this method, a hot alkaline-detergent extraction buffer (Buffer A) is used to extract the gDNA from the desiccated needle. After a 10 min incubation, Buffer A is neutralized with an equal volume of an acidic chelating buffer (Buffer B).

The procedure is as follows:

1) Place 5 mm section of desiccated needle(s) in test tube(s) or 96-well plate.
2) Add 50 uL to 100 uL of stock Buffer A (100 mM NaOH, 2% Tween-20)
3) Incubate at 95 °C for 10 min. This can be performed in a heat block, water bath, or a thermal cycler. The sample is brought to 4 °C prior to proceeding to the next step.
4) Add equal volume of freshly made ambient temperature Buffer B (2 mM EDTA, 100 mM Tris-HCl). There is no need to adjust the pH of Buffer B if Tris-HCl is used in the preparation instead of Tris base.
5) Store extracted sample at –20 °C or proceed immediately to PCR.

There is no need to macerate the sample(s) or to remove the needle section from the extract during storage or manual PCR setup. However, if using an automated liquid handling method, it may be advisable to manually aliquot to a compatible reservoir or plate format. Removing the needle section is advisable to prevent blockage of liquid handling robotic pipette tips for successful gDNA transfer to the PCR reaction vessel.

No observable physical change to the needle occurred. The extract buffer changed color ranging from nearly clear to a dark brown. It was observed that the darker extracts tended to amplify less frequently. Longer incubation times in the 95 °C extraction step were not beneficial and often resulted in less successful amplification.

Amplification of DNA

Amplification was performed (Xin et al., 2003) using the five *Sequoia*-specific microsatellite primer pairs and conditions (Table 2) specified by Bruno and Brinegar (2004) with modification to allow compatibility with 384-well format and liquid handling robotics (BiomekFX, Beckman Coulter, Palo Alto, California). The reactions were carried out as singleplexes.

Final PCR conditions were an initial 10 min DNA polymerase activation step at 95 °C, 35 cycles at 95 °C for 30 s, the optimized annealing temperature (55-60 °C) for 1 min, and 72 °C for 2 min followed by a final extension at 72 °C for 6 min.

Table 2. *Sequoia sempervirens* microsatellite primer sequences and optimized annealing temperatures (from Brinegar and Bruno, 2004)

Sequence Name	Annealing Temperature	Sequence
*Seq*8E8	55 °C	F: ATACTCACCCTTACACGGGC R: AAATGCCTTGATGAAGCAAAA
*Seq*20E5	55 °C	F: GATGCGGTTTGGGAATTG R: GCAAGCATTCTGACAATGAAC
*Seq*37G5	60 °C	F: GAATTCTCGCACGGACTTTC R: CATGTGACCATGTACCGCTT
*Seq*18D7-3	55 °C	F: GCAAAAAGGGAATTGTAATTGGGTTCA R: CCCTAGGTCTAGGCTACGCGACTTG
*Seq*21E5	57 °C	F: GCTACTCGAGCTCCATTGGTT R: TTCGGTGTTCAGCTTTGAGC

Forward primers were fluorescently labeled with 6-FAM for capillary electrophoresis data acquisition. All primers were HPLC purified and synthesized by Applied Biosystems. Total PCR volume was 5 uL containing 1 uL 0.25X gDNA

template diluted in HPLC grade water, 0.5 uL 10X AmpliTaq Gold PCR Buffer II (Applied Biosystems, Foster City, California), 0.3 uL 25 mM $MgCl_2$, 0.1uL 10 mM each dNTP (dATP, dCTP, dGTP, dUTP), 0.25 uL each of 10 uM forward and reverse primers, 0.5 uL 10% polyvinylpyrrolidone (PVP-40) (w/v), 0.5 uL 1% BSA (w/v), and 0.05 uL 5 U/uL AmpliTaq Gold DNA polymerase (Applied Biosystems). The reaction was brought to 5 uL with HPLC grade sterile water.

The stock solutions of PVP-40 and bovine serum albumin (BSA) were made up in bulk prior to PCR and stored at 4 °C. Freezing either of these stock solutions impaired PCR unless they were completely redissolved for several hours at ambient temperatures.

Aliquoting and PCR setup was performed with a BiomekFX fitted with a 96-tip pod. A master mix without primers was created in bulk using common manual tools (Fig. 3). Five different PCR singleplex master mixes were created for each of the loci (Fig. 4). Each respective master mix was aliquoted to the reaction plates and spun down to remove air bubbles. The template DNA was subsequently added and then quick spun (1000 rpm, 30 s) to remove air bubbles prior to thermal cycling. Amplification was carried out in 384-well optical reaction plates sealed with optical adhesive covers (Applied Biosystems) and run on multiple calibrated dual 384-well aluminum block 9700 thermal cyclers (Applied Biosystems) with compression pads (Applied Biosystems) (Fig. 5). All thermal cyclers were brought to within 5 °C of operating temperature prior to placement of the reaction plates and beginning of the thermal cycling method.

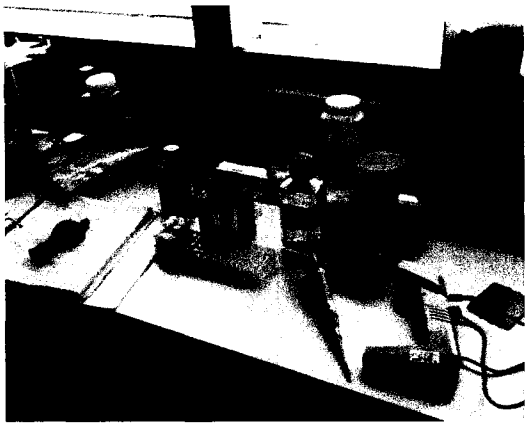

Figure 3. Typical bench tools required for manual setup of a high throughput robotic method: multichannel pipettor, tube racks, single channel pipettors, high volume pipettes, and pipette bulb.

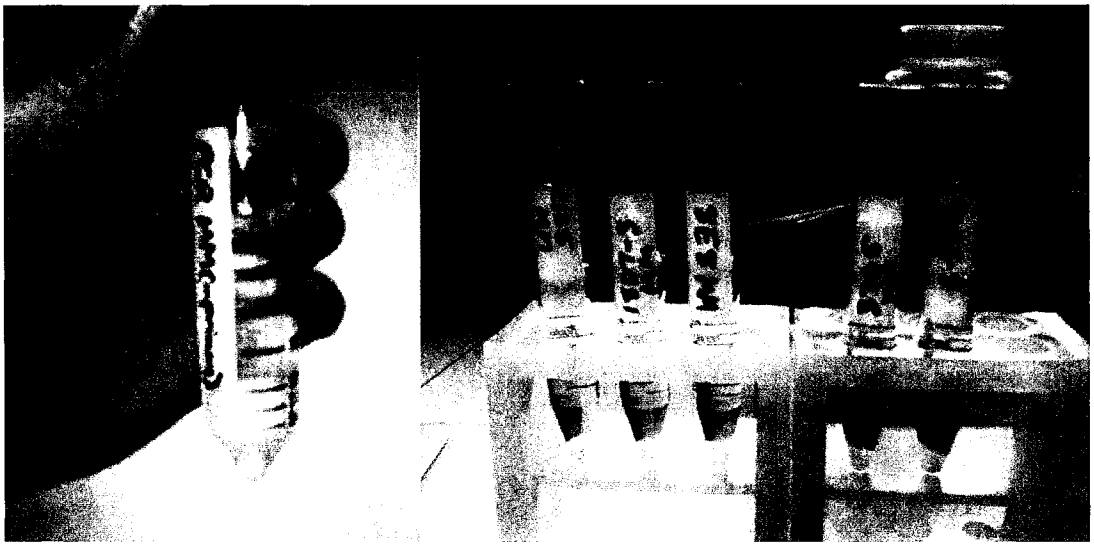

Figure 4. Volumes of PCR master mix used both with primers (15 mL Falcon tubes) and without (50 mL Falcon tube). The 50 mL volume was aliquoted then spiked with appropriate primers for automated PCR setup.

Figure 5. Bank of nine Applied Biosystems 9700 Thermal cyclers with dual 384-well aluminum head (left). Detail of a thermal cycler with two 384-well plates loaded (right). All instruments were calibrated within the past six months to ensure reproducibility.

Analysis by capillary electrophoresis

Sample set up for capillary electrophoresis (CE) was performed on a BiomekFX (Fig. 6). Data were collected with a 36 cm 96-capillary 3730xl Genetic Analyzer (Applied Biosystems) (Fig. 7). All samples were prepared in 384-well optical reaction plates (Applied Biosystems). The singleplex reactions were diluted 1:9 in HPLC grade sterile water, mixed and spun down, then diluted again 1:4 in Sample Loading Reagent (Applied Biosystems) spiked with GS-500LIZ Size Standard (Applied Biosystems) for injection on the CE device. Total CE sample volume was 10 uL containing 0.02 uL 1X PCR product, 0.04 uL GS500-LIZ Size Standard. The sample volume was brought up to 10 uL with Sample Loading Reagent (Applied Biosystems).

Plates were spun down and sealed with septa covers (Applied Biosystems) prior to injection. Injection parameters were defined by the GeneMapper36_POP7 run module provided by Applied Biosystems for fragment analysis on the 3730xl.

Figure 5. Bank of nine Applied Biosystems 9700 Thermal cyclers with dual 384-well aluminum head (left). Detail of a thermal cycler with two 384-well plates loaded (right). All instruments were calibrated within the past six months to ensure reproducibility.

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Plates were spun down and sealed with septa covers (Applied Biosystems) prior to injection. Injection parameters were defined by the GeneMapper36_POP7 run module provided by Applied Biosystems for fragment analysis on the 3730xl.

Automated fragment analysis was performed with GeneMapper v3.7 (Applied Biosystems). Default autobinning was used. Four bases were added to the default size standard to correlate with previously verified gel optimizations and the sequence-verified sizes referenced by Bruno (2002). The 35 bp and 250 bp markers were excluded from the size standard as suggested by Applied Biosystems for this application.

Figure 6. BiomekFX (left) used for both PCR and CE setup and detail of robot in action (right). This instrument is equipped with both a 96-channel removable tip head (left side) and 8-channel fixed tip head (right side). The fixed tips were not used due to unreliable volume dispense and possible system liquid contamination. Total time to set up 510 samples for 5 uL PCR at five loci was 30 min.

RESULTS

A total of 510 trees were screened at five different microsatellite loci, one of which was a chloroplast marker. Given that *S. sempervirens* has a hexaploid genome, statistical analysis of allele frequencies was not possible for the non-chloroplast markers.

Chloroplast marker

The chloroplast marker, 21E5-7, proved to be of limited polymorphism with 68 - 88% of the trees at each site having the 112 bp allele (Table 3). Of all 510 trees tested 74% had the 112 bp allele. The next most abundant allele was at 104 bp with a frequency of 6 – 29% across the sites. Only site 10 had any noticeable frequency increase in a different fragment size, 120 bp, with a frequency of 15% that is equivalent to the 104 bp frequency for this site.

Table 3. Allele frequencies for marker 21E5-7 sorted by site.

21E5-7 allele (bp)	Site 1 n=40	Site 2 n=40	Site 3 n=60	Site 4 n=80	Site 5 n=40	Site 6 n=40	Site 7 n=60	Site 8 n=90	Site 9 n=40	Site 10 n=40
96	-	-	-	-	-	-	-	0.01	-	-
100	-	0.03	-	-	0.03	-	-	-	-	-
104	0.16	0.06	0.18	0.25	0.23	0.14	0.25	0.29	0.25	0.15
108	0.03	-	-	-	-	-	-	-	-	-
112	0.75	0.88	0.78	0.69	0.71	0.83	0.72	0.68	0.75	0.65
116	-	-	0.04	0.04	-	-	-	-	-	0.06
120	0.06	-	-	0.02	0.03	-	0.02	0.01	-	0.15
124	-	-	-	-	-	-	-	-	-	-
128	-	0.03	-	-	-	-	-	-	-	-
140	-	-	-	-	-	0.03	-	-	-	-
144	-	-	-	-	-	-	0.02	-	-	-

Genomic markers

Given the limitation of statistical analysis on hexaploids due to the presence of null alleles, a count of allele instances, instead of frequencies, was performed on the genomic markers. Only one tree had five different alleles at one locus (tree 02NL07, marker 37G5 – 112, 114, 120, 124, 130 bp). No occurrence of six instances for any marker was recorded despite the hexaploid genome. The total number of instances of a given fragment length (allele) was tabulated and sorted by site.

Anywhere from one to four instances per locus were observed per sample for any given marker (Tables 4-7). Marker 20E5-8 (Table 4) showed a bimodal distribution focused on 156 bp and 194 bp. Marker 8E8 (Table 5) is highly polymorphic and shows a broad distribution of alleles starting at 116 bp. Marker 18D7-3 (Table 6), also highly polymorphic, showed the majority of its fragments at 141 bp and 144 bp. Marker 37G-5 (Table 7) showed amplification primarily at 120 – 128 bp, but with no or limited amplification at sites 4, 9, and 10.

Table 4. Allele instances for marker 20E5-8 sorted by site.

20E5-8 allele (bp)	Site 1 n=40	Site 2 n=40	Site 3 n=60	Site 4 n=80	Site 5 n=40	Site 6 n=40	Site 7 n=60	Site 8 n=90	Site 9 n=40	Site 10 n=40
104	-	-	1	-	-	-	-	1	-	1
148	-	-	-	-	-	-	1	-	-	-
152	2	1	1	1	3	-	2	8	4	4
154	5	2	9	3	4	2	3	13	12	1
156	6	20	22	16	14	14	18	15	5	9
190	-	1	-	-	-	-	1	-	-	1
192	4	2	9	7	-	5	2	8	4	1
194	19	27	33	34	26	21	35	42	19	22
196	-	2	5	-	1	1	2	2	-	1
200	3	2	3	1	1	-	6	3	4	1
300	-	-	-	-	-	-	-	1	-	-
392	-	1	-	-	-	-	1	-	-	-

Table 5. Allele instances for marker 8E8 sorted by site.

8E8 allele (bp)	Site 1 n=40	Site 2 n=40	Site 3 n=60	Site 4 n=80	Site 5 n=40	Site 6 n=40	Site 7 n=60	Site 8 n=90	Site 9 n=40	Site 10 n=40
104	-	-	-	-	-	-	1	-	-	-
116	7	3	11	11	7	5	14	18	6	6
118	-	4	7	11	-	4	11	10	7	8
120	6	7	21	7	10	16	12	19	7	7
122	6	4	4	6	3	2	6	10	1	2
124	-	1	-	-	1	-	-	-	-	1
126	-	-	4	2	3	-	1	-	-	1
128	-	-	-	1	-	-	1	-	-	-
130	2	1	3	7	3	-	2	2	-	3
132	3	1	-	3	2	1	2	2	2	3
134	-	3	4	6	1	2	3	2	1	3
136	1	-	-	1	2	8	1	7	-	1
138	1	2	3	4	1	9	4	1	-	1
140	1	-	-	5	2	1	8	3	2	-
142	-	-	-	1	-	-	-	1	-	-
144	-	6	-	-	-	-	-	1	-	-
146	-	-	-	1	-	-	-	-	-	1
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170	-	-	-	-	-	-	-	1	-	-
172	6	-	2	-	-	-	2	-	-	-
174	2	-	-	-	-	-	-	1	1	-
176	2	-	4	2	1	2	-	1	-	-
178	1	1	1	2	-	1	-	2	-	-
180	-	-	-	-	-	-	1	2	-	-
182	1	1	-	3	-	-	-	-	-	-
184	-	-	2	-	-	-	-	-	-	-
276	-	-	-	1	-	-	-	-	-	-

Table 6. Allele instances for marker 18D7-3 sorted by site.

18D7-3 allele (bp)	Site 1 n=40	Site 2 n=40	Site 3 n=60	Site 4 n=80	Site 5 n=40	Site 6 n=40	Site 7 n=60	Site 8 n=90	Site 9 n=40	Site 10 n=40
90	1	-	2	2	1	2	-	2	-	-
93	-	-	-	-	-	-	-	-	-	-
96	-	-	1	-	1	-	-	1	1	-
117	-	-	-	-	-	-	-	-	1	-
123	1	-	-	-	1	-	1	-	-	1
126	3	-	2	2	-	-	3	5	2	-
129	-	-	-	-	-	-	-	-	-	-
132	5	1	-	5	2	1	1	-	1	2
135	-	-	-	-	1	-	-	-	1	1
138	6	11	15	14	8	7	13	16	8	6
141	14	14	-	17	13	6	30	26	11	18
144	6	2	-	13	13	14	7	17	4	7
147	-	-	-	1	2	-	1	2	-	-
150	3	8	8	-	3	3	4	8	3	7
153	5	13	3	5	5	2	5	8	3	3
156	6	4	4	2	-	1	4	8	1	4
159	1	1	5	8	1	-	2	5	3	4
162	1	2	-	4	-	-	2	1	-	-
165	-	2	-	-	1	-	1	1	-	-
168	-	4	-	1	-	-	-	-	-	-
171	-	-	-	-	-	-	2	-	-	-
174	-	-	1	1	-	-	-	-	-	-
177	-	-	1	-	-	-	-	-	-	-
180	1	-	4	-	-	-	-	-	-	-
657	-	-	-	-	1	-	-	-	-	-

Table 7. Allele instances for marker 37G5 sorted by site.

37G5 allele (bp)	Site 1 n=40	Site 2 n=40	Site 3 n=60	Site 4 n=80	Site 5 n=40	Site 6 n=40	Site 7 n=60	Site 8 n=90	Site 9 n=40	Site 10 n=40
104	-	-	-	1	-	-	-	-	-	-
106	-	-	1	-	-	-	-	-	-	-
108	-	-	1	-	-	-	-	-	-	-
110	-	1	-	-	-	-	-	-	-	-
112	-	1	-	-	-	-	-	-	-	-
114	-	1	-	-	-	-	-	-	-	-
118	4	-	1	-	1	-	1	3	-	-
120	14	9	3	-	15	22	22	2	-	-
122	9	19	1	1	11	4	22	5	-	-
124	14	8	2	-	9	12	16	5	-	-
126	1	3	2	-	1	1	-	-	-	-
128	3	5	-	-	-	-	-	-	-	-
130	-	8	-	-	-	-	-	-	-	-
136	-	-	-	-	-	-	-	1	-	-
160	-	-	-	-	-	-	-	1	-	-
484	-	1	-	-	-	-	-	-	-	-
612	-	1	-	-	-	-	-	-	-	-

Sampling was designed to avoid clones, however some clones were inevitably collected (Table 8). A few potential clones were found immediately neighboring (e.g., 02NR08 and 02NR09). Other potential clones were found in the same site, but at greater distances (e.g., 08EL07 and 08NR03). Finally, cross-site analysis detected trees with the same genotypes (e.g., 09SR08 and 10ER04), but at such a distance the trees may not be clonal.

Table 8. Trees with identical genotypes at all five loci. Bold indicates potential clones at different sites. Italics indicates likely clones immediately neighboring. Normal text indicates likely clones found in the same site, but not immediately neighboring. Sampling techniques were designed to minimize the amount of clonal sampling.

1) *02NR08, 02NR09*
2) **04NR07, 04WR10, 09SR10**
3) **04NR03, 04SR10, 08NWL06**
4) *08NR06, 08NR07*
5) 08EL07, 08NR03
6) *10WR07, 10WR08*
7) **09SR08, 10ER04**
8) 10EL02, 10WR06
9) 03SL03, 03NR03
10) **06ER05, 07SR07**
11) 06EL05, 06EL02
12) *10WR10, 10WR09*
13) *03SR09, 03SR10*

Amplification trends in 37G5

The only marker that showed a geographical trend within the study area was marker 37G5. This was observed by instances of amplification when compared to instances of non-amplification (nulls) across sites. When correlated, a gradient was discovered separating northern and southern sites by percent amplification ranging from 0% to 75% (Figs. 8, 9, 10). Previous studies (Bruno and Brinegar, 2004; Brinegar et al., 2006) have shown 0% to 90% amplification at this locus.

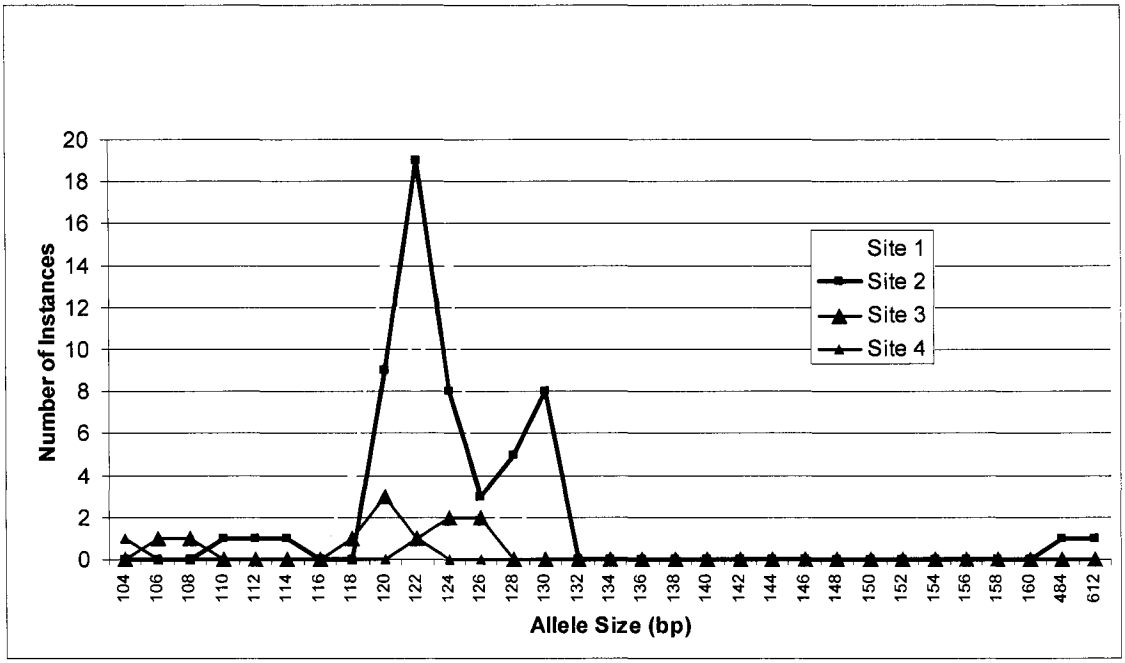

Figure 8. Allele sizes of marker 37G5 versus number of instances by site (1-4). Note the lack of amplification for sites 3 and 4 (n=120) with respect to sites 1 and 2 (n=80).

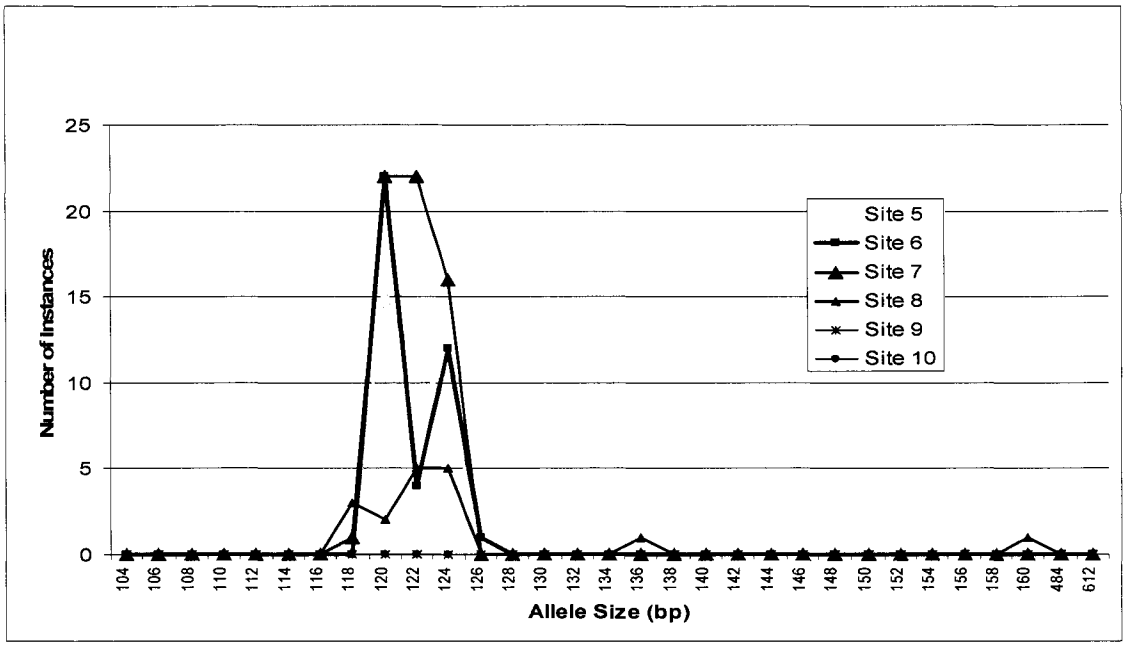

Figure 9. Allele sizes of marker 37G5 versus number of instances by site (5-10). Note the lack of amplification for sites 8-10 (n=170) with respect to sites 5-7 (n=140).

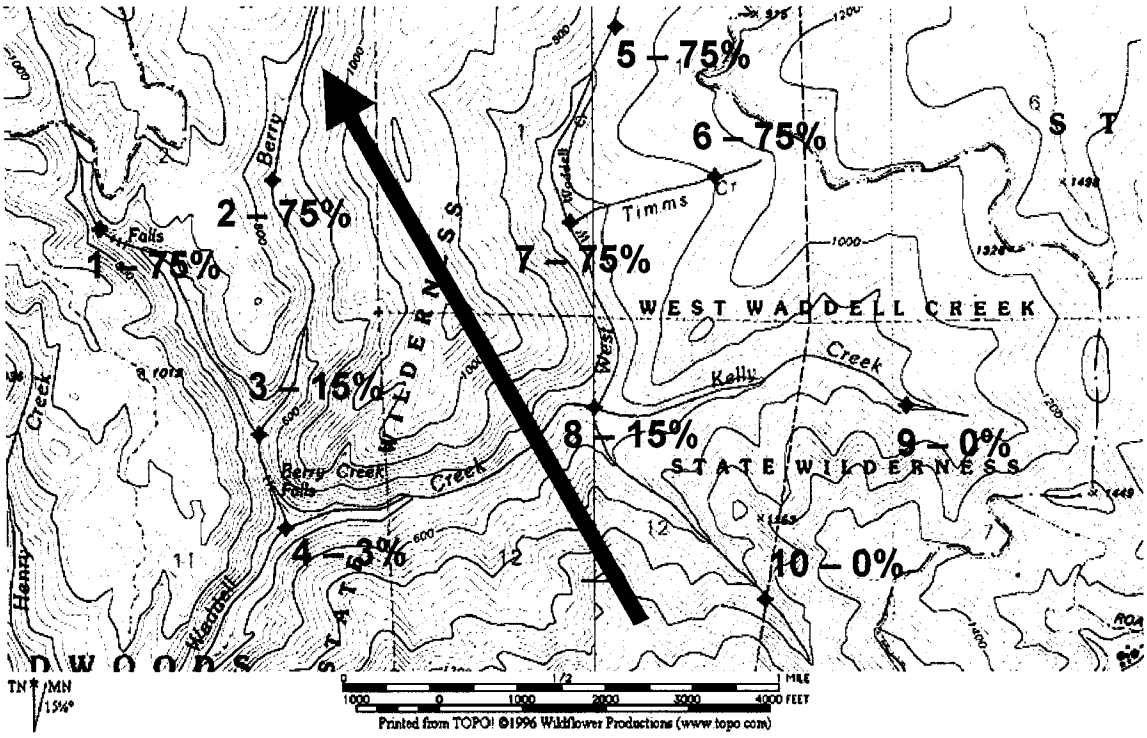

Figure 10. Percent amplification of marker 37G5 as a gradient across the study area. Sites 1, 2, 5, 6, and 7 had 75% amplification; sites 3 and 8 had 15% amplification; sites 4, 9, and 10 had 3% or less amplification of marker 37G5 as noted on the map.

DISCUSSION

Comparison to other population studies

This study represents a first attempt at elucidating the relationship of the study site population to previously characterized populations. A study of individuals in a southern second growth stand does not show a gradient (Bruno and Brinegar, 2004). However another small population study covering old growth northern stands (Brinegar et al., 2006) showed a greater overall diversity than demonstrated in this study. How this data correlates to the current study is uncertain without a direct study of an old growth southern population.

Allele fragment sizes differ when the allele sizes of this study are compared to those reported in the smaller (n=25) Bruno and Brinegar (2004) study in the second growth San Lorenzo River Redwoods (15 miles away). This could be attributed to the smaller sample size in the first study. The chloroplast marker, 21E5-7 (Table 3), showed a range of 96-144 bp with the large majority of all samples being either 104 or 112 bp. This shows a larger range of alleles than the 104-128 bp previously reported. Allele 20E5-8 (Table 4) showed a different fragment range of 104 to 200 bp with a bimodal distribution at 152-156 bp and 190-200 bp with two outlier sizes of 300 and 392 bp. In comparison, Bruno and Brinegar (2004) reported a limited range between 190 and 198 bp. The 8E8 (Table 5) allele fragments also fell within a 116-184 bp size range with two outliers at 104 and 276 bp that appear to be artifacts. The trinucleotide repeat motif, 18D7-3 (Table 6), showed a range of alleles from 90-180 bp with an

outlier at 657 in this study. This marker shows a much higher polymorphism than the 126-159 bp range previously reported. The unique 37G5 (Table 7) marker had a larger range with many small fragments from 104 to 114 bp, but the bulk of the amplified fragments occurred between 118-136 bp with outliers at 160, 484, and 612 bp. Bruno and Brinegar's 37G5 alleles were sized 118-124 bp.

In the Brinegar et al. (2006) study, some of the southern range second growth 21E5-7 marker data from this thesis and the Bruno and Brinegar (2004) study were compared to data from northern old growth forests. Only four alleles were found in each of the second growth forests of Big Basin Redwoods State Park (n=77) and the San Lorenzo River Redwoods (n=40), whereas eight and 11 alleles, respectively, were found in the old growth forests of Prairie Creek Redwoods State Park (n=39) and Humboldt Redwoods State Park (n=38). The authors attributed the apparent decreased allelic diversity in the southern second growth forests to several potential factors: loss of alleles due to random genetic drift, less seedling establishment (i.e., less sexual reproduction) in the drier southern region (Viers, 1996), or loss of genotypes from old trees (over 400 years old) which do not sprout as robustly as the younger ones after clearcutting (Powers and Wiant, 1970).

In contrast to the preliminary Big Basin study (n=77; 4 alleles), the final data from this thesis show that a much larger sample size (n=510) detects 10 alleles in this second growth population a number more in line with the northern old growth populations. However, the "extra" six alleles found in the larger sampling all have frequencies much lower than those same alleles in the northern populations.

Additionally, recent data (C. Brinegar, personal communication) indicate that there is no significant difference in genetic diversity at the 21E5-7 locus in adjacent second growth vs old growth populations in Prairie Creek Redwoods State Park, putting into question the hypothesis that old growth forests are more diverse than second growth forests.

Taken together, these results imply that the southern populations of redwoods have extremely low frequencies of all but the 104 and 112 bp alleles compared to the northern populations. Whether these genotypes were once more plentiful in the south and have been become less abundant through genetic drift, or were never well established due to some unknown selection process, has yet to be investigated.

Homogeneity of study site

Genomic and chloroplast markers

This study indicates that the genetic structure of the study site is likely homogeneous. There is no significant difference between the various sites within the study area with respect to the genomic and chloroplast markers.

The gradient of 37G5 amplification does not definitively suggest homo- or heterogeneity. The study site, being limited to a second growth southern *S. sempervirens* population in a 6-square mile region, may not be large enough to definitively say whether or not a border between two 37G5 populations has been found or even if the observed 37G5 gradient is accurately representing the populations as described below.

More importantly, given the characteristics of microsatellites, the existence of null alleles may either be a result of a mutation at the primer site or a total deletion of the marker. The only way to fully characterize the 37G5 locus in the given population is though sequencing the region of interest.

Identified clones

The collection methodology was well suited for avoiding the sampling of clones. As referenced in Table 8, the list of identified potential clones is a short one. There were only five likely clones and four possible clones collected at the same sites. This suggests clones do not easily propagate over long distances, e.g. site to site, given the extensive testing in the study site.

Polyploid structure

In only one tree (out of 510), and only at one nuclear locus (out of four), was there more than four alleles present. This observation strongly supports the idea that *S. sempervirens* is an autoallohexaploid with an AAAABB genome (Saylor and Simmons, 1970) and that the nuclear microsatellite loci characterized by Bruno (2002) are present in the AAAA part of the genome.

It would be of interest to develop microsatellites for *Metasequoia* and thus test the hypothesized ancestral hybridization event to determine definitively the origin of the *S. sempervirens* genome's donor BB portion. This also assumes a limited deviation of the contemporary BB portion of *S. sempervirens* from *Metasequoia* genome versus the ancient original BB donor genome. These markers could then be used cross species in this regard and for further conservation studies in both *S. sempervirens* and *Metasequoia*.

High throughput methodology

The benefits of the high throughput methodology are clear: increased throughput will provide more data and the potential of a faster understanding of a given population. However, this desire for an increased throughput must be justified by not only budgetary concerns, but also the skill set of the principle investigator.

The set up costs of a fully automated laboratory can easily cost $1 000 000. The latest 384-well compatible technology generally begins in the $200 000 to $300 000 range. The same applies to industry standard CE (capillary electrophoresis) devices.

Consumables, reagents, buffers, and dyes are generally proprietary and are also priced in line with the instruments for which they are compatible. Beyond the main instrumentation, one will find the number of thermal cylcers, wash stations, ancillary pipettors, laboratory and office space, site preparations and even power requirements required to supply high throughput instrumentation staggering. All of the aspects add to the overall cost of setting up a laboratory. It is not a simple undertaking in setting up something equivalent to a small-scale genome center. The required knowledge and skills of a single individual will easily be pushed to the limits – this type of undertaking requires an experienced team.

After setting up the laboratory to a fully functioning and optimized state, there is the question of providing the number of samples required to keep the instrumentation running at full capacity. Sample collection is highly manual and is highly dependent on weather, terrain, permitting, and GPS signal strength. Typically, 75-150 samples can be collected per day. With a 5000-sample per day capacity, a 96-capillary CE device would require 264-528 man-hours per day of sample collection. If running 365 days a year, then 1 825 000 individuals could theoretically be fully genotyped at 16 loci. Currently, with only five known loci for *S. sempervirens*, genotyping of the entire redwood forest begins to sound like an attainable goal with a single 3730xl.

Manual multichannel option

A more feasible method commonly available to the laboratory with a more restrained budget would be the manual multichannel option. By choosing a set of

multichannel pipettors over a liquid handling robot, significant upfront costs and ongoing maintenance and proprietary consumables costs are avoided.

The general methods used in an automated method would still be used since manipulation of individual sample vessels is not easily accomplished with multichannel pipettors. Generally, given the small well size of the 384-well plate, a 96 well plate is suggested. By using a multichannel pipettor, compatibility with a multicapillary electrophoresis device is retained. Alternatively, the samples can be run on a wide format gel.

Capillary versus gel electrophoresis

When considering which platform to analyze samples with, price, throughput, and sensitivity are the considerations that have the largest effect on results. Each platform does have its benefits and detractions.

The common availability and low cost of a gel electrophoresis unit makes this method very attractive to the majority of researchers. The flexibility of the unit allows the user to choose how many lanes to run, samples to load per well, and to manipulate the physical gel if needed. The drawbacks are the lack of viable choices of single base pair resolving gels on the market, run time of 2-3 h, staining time, required imaging equipment, and lack of sensitivity regardless of dye used. Gels are insensitive, sources of hazardous waste, and labor intensive.

Conversely, capillary electrophoresis is expensive, not quite commonplace, yet extremely fast, clean, and sensitive. What would normally take 10 uL of PCR product to get a potentially adequate signal via gel-based analysis can be done with 0.02 uL

product. Even with such a small amount of product, the robust 21E5-7 marker was verging on signal saturation. Capillary electrophoresis was able to detect more data from the weaker amplifying samples. This increased sensitivity allows the researcher two benefits: decreasing reagent costs and discovering previously undetectable data points.

Although a 96-capillary 3730xl was used for detection, another platform may be better suited for this type of research. Applied Biosystems offers another product, the 4- or 16- capillary 3130 Genetic Analyzer. This newly released platform is even more sensitive than the 3730 series and allows the principal investigator to further optimize the upstream reactions thus reducing reagent costs even further. An added benefit is the possibility of running both a sequencing and fragment analysis reaction on the same instrument at the same time without any physical changes to the instrument – only software settings need be modified. This does not apply to the predecessor 3100 or even the current 3730 platforms.

Analysis of gel and capillary electrophoresis data

Some additional drawbacks of a gel apparatus involve the data collection itself. Manual scoring of gels is not uncommon and introduces subjective errors. Gel images are either printed out or stored as image files. Some software is available for identifying bands on a gel image and converting this to a numerical value. This step becomes time consuming and problematic, especially when considering stutter associated with dinucleotide repeats and the high number of alleles in *S. sempervirens*. Three of five markers are dinucleotide repeats, thus quite difficult to analyze even with a well-trained

eye. The only positive aspects of this approach would be cost outlay and that those less comfortable with technology would feel more comfortable working with data requiring minimal computer use. However, the manual entry of data points into the spreadsheet or statistics package is prone to error.

The method of analyzing the data acquired by CE is generally automated. When using fragment analysis software, specifically GeneMapper v3.7 and above, some human intervention is required in the initial phases of data analysis that enable later steps to be essentially fully automated. The software algorithm is based on identifying peak heights relative to size of a given fragment. User defined fragment bins, or migration distances, are correlated with the relative sizes as determined with the size standard. The software then compares the peaks collected with the calculated standard size. Overlapping peaks, stutter, A-addition, capillary crosstalk, and many other common raw data problems affecting data analysis are automatically and reliably corrected. Once a study's bins, panels, and markers are defined, analysis essentially becomes a matter of clicking the "analyze" button.

Care must be taken, however, to realize fragment mobility of any kind varies platform to platform. What this means is if a study is begun on one platform – e.g. a different gel or CE instrument or even the switching of a capillary or run polymer in the same instrument – it should be finished on the same instrument with the same settings if the researcher is not going to be cross checking actual versus relative fragment sizes. If one will be switching to a new platform, as was the case in this study, one can correlate the data of a well-characterized group of individuals on multiple platforms. The

observed mobility difference can then be used to adjust the new data points to the old data points. This is a simple matter of redefining the size standard fragment lengths in the analysis software.

In this study, 2550 CE runs were analyzed for their respective peak heights. In all, once optimized, the extraction of data points for each individual tree took approximately 3-4 h unattended. These data were then exported to a statistics package of choice as a comma delimited file for further manipulation as would be the case in the manual gel method. Manual scoring of gels and the manual, error prone data entry associated with gel data were avoided.

Suggested improvements: a compromise approach

Some improvements for the method used can be made in various aspects from the PCR set up through the data acquisition. Clearly, a decrease of throughput is needed to fit with available sample collection resources, but some other options for decreasing the overall cost of data acquisition can be achieved through various means. One positive aspect, in the opinion of most field biologists, is the decreased amount of time needed in the laboratory and the increased time required in sample collection.

No robotics of any kind is needed for *S. sempervirens* microsatellite analysis. A simple 8- or 12- channel pipettor would serve all the fluid handling needs from DNA extraction through CE setup. This would significantly minimize the dead volume associated with robotics platforms while increasing throughput to a reasonable speed.

Multiplexing, though extremely difficult to optimize, is an option for increasing throughput. Only three of the primer sets used in this study may have the potential to be

multiplexed based solely on their annealing temperatures. Perhaps some primer redesign or some other modification is in order. It is worth the effort to multiplex since it will decrease PCR reagent costs and reaction times, but is commonly, and rightly so, moved to the bottom of the list in optimizing throughput.

Multiplexed dye labeling is another method of increasing throughput and reducing costs that was not addressed in this study. It is an excellent method to decrease the number of CE runs required for any given individual. On current Applied Biosystems CE devices, the instrument is capable of collecting data for up to five dyes. One dye is required for the size standard (i.e., orange, as in this study), but the remaining four could be used for microsatellite markers. In this study, a good approach would be to have genomic 20E5 and chloroplast 21E5-7 markers labeled with the same color (i.e., blue, as in this study) given their fragment size difference, while the remaining three loci would one of three remaining colors (i.e., red, yellow, green). The dyes chosen would have to be compatible with the instrument chosen. What multiple dye labeling allows the researcher to do is to pool aliquots of any given singleplex PCR reaction into a multiplexed CE run. The researcher can then optimize the peak heights of a given locus by aliquoting more or less of the respective PCR product to bring the signal to the desired level. In this study, for example, all loci could have been run at the same time decreasing CE costs and runtime by 80%.

If a researcher has GeneMapper v3.7 or above, a good option available is to purchase the services of a core facility that has a 4-or 16-capillary 3130. The major expense of the instrument and its upkeep could be avoided. What this allows is

sensitive data collection with the benefits of reliable automated analysis not afforded by gel electrophoresis. The 2-3 h running time per gel, extended staining, imaging, and manual scoring is also completely sidestepped. The only drawback is the ongoing costs of purchasing the services, often charged on a per run basis, which can be mitigated by pooling PCR products and significantly decreasing upstream PCR reaction volume. There are other benefits associated with the time saved, but that would be up to the individual to decide how to spend it.

Depending on which commonplace tools the researcher has, the costs from the single test tube and gel method to this more sensitive approach are not drastic. The major equipment and reagents needed would be a 96-well compatible centrifuge and thermal cycler, a multichannel pipettor, dye labeled primers, and a service for running samples. The researcher could spend several weeks in the field on the front end with an equivalent time measured in days – not weeks – in the lab preparing the samples for analysis on the back end. By minimizing lab time, high throughput tools allow the biologist more time in the field while increasing the quality and amount of data at a reasonable cost.

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