Clonal Diversity, Patterns, and Structure in Old Coast Redwood Forests

by

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Abstract

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Coast redwood (*Sequoia sempervirens* (D. Don) Endl.) is a valuable endemic and important source of timber and carbon storage in California. This species is unique among conifers in its ability to reproduce clonally through prolific sprouting. Due to extensive timber harvesting, only ~5% of the native range of old growth redwood forest remains uncut. Knowledge about clonal diversity and patterns throughout the range of coast redwood may allow us to better understand the reproductive ecology of this species and identify populations that may be at risk due to low genetic or genotypic diversity. In this dissertation, clonal diversity and patterns are described in two paired one-hectare plots at each of three old-growth redwood forests located at different latitudes within the native the range of coast redwood. The impacts of clonal reproduction on spatial patterns in old redwood forests are also explored. High levels of genetic and genotypic diversity were present at all three study sites. Clonal diversity and structure did not seem due vary by geographic location. Instead, variation between study plots may have been due to a combination of local environmental factors and disturbance history. Clones were spatially aggregated at all study plots, and clonal reproduction generally led to significant spatial clustering at scales less than 10 m. The finding of high genotypic diversity suggests that despite prolific sprouting, sexual recruitment still plays an important role in the reproductive ecology of coast redwood. In order to emulate old forest reference conditions, second growth forests should be managed to maintain high levels of clonal diversity. Additionally, forest managers seeking to restore old forest characteristics should plant and thin to create spatial patterns that mix single seed-origin trees and clonal clusters.

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Chapter 1: Introduction

1.1 Background

Coast redwood (*Sequoia sempervirens* (D. Don) Endl.) is the tallest tree species in the world, and is a charismatic endemic in northern California (Noss 2000). It is also an important source of timber production and carbon storage in its native range and elsewhere where it has been introduced. It is unique among conifers in that vegetative reproduction is common, resulting in natural patterns of clonal reproduction. Due to the extensive logging that started during the California gold rush, only about five percent of the pre-European range of coast redwood remains uncut (U.S. Fish and Wildlife Service 1997). Understanding the reproductive ecology and patterns of genetic diversity in coast redwood may help to identify potential threats to its persistence in a changing environment. Additionally, given that so much of coast redwood's current distribution is made up of second-growth forests, there is a great deal of interest in determining the most effective ways to accelerate restoration of cut stands to old forest conditions. This dissertation seeks to inform the conservation and restoration of coast redwood forests by describing patterns of genotypic and genetic diversity in old redwood forests and determining the impact of clonal reproduction on spatial patterns of coast redwood trees in old forests.

Given that clonal sprouting plays an important role in the reproductive ecology of coast redwood, it is important to understand how clonal and genetic diversity varies throughout the range of coast redwood. While clonal reproduction benefits plants by allowing them to share resources (Jóhnsdóttir and Watson 1997), reduce their reliance on seeds for reproduction (Bond and Midgley 2001), and produce offspring quickly after disturbance (Peterson and Jones 1997), it may have negative consequences as well. Heavy reliance on clonal reproduction may reduce the production of new allelic and gene combinations in clonal stands, making it more difficult for clonal communities to adapt to rapidly changing environments (Eckert 2002). In an extreme case, reliance on clonal reproduction could lead to the gradual accumulation of deleterious mutations and increase a tree population's susceptibility to the negative effects of inbreeding depression and genetic drift. Thus, knowledge about variation in clonal reproduction and genetic diversity throughout the range of this species is crucial for identifying populations of this species that may experience declines due to climate change.

Studying clonal patterns in coast redwood forests can also provide information that is critical for the restoration of second-growth coast redwood forests and management of commercial timber plantations. One aspect of old forest condition that can be controlled through management is the spatial arrangement of trees on a landscape (Pommerening 2002, Larson and Churchill 2012, Churchill et al 2013). In coast redwood, clonal reproduction may cause trees to be spatially aggregated. Quantifying spatial patterns and clonal patterns in old coast redwood forests can provide reference conditions for forest restoration and management. Additionally, studying the relationship between spatial and clonal patterns helps us understand the underlying processes generating the spatial distribution of coast redwood trees.

1.2 Components of the Dissertation

Chapter 2 describes the development and testing of a genotyping protocol for coast redwood using microsatellite markers. Genotyping coast redwoods using microsatellites presented a significant challenge for two reasons. First, coast redwood is hexaploid, having three copies of each pair of homologous chromosomes. Hexaploidy complicates the identification of alleles in microsatellite scans and the calculation of the probability of an identical genotype occurring in two sexually derived individuals. Second, the immense size of coast redwood makes it difficult to obtain foliage samples for many trees. To genotype coast redwoods with inaccessible foliage, we developed a protocol for extracting DNA from cambium/sapwood tissue and tested it to ensure that results from cambium/sapwood matched results from foliage samples. A combination of laboratory and simulation tests was used to validate our genotyping protocol.

Chapter 3 compares clonal diversity and structure, genetic diversity, and spatial genetic structure between study plots sampled in different regions of the range. Tests for population structure were also conducted to determine how genetic variation in coast redwoods is partitioned between individuals, study plots, and geographic locations. Results are discussed in the context of both previous work looking at clonal diversity and structure and range-wide genetic variation in coast redwood.

Chapter 4 describes clonal patterns and spatial patterns of coast redwood trees in old forests (i.e. old growth forests, (O'Hara et al. 1996)). This chapter also looks at the impact of clonality on the spatial distribution of coast redwood trees in old forests. In addition to traditional spatial statistics, a continuum percolation algorithm is used to investigate scales of spatial clustering and to identify the sizes of clonal clusters in different study plots. Recommendations for thinning and planting in second-growth stands are made based on the results of this chapter.

Chapter 2: Genotyping Protocol for Coast Redwood

2.1 Introduction

Coast redwood (*Sequoia sempervirens* (D. Don) Endl.) is an iconic species and important source of timber production and carbon storage in northern California. It is also one of few conifer species able to produce basal sprouts as a form of natural clonal reproduction. Redwood trees commonly regenerate from cut stumps, fallen logs, or roots (Neal, 1967; Del Tredici, 1998). This vegetative reproduction may lead to the dominance of a small number of clones over a large area and the long-term persistence of genotypes. In the case of redwoods, which are extremely long-lived as individual stems, clonal reproduction could theoretically lead to the persistence of single genotypes for tens of thousands of years. Given the role of coast redwood as a valuable endemic and timber species, surprisingly little is known about the extent of clonal reproduction and patterns of genotypic diversity throughout its range.

Previous studies of clonal patterns in old growth (Rogers, 2000; Rogers and Westfall 2007) and second-growth (Douhovnikoff et al., 2004) coast redwood forests using allozyme markers and amplified fragment length polymorphisms, respectively, found that multiple genotypes were often intermingled, and that members of the same clone could be found up to 340 meters apart. Due to the challenge of collecting foliage from the canopy of dominant redwood trees, no study to date has been able to comprehensively sample all trees in a forest area. Microsatellite markers may facilitate genetic studies of trees where high-quality foliar tissue is not available because their use requires relatively low concentrations of template DNA. Additionally, microsatellites are generally species-specific, which eliminates potential interspecific contamination in samples with low concentrations of DNA from the species of interest.

One factor that complicates genotyping coast redwoods using microsatellite markers is its hexaploid condition. In genetic analyses of polyploid organisms it is difficult to 1) discern copy number of alleles in microsatellite scans; and 2) accurately score microsatellite scans with potentially higher numbers of alleles. For coast redwood, copy number can be determined for a homozygote (1 allele) or a full heterozygote (6 alleles), but for partial heterozygotes, copy number is impossible to determine with certainty. One method for polyploid organisms is to estimate copy number using the peak size on microsatellite scans (Esselink, 2004). However, implementing this method becomes more challenging with increasing ploidy, and not all marker sets have consistent enough amplification to confidently employ this method.

Additionally, testing the fidelity of amplification products is complicated in polyploids. Since allele copy number cannot typically be resolved exactly, tests for null alleles and other PCR artifacts that require calculation of exact allele frequencies cannot be used on polyploid organisms (Dufresne et al., 2014). Microsatellite scans with many alleles make it more challenging to determine the presence of stutter bands. For coast redwood, it is possible to observe between one and six alleles in a microsatellite scan. If the size difference

between alleles is within several base pairs, it can be difficult to distinguish between stutter and true alleles.

Another challenge in determining the genotypic identity of clonal plants regardless of ploidy level is the possibility of somatic mutation, where a mutation occurs that changes the genotype of an individual in a clonal lineage. For coast redwood, basal sprouting often occurs as response to disturbance, such as fire or timber harvesting (Neal, 1967; Lorimer, 2009, Ramage et al. 2010). Somatic mutation in basal sprouts has the potential to confound genotyping studies seeking to identify the origin of shoots, particularly in the cases where different tissue types are being sampled for clonal identification.

Given that two ramets from a clonal plant may differ in their genotype due to the presence of null alleles, scoring errors, or somatic mutation, the concept of identifying clones that belong a multi-locus lineage (MLL) has been proposed to identify clonal lineages that may not be identical in genotype (Arnaud-Haond et al., 2007a). Here, we used microsatellite data to identify MLLs using multiple tissue types from coast redwood. Existing protocols were modified in order to extract and amplify DNA from redwood cambium, and samples of cambium and leaf tissue from the same trees were compared to ensure consistency between tissue types in our genotyping protocol. We also developed a novel protocol to improve accuracy of microsatellite scoring. MCMC simulations were used to calculate probability of identity and explore the effect of null alleles on genotyping accuracy. In addition to providing genotyping methods for future studies of coast redwood, these protocols should be applicable to genotyping other polyploidy species.

2.2 Methods

Sample collection

Samples were collected in square one-hectare plots in old-growth redwood forests in northern California. Plots were located in areas classified as "old-growth" on Save the Redwoods League maps where coast redwood was the dominant species. Two one-hectare plots were located at Big Basin Redwoods State Park (N 37.18056, W 122.23278; N 37.18528, W 122.21444), two at Humboldt Redwoods State Park (N 40.34833, W 123.92444; N 40.34028", W 123.94833), one at Redwood National Park (N 41.30750, W 124.02667), and one at Prairie Creek Redwoods State Park (N 41.37250, W 124.02528). All trees over ten cm in diameter at breast height were mapped, measured for diameter, classified by canopy position and strata (Oliver and Larson, 1996) and identified to species. All coast redwood trees were cored for cambium/sapwood samples using a 5.15 mm diameter increment borer. The increment borer was dipped in and sprayed with 10% bleach, rinsed, and dried with several lengths of clean yarn between trees. Cambium samples were preserved in bags of silica gel. Wherever foliage, epicormic sprouts, or basal sprouts (hereafter referred to collectively as "leaf" samples) were accessible, they were collected in a ziplock bag with a few drops of distilled water. All samples were stored in a 4 °C freezer within two weeks of collection.

DNA extraction

Leaf samples were cut and ground for one minute (min) in a Mini Beadbeater (Biospec Products, Bartlesville, Oklahoma, USA) using a combination of 2.5 mm and 6.35 mm glass beads in XXTuff Reinforced 2mL Microvials (Biospec Products). Cambium samples were freeze dried for at least 72 hrs using a FreeZone 12 Freeze Dry System (Labconco, Kansas City, Missouri, USA) then ground to a powder in XXTuff Reinforced 2mL Microvials using 6.35mm chrome-steel beads (Biospec Products). Cambium samples were ground in three one min intervals. Between grinding intervals, samples were placed on ice for five min to prevent degradation from overheating. DNA was extracted from both leaf and cambium samples using a modified CTAB method (Cullings, 1992).

Primer development

We tested primers that were developed from genomic libraries by Bruno and Brinegar (2004) and Douhovnikoff and Dodd (2011) for use in this study. To test primers, we used a set of 21 samples from Humboldt Redwoods State Park (HRSP) and a control tree from the University of California Berkeley (UC Berkeley) campus. Samples from HRSP were in sets of three that included foliage, epicormic, and basal samples from the same tree. We initially screened primers by amplifying fragments from our test samples and visualizing the product using gel electrophoresis. If a primer amplified fragments showing consistency within trees and polymorphism between trees, we ran PCRs with fluorescent-labeled primers with different salt concentrations and temperature cycling protocols to see which were polymorphic and amplified well. We found that primers "seq8e8" (dinucleotide repeats) and "seq18d73" (trinucleotide repeats) from Bruno and Brinegar (2004) and "rw28" and "rw39" (tetranucleotide repeats) from Douhovnikoff and Dodd (2011) amplified well and were polymorphic. In addition to the four previously developed primers, we also developed two new primers, "rw56" and "rwdi11". Cloning and sequencing followed Douhovnikoff and Dodd (2011). From these sequences, we developed an additional primer for a tetranucleotide repeat region ("rw56") and an additional primer for a dinucleotide repeat region ("rwdi11").

PCR optimization

For all primers, we optimized amplification by testing magnesium chloride ($MgCl_2$) concentrations between 1.5 mM and 3.0 mM and by modifying the number of cycles and range of annealing temperatures in the thermocycling protocols. 3.0 mM $MgCl_2$ was the optimal salt concentration for all primers. Optimal annealing temperature range and number of cycles differed between primers, but all protocols were touchdown protocols that consisted of: 1) an initial denaturing period of 3 min at 94 °C; 2) 27-35 cycles of denaturing for one min at 94 °C, one min of annealing, where the annealing temperature was lowered each cycle, and one min of extension at 72 °C; 3) one cycle of denaturing for one min at 94 °C, one min of annealing at 45 °C, and one min of extension at 72 °C; and 4) a final extension at 72 °C for two min. Number of cycles and annealing temperatures for each primer are given in Table 2.1. PCRs took place in 10 μL volumes consisting of 1 μL of 1:10 diluted template DNA, 1x PCR Buffer (Invitrogen Life Technologies, Carlsbad, California, USA), 3.0mM $MgCl_2$ (Invitrogen), 800μM dNTPs, 0.6 μM each forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa, USA), 0.25 μg/μL bovine serum albumen (New England Biolabs, Ipswich, Massachusetts, USA), 0.25 Units Taq Polymerase (Invitrogen), and water to bring the final volume to 10 μL. Forward primers were labeled

with either 6FAM or HEX fluorescent dyes (Table 2.1). For the marker "seq18d73" the reverse primer was labeled instead of the forward primer. Leaf and cambium PCRs were always separate, to prevent contamination of cambium samples, which could potentially have a lower concentration of template DNA due to fewer living cells in woody tissue than in leaf tissue. PCR product was diluted 1:10 and fragments were analyzed with GeneScan 500 LIZ Size Standard (Applied Biosystems, Foster City, California, USA) on an ABI 3730 DNA Analyzer (Applied Biosystems) at the Evolutionary Genetics Lab in the Museum of Vertebrate Zoology at UC Berkeley. A positive control sample from a tree on UC Berkeley campus and a blank were included on each plate.

Allele scoring

Microsatellite data were analyzed with GeneMapper v4.0 software. To make our allele scoring protocol more robust against the accidental scoring of stutter peaks or noise, we created bins only for alleles that amplified in at least two different tissue types from the same tree. For example, if we found a new allele in a cambium sample, we extracted and amplified a second sample from an alternate tissue type (foliage, epicormic, or basal) collected from the same tree to verify the allele. If an allele did not amplify in multiple tissue types, a bin was not added for that allele. We used the GeneMapper software to score alleles and manually checked and re-scored samples as necessary.

Given the quality of our primers and our comparison of different tissue types, we did not think that we would be able to accurately estimate copy number in partial heterozygotes from allele peak size as described in Esselink et al. (2004). Instead, alleles were recorded as either present or absent in each sample.

Clonal assignment protocol

To determine which trees were part of the same multi locus lineage (MLL) we used a protocol described by Arnaud-Haond et al. (2007b). We calculated the pairwise genetic distances between samples at each site (Big Basin Redwood State Park, Humboldt Redwoods State Park, and Redwood National Park/Prairie Creek Redwoods State Park) using *polysat* (version 1.3-2 – 1.3-3; Clark and Jasieniuk, 2011) in R (version 3.1.1; www.R-project.org). We used both the Bruvo distance metric (Bruvo et al., 2004), which takes into consideration that alleles similar in size could be closely related by mutation, and the Lynch distance metric (Lynch, 1990), which is a simpler band-sharing measure. As results from both metrics were very similar, from here forward we present results using the Bruvo metric. For a non-clonal organism with random mating, we would expect a histogram of the pairwise genetic distances between individuals to show a roughly normal distribution. For a clonal organism, we would instead expect the histogram of pairwise genetic distances to have a bimodal distribution, with one peak centered on the mean genetic distance between non-clonal individuals, and a second peak very close to zero, consisting of pairwise genetic distances between samples from the same MLL. If the genotypes of all clonal pairs are perfectly identical, the genetic distance between these samples should be zero. However, due to scoring errors, null alleles, and somatic mutation, the genetic distance between clones may be greater than zero. We planned to set the genetic distance threshold for clonal assignment at the anticipated trough between the clonal and non-clonal peaks in histograms of pairwise genetic distances.

Probability of identity calculation

To calculate the probability of identity (p_{id}), we used Monte Carlo simulations to determine the probability of drawing two indistinguishable genotypes given the overall allele frequencies from sampled individuals. Calculating p_{id} for a 7olyploidy is complicated by copy number ambiguity, since for partially heterozygous allelic 7olyploidy, many allelic configurations are possible. Instead of calculating p_{id} based on the presence or absence of alleles, we developed a protocol to account for the multiple different genotypes that could result in an identical allelic phenotype.

We used the "round robin" method developed by Parks and Werth (1993) to calculate allele frequencies in populations of clonal plants. To calculate the allele frequency for a given primer, clonal identity of each individual was determined without data from the marker for which allele frequencies were being calculated. The dataset was then trimmed to include one individual per clone, and allele frequencies were calculated using the remaining individuals. This process was repeated for each marker. Since allele frequencies could not be calculated exactly due to uncertainty in allele copy number, we used the simple allele frequency estimator in *polysat*. The use of the simple allele frequency estimator assumes that, in a partially heterozygous sample, all alleles have an equal probability of being present in multiple copies. This estimator did not allow us to account for inbreeding or departures from Hardy-Weinberg equilibrium, which are likely given coast redwood's clonality and non-continuous geographic distribution. However, given the complexity of accounting for these factors in polyploid organisms, we chose to use a simple allele frequency estimator that did not require us to make any assumptions about the evolution of polyploidy in coast redwood or levels of selfing in this species.

Initially, we attempted to calculate probability of identity by a "brute force" method where we first created a matrix of every possible genotypic permutation. Next, we added a column to describe the allelic phenotype of each genotype. For example, a genotype with alleles *aaabbb* would have an allelic phenotype of *ab*. Then, we summed the probability of all permutations that would yield a given allelic phenotype. For a hexaploid, this would mean that we summed the probability of all sixty-two genotypes that yielded the allelic phenotype *ab*. Finally, we summed the squared probabilities of each allelic phenotype to find the probability that an identical allelic phenotype would appear in two successive draws.

Unfortunately, this brute force method resulted in extremely large matrices of possible allelic configurations. For our most diverse locus, which had 69 alleles, there were greater than $1x10^{11}$ permutations. Instead of calculating the probability of every possible genotype, we instead used Monte Carlo simulations to approximate the probability of drawing an identical allelic phenotype twice for a given locus. To do this, we simulated genotypes for 100,000 pairs of trees based on our allele frequencies. We then assigned the appropriate allelic phenotype to each tree, and counted the number of times out of 100,000 that the paired trees had a matching allelic phenotype to estimate the probability of identity for each allele. To find the overall probability of identity, we multiplied the p_{id} estimates from each locus, then multiplied that number by 32,942, the number of comparisons in the plot with the maximum number of sampled redwood trees (182). We were able to verify the accuracy of this method by comparing results of the Monte Carlo simulations to our brute force results from our least diverse primer, "seq18d73."

Null allele trials

While probability of identity calculations gave the probability of finding an exact match in our genotyping data between sexually reproduced samples, our clonal identification protocol allowed individuals with slightly different allelic phenotypes to be assigned to the same MLL. To test the sensitivity of our genotyping protocol to null alleles, we created simulated data sets with increasing numbers of missing alleles to see how this impacted the probability of assigning sexually generated genotypes to the same MLL. In each simulation, genotypes of 182 trees were randomly generated using allele frequencies from the original data as the probability of sampling each allele. Alleles present more than once in an individual were deleted to reduce the genotype data down to allelic phenotype data, to match the allele copy number ambiguity present in the original data. Next, null alleles were deleted from individuals in roughly the same number from each marker so that each marker had an allele deleted in 30 to 31 individuals. Within each marker, alleles were deleted randomly with equal probability. If a marker only had one allele present during a round of deletions, it would be skipped, and its single allele would not be deleted, since in the actual data collection, microsatellite scans that showed no alleles were re-run. Once a data set had been simulated and alleles deleted, we used the same clonal assignment protocol that was used on the original data, and determined whether any individuals had been classified into the same MLL. We simulated 100 data sets of 182 trees for each number of rounds of deletions (0-30), and counted 1) the number of simulations out of 100 that had false positives and 2) the total number of false positives present in all 100 simulations. We also calculated the average number of deletions per tree for each number of rounds of deletions, since deletions were skipped for markers with only one allele present.

Test samples

To check our genotyping protocol, tested it on 88 sets of paired samples of different tissue types from the same trees. Of these 88 sample sets, we had similar numbers of comparisons between foliage-epicormic samples, foliage-basal samples, epicormic-basal samples, and cambium-basal samples, which allowed us to compare the average genetic distance between different tissue type pairs using an analysis of variance (ANOVA). We also assessed the effect of variation between amplification plates on genetic distance between paired samples. Pairwise genetic distance between duplicate samples was regressed on proportion of loci amplified on the same plate using a linear model. As pairs consisting of cambium and leaf samples were always run on separate plates, we excluded them from this analysis to eliminate the potentially confounding effect of tissue type on our assessment of whether variation between plates affected genetic distances between duplicate samples. Prior to these analyses, the dataset was checked for outliers and any outliers were removed from the analysis.

2.3 Results

Pairwise genetic distances

We plotted histograms of pairwise genetic distances for each one-hectare plot, which generally showed one peak around 0.6 and a second peak close to zero. The second peak

likely resulted from scoring errors or somatic mutations causing slight variation between the genotype of clones (Figure 2.1). The histograms consistently showed a trough around 0.2, so we set this as our genetic distance cutoff for clonal assignment. Using this criterion, 449 clones were identified in the 770 trees genotyped.

Probability of identity calculation

We compared the estimate of p_{id} generated from Monte Carlo simulations to our "brute force" calculation from our least diverse primer, "seq18d73," which had thirteen alleles, and found that 100,000 simulations were enough to give us an estimate that was accurate within 10^{-3}. When we calculated the p_{id} from all six primers, the product, or overall p_{id}, was less than 1.1 x 10^{-18}. Correcting for the number of comparisons being made in the plot with the most trees resulted in a p_{id}<3.6 x 10^{-14}.

Null allele trials

Our null allele trials showed one or fewer false positives in sets of 100 simulations up to twenty rounds of deletions, an average of eighteen actual deletions (Figure 2.2). When we deleted twenty rounds of alleles from allelic phenotypes in our simulations, three out of 100 simulations contained one false positive, giving an error rate of 0.03. In further simulations with increasing numbers of alleles deleted, both the number of simulations out of 100 that had false positives and the total number of false positives present in all 100 simulations continued to increase.

Test samples

Of our 88 sets of paired samples, only one pair of samples from the same tree was identified as clonally distinct. This pair had a genetic distance of 0.60, and consisted of a cambium sample and a basal sample. Excluding this sample, the mean genetic distance between paired samples was 0.03 and ranged from 0-0.17. 49 out of 87 remaining pairs had a genetic distance of zero. Of the duplicate pairs with a genetic distance greater than zero, most of these differences were due to one or two alleles being present in one sample but not the other. 11 out of 87 pairs had alleles that were one base pair different. In all of these cases, the mismatching alleles were from the primer "rwdi11" which amplified a dinucleotide repeat region.

An ANOVA comparing genetic distances between paired samples of different tissue type combinations showed a modest statistical difference between sample types ($F(3,82)$=2.93, p = 0.03, Figure 2.3). A Tukey's Honestly Significant Differences test showed that the genetic distance between foliage-epicormic samples was, on average, lower than cambium-basal samples (p=0.02), but there were no other differences between tissue type combinations. The regression of genetic distance between duplicate samples on proportion of loci amplified on the same plate showed a small but significant negative correlation (slope = -0.040, $t(65)$ = -2.31, p = 0.02).

2.4 Discussion

Results from our probability of identity calculations, null allele simulations, and test samples suggest that our genotyping protocol was able to consistently identify multi-locus lineages (MLL). Optimizing PCR conditions and confirming consistent amplification of alleles before scoring allowed us to generate histograms with a consistent trough in the distribution of genetic distances between clonal and non-clonal trees. Using the genetic distance value at this trough as the threshold in our clonal assignment protocol, trees were assigned into MLLs in a way that accounted for non-zero genetic distances. Our protocol distinguished between clones collected in close physical proximity, which might be more genetically similar than individuals sampled at random from a population.

Null allele trials also suggested that our genotyping protocol was robust to the presence of null alleles. In our simulations, randomly generated allelic phenotypes were identified as clones in one in a hundred or fewer simulations, with up to eighteen deleted alleles. Our protocol for clonal identification may be useful for other studies of polyploid plants where null alleles are an issue, although consideration should be given to the fact that, in studies with less diverse primer sets than ours, null alleles may present more of a challenge than they do here. For both probability of identity estimation and null allele trials, we found simulations to be extremely useful. Our simulations for calculating probability of identity and investigating the robustness of our genotyping protocol could also be applied earlier in a clonal identification study to determine 1) how many markers are needed for reliable genotyping of an organism; or 2) whether a highly conservative microsatellite scoring protocol that had the potential to generate null alleles would be appropriate for a given set of markers.

Results from test samples showed that our genotyping protocol was robust to the use of different tissue types. We found only one case where two samples from the same tree were not assigned to the same MLL. In this case, the samples were a basal sprout and cambium sample from the same tree, with a genetic distance of 0.6. Given this genetic distance, it seems extremely unlikely that these two samples came from the same MLL. Instead, it seems more likely that the basal and cambium samples in this pair came from different trees. During sample collection, some basal samples collected were sprouting out of the ground near the presumed parent tree, so there was some potential for mis-identification. To confirm that lab contamination was not the reason for this mismatch, both samples were re-analyzed for all loci, but results remained the same.

Although our protocol for assignment into MLLs was robust to the use of different tissue types, different tissue type pairs varied in their average pairwise genetic differences. Pairs of duplicate samples consisting of basal and cambium tissue from the same tree had the highest average genetic distance while foliage-epicormic pairs had the lowest. Most non-zero pairwise genetic distances between samples from the same tree were due to null alleles in one of the samples. While it is possible that somatic mutation in the microsatellite primer regions is responsible for some missing alleles, it seems unlikely that this is responsible for the number of null alleles we observed in duplicate samples. Instead, these are probably due to amplification and scoring inconsistencies. It is possible that certain tissue types are more likely to have amplification failure than others. For example, some types of leaf tissue could have higher concentrations of PCR-inhibiting secondary metabolites. Given our result that basal-cambium samples from the same tree had higher genetic distances on average than other tissue type pairs, we wondered if samples from cambium tissue were more prone to

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null alleles due to lower template DNA concentrations in the PCRs. However, when we looked at the allelic phenotypes of samples in cambium-basal pairs with non-zero genetic distances, we found that only four cambium samples were missing peaks that were present in the corresponding basal sample, whereas seven basal samples were missing peaks that were present in the matching cambium sample.

Another explanation for the greater genetic distances between basal-cambium pairs could be that, unlike paired leaf tissues, basal and cambium samples were always run on separate PCR plates. Our analysis showed that amplification on different plates did cause slightly greater genetic distances between samples. This result underscores the importance of optimizing PCRs for different primers. It also provides an argument for randomizing the order of samples during DNA extraction and amplification to prevent bias due to the grouping of samples collected in close geographic proximity. In this study, the effect of amplification differences between plates was not enough to cause genotyping inaccuracy, as duplicate sample pairs consistently had genetic distances below our threshold of 0.2 and our positive control sample had a consistent genotype in all runs.

In terms of detecting differences in somatic mutation rates between tissue types, our results were inconclusive. We only detected microsatellite repeat regions that seemed to vary in length between duplicate samples in rwdi11, a marker that amplified dinucleotide repeats. In this marker, the only shifting in length of microsatellite repeats occurred where several different alleles were only one base pair apart. Rather than somatic mutation, we believe that single base pair differences in the size of microsatellite repeat regions in samples from the same tree were due to slight error in the measurement of DNA size fragments with respect to size standards. If we had seen alleles in duplicate samples shifting up or down by one repeat length in other markers as well, this would have been stronger evidence for somatic mutation.

While our microsatellite data from different tissue types from the same tree allowed us to verify the effectiveness of our genotyping protocol, it was not ideal for measuring rates of somatic mutation. Since microsatellite data only provides information on fragment length, and null alleles are often present, it was impossible to distinguish between somatic mutation and scoring error. Single-nucleotide polymorphism (SNP) or sequence data, where single base-pair changes in the genome can be detected, would be a more appropriate way to test for somatic mutations between tissue types. It would also be useful to conduct a study using all four tissue types (foliage, epicormic sprouts, basal sprouts, and cambium) from every tree sampled, which was not possible at our collection locations.

One approach not used in this study is to sample megagametophye tissue, which would have allowed us to look at the maternal haplotype contributing to zygotes. Sampling of megagametophyte tissue may have the potential to improve allele frequency estimates, since the megagametophytes of coast redwood should be triploid, rather than hexaploid. However, triploid megagametophytes would still have some allele copy number ambiguity, making this approach less useful than it might be in a tetraploid organism. While it is possible to separate megagametophyte tissue from embryo tissue in redwood seeds (Rogers 1997), we chose not use megagametophytes for the development of a clonal identification protocol for coast redwood. Scoring haploid tissues instead of the full hexaploid genome may have caused us to lose some of the power of our microsatellite markers, and issues

caused by copy number ambiguity would remain. Due to the immense height of coast redwood trees, it would be very difficult to get seeds from every tree in a one-hectare plot, particularly if the exact locations of genotypes were desired. Although analyzing megagametophyte tissue did not seem like a viable option for genotyping coast redwood, it may be an extremely useful tool in parentage and population genetic studies of this species.

In summary, a combination of optimizing PCRs, developing a conservative allele scoring protocol, and allowing for non-zero genetic distances in clonal identification allowed us to effectively identify multi-locus lineages from multiple types of coast redwood tissue. We confirmed the effectiveness of our protocol using simulations and paired samples from the same trees. The techniques described in this paper will allow us to accurately identify coast redwood clones from available tissue types and have broad applicability to genetic studies of polyploid organisms, particularly where multiple tissue types are being sampled.

Primer Name (label)	GenBank accession no.[1]	Primer sequences (5'-3')	No. PCR extension cycles	PCR temp[2] range (°C)	Size range (bp[3])	No. alleles	H_o[4]	Nos. of individuals with 1/2/3/4/5/6 alleles detected
RW28 (FAM)	GU969047	F: GATAGATAAATAGATGGATAG R: TTTTTAAGGTTTCATGGATAAGTACAA	35	65-50	187-342	19	0.39	219/106/29/5/0/0
RW39 (FAM)	GU969046	F: CCATAAGGTTGAAATGAAGAAAAA R: GTTGATTGATCGTTGGTTGG	30	65-50	240-470	69	1.00	2/9/52/138/149/97
SEQ18D7-3 (HEX)	AY562168	F: GCAAAAAGGGAATTGTAATTGGGTTCA R: CCCTAGGTCTAGGCTACGGCGACTTG	27	67-52	124-183	13	0.68	136/180/88/15/5/0
SEQ8E8 (FAM)	AY562169	F: ATACTCACCCTTACACGGGC R: AAATGCCTTGATGAAGCAAAA	28	67-52	112-185	21	0.24	246/65/9/2/0/0
RW56 (HEX)	KP055095	F: CTTGACATCATCCATAGCT R: AAATTGCAAGGGGTGCAA	30	69-54	189-259	18	0.89	51/116/166/89/23/1
RWDI11 (HEX)	KP055096	F: GGACCAAATGCCCTGAAC R: GCCAAGCCATATGGGTTTG	30	63-48	215-268	29	1.0	1/19/65/174/142/48

Table 2.1. Primer information. Number of alleles, observed heterozygosity, and numbers of individuals with each allele possible allele count were calculated using combined data from all six study plots. Notes: [1]No = number; [2]temp = temperature; [3]bp = basepairs, [4]H_o = observed heterozygosity.
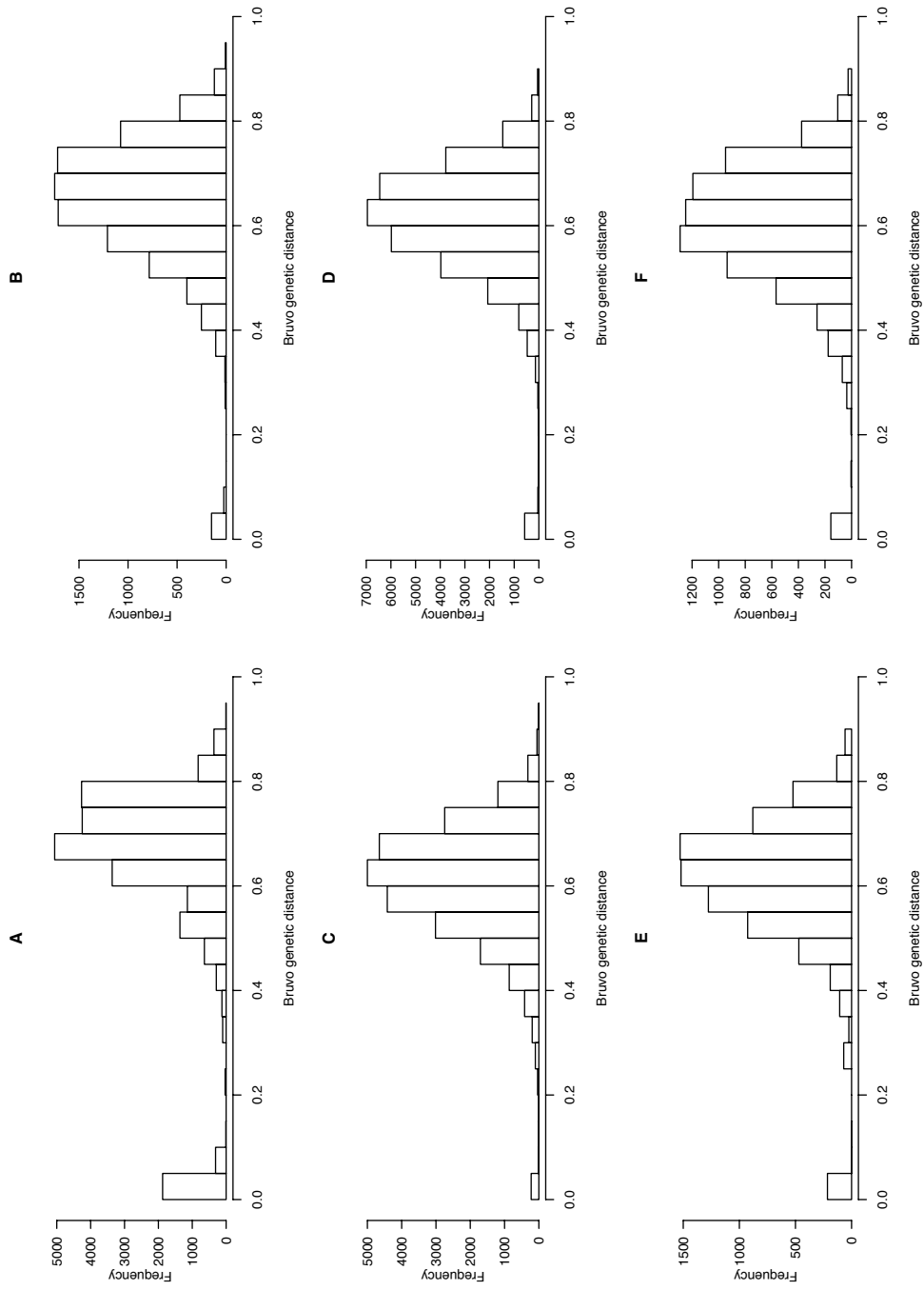
Figure 2.1. Plot of pairwise Bruvo distances for A) Big Basin Redwoods State Park 1; B) Big Basin Redwoods State Park 2; C) Humboldt Redwoods State Park 1; D) Humboldt Redwoods State Park 2; E) Redwood National Park; and F) Prairie Creek Redwoods State Park.
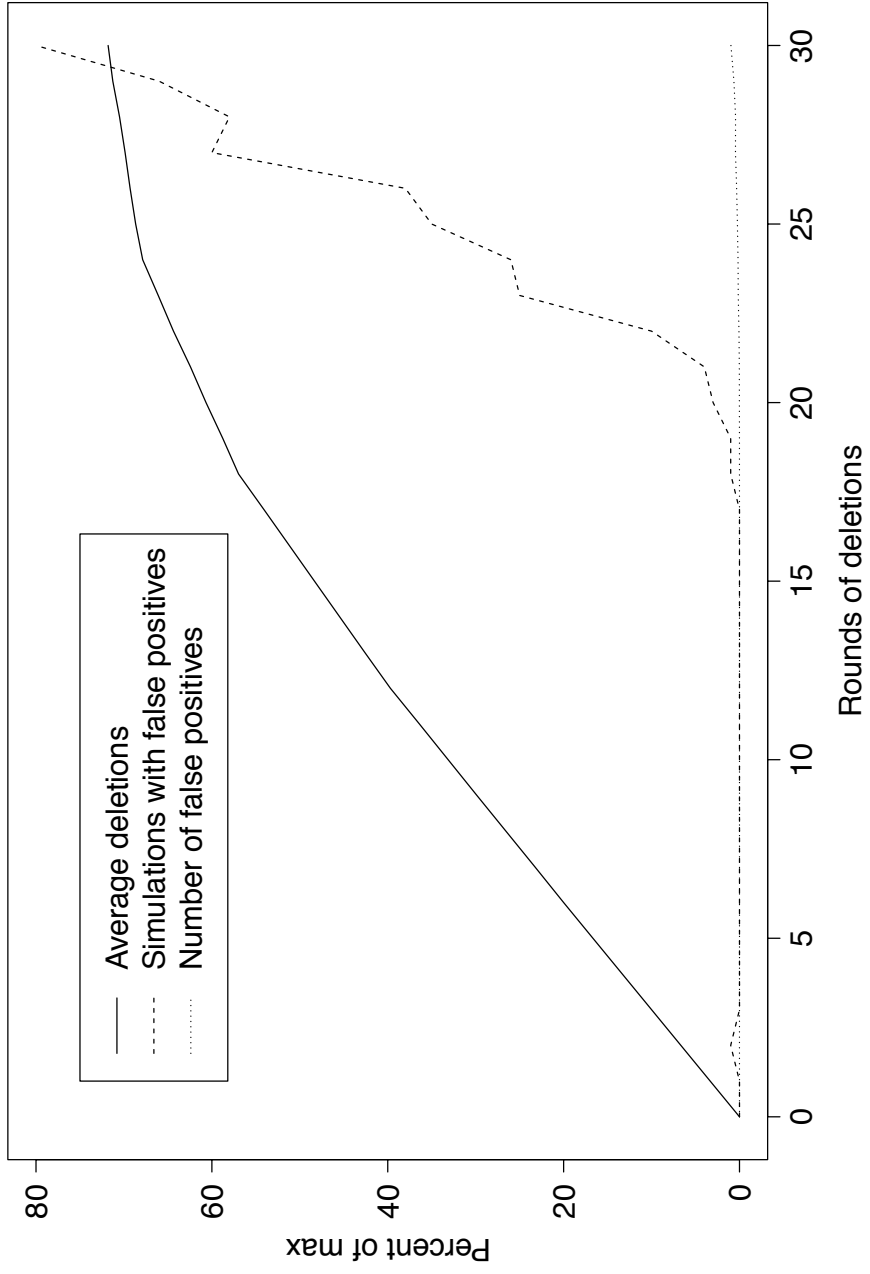
Figure 2.2. Results of null allele trials. One hundred data sets were simulated at each number of rounds of deletions. Lines show average deletions, simulations with false positives, and the total number of false positives for all simulations with a given number of rounds of deletions. Results are shown as a

Figure 2.3. Bruvo genetic distance between test samples of different tissue-type pairs. Circles are scaled to show the number of sample pairs with each genetic distance.

Chapter 3: Regional comparison of clonal diversity and structure, genetic diversity, and spatial genetic structure

3.1 Introduction

Coast redwood (*Sequoia sempervirens* (D. Don) Endl.) is an iconic species and important source of timber production and carbon storage in northern California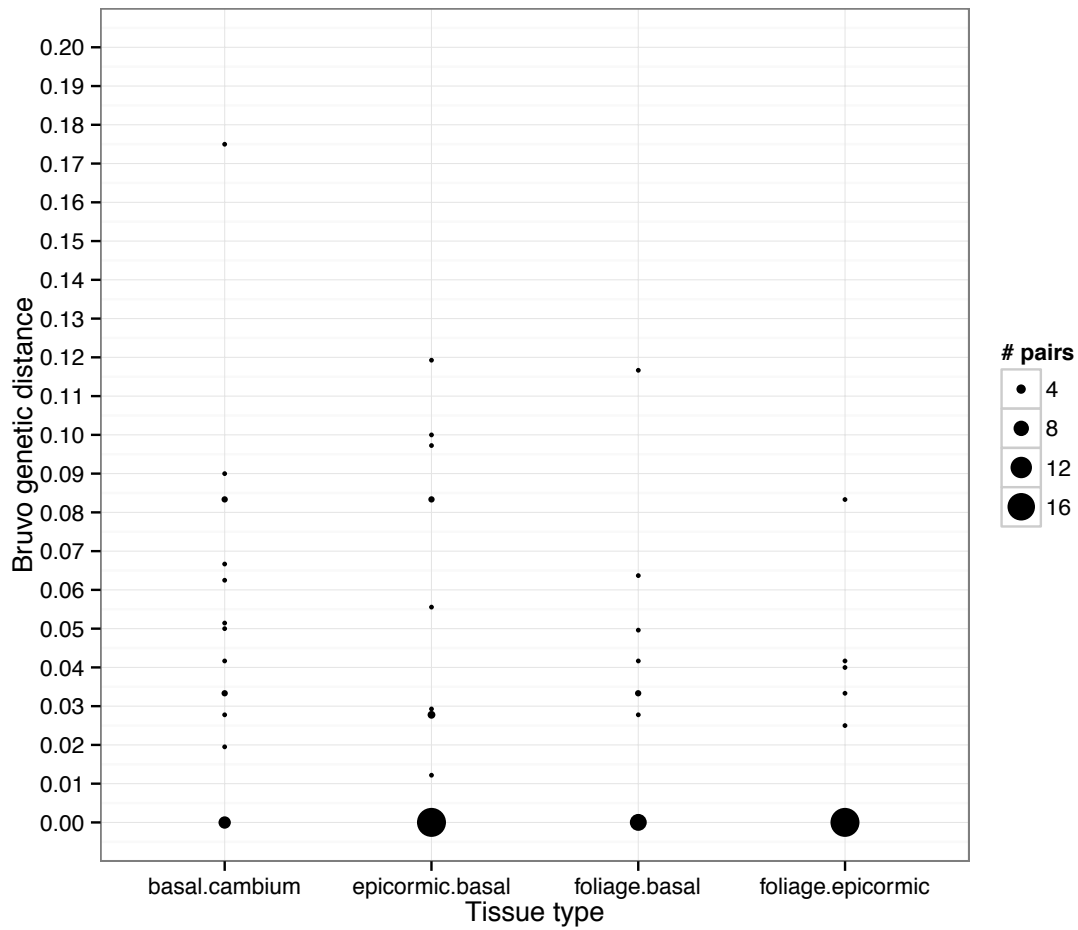 (Noss 2000, Sillet et al. 2010, Jones and O'Hara 2012). This species can reproduce through both sexual and asexual reproduction. Clonal reproduction plays an important role in the reproductive ecology of coast redwood, as coast redwood has low seed viability, high seedling mortality, and the capacity for prolific sprouting (Davidson 1970, Becking 1996, Ornduff 1998, Jacobs 1987, Olson et al. 1990, Neal 1967, Noss 2000, O'Hara et al. 2010). Knowledge about the patterns and prevalence of clonal reproduction in coast redwood is essential for the effective management of coast redwood in both managed forests and natural reserves.

Despite its status as a valuable endemic and timber commodity, little is known about patterns of clonal structure throughout the range of coast redwood. A study of four mapped plots at Humboldt Redwoods State Park found 15-34% of redwood clones to be multi-stemmed, within averages of 1.17-1.53 stems per clone (Rogers 2000). Rogers also found some instances of spatially disjunct clones, with a maximum between-ramet distance of 340 meters. In comparing upland and lowland sites, average number of ramets per clone was slightly greater at the two upland sites, due to clones on upland sites being more likely to be multi-stemmed. In a study in second-growth stands at Jackson Demonstration State Forest in Mendocino County, Douhovnikoff et al. (2004) found a similar proportion of clones to be multi-stemmed (roughly twenty-four percent), but that clone sizes were greater in second growth coast redwood forests than in Rogers' old growth plots.

No study to date has compared clonal structure across the range of coast redwood. Based on the past and current distribution of coast redwood and underlying theory about the benefits and drawbacks of clonal reproduction, predictions can be made about how coast redwood forests might vary in amount of clonal reproduction throughout the geographic range of this species. Previous theoretical and experimental work on the evolutionary advantages and patterns of clonal and sexual reproduction have suggested that 1) reduced sexual reproduction should be common at the geographic margins of a clonal species' range, where environmental conditions are too extreme for sexual reproduction to reliably occur; 2) in small, geographically isolated populations, where sexual reproduction could be inhibited by low genetic diversity; and 3) there should be greater clonality in older populations of terrestrial plants, as vegetative reproduction is generally a less effective method of colonization than seed dispersal (Eckert 2002, Silvertown 2008).

It is generally believed that the current distribution of coast redwood is limited by moisture at the southern end of its range and by temperature at the northern end. At the southern end of its range, coast redwood stands are confined to riparian areas (Zinke 1988), suggesting that moisture may be a limiting factor. A study of temperature variation in Humboldt County found that redwood dominated forests with a mean annual temperature

of greater than 8°C and low variation between summer and winter surface soil temperatures (Hauxwell et al. 1981). If drought or cold make it difficult for coast redwood to reproduce sexually in certain parts of its range, theoretical work suggests greater levels of clonality in these areas. However, geographic variation in patterns of clonality might also depend on the specific ways in which environmental pressures impact the reproduction and survival of coast redwoods. For example, if seedling establishment were limited by drought in southern populations of coast redwood, less clonal diversity might be observed in these populations because unique genotypes that died out might be replaced by progeny of the remaining clones rather than new sexual recombinants. Similarly, less clonal diversity would be expected if the production of viable seed was lower due to poor tree vigor in dry environments. Conversely, if environmental stress limits tree survival rather than establishment, clonal diversity should be higher in areas where individual stems are less likely to survive and produce clonal offspring, as these populations might be composed of trees from more recent seed-dispersal events.

Regarding the theoretical prediction of reduced sexual reproduction at the edges of a species' range due to decreased genetic diversity, this seems like an unlikely driver for patterns of variation in clonal reproduction throughout the range of coast redwood. Although coast redwood has a very limited distribution, previous work has documented extremely high levels of within-population diversity in coast redwood. Coast redwood is a hexaploid species (Ahuja and Neale 2002), and polyploid plants have more potential sites for mutation and reduced susceptibility to genetic drift (Meirmans and Van Tienderen 2013). Coast redwood also occupied a historic range much larger than its present distribution which may also contribute to genetic diversity (Sawyer et al. 2000). Rogers' (2000) study of old growth forests using isozyme markers showed high levels of within-population diversity typical of wind-pollinated conifer species. High levels of within-population diversity were also found in a 2011 study of coast redwoods using microsatellite markers (Douhovnikoff and Dodd 2011). Douhovnikoff and Dodd sampled 135 non-clonal individuals from 81 stands throughout the range of coast redwood and looked for patterns of genetic divergence between watersheds. While their results showed some variation between geographic regions, most of the variation occurred within watersheds.

Finally, clonality in coast redwood might vary between populations due to variations in migration patterns throughout the range. During the last Glacial Maximum, approximately 18,000 years ago, the distribution of coast redwood was probably confined to small, protected coastal areas in northern California and coastal regions farther south in California than coast redwood's current range (Sawyer et al 2000). Based on pollen records, at the peak of its abundance, coast redwood populations in coastal areas of Santa Cruz and Monterey Counties were more widely distributed and abundant than they are today. It is therefore likely that redwood populations in the southern part of the range are generally older, and may therefore have greater amounts of clonal than sexual reproduction.

In this study, we explored the hypothesis that clonal diversity and structure differ between populations throughout the range of coast redwood. Two one-hectare plots at each of three sites spanning approximately 380 km within the native distribution of coast redwood were mapped and comprehensively sampled. Although the small number of study plots limits statistical inference, having two study plots at each site provides some information about whether clonal diversity and structure differed due to geographic location

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or due to local site factors. Study plots were also compared in terms of their genetic diversity, tested for within-plot spatial autocorrelation, and tested for genetic differentiation between plots and between the geographic areas in which our plots were located. Doing these tests added to the existing knowledge about genetic diversity and divergence in coast redwood, and provided additional context for our comparison of clonality between sites.

Understanding patterns of clonal reproduction and genetic diversity throughout the range of coast redwood has important conservation implications. In some commercial timber forests, genetically identical laboratory-propagated coast redwood clones have replaced seed-origin seedlings as the primary source of regeneration to supplement vegetative reproduction. One justification for clonal planting is that clonal reproduction occurs naturally in redwood forests, and that redwood clones might be spatially extensive. Having information about the patterns and prevalence of clonal reproduction throughout the range of this species can provide guidance for land managers looking to maintain forests within the range of clonal and genetic diversity that is present in old growth stands. Lastly, knowledge about the amount of genotypic and genetic diversity in different redwood forests throughout the range of this species is crucial for understanding the potential for gene flow between populations and identifying populations of this species that may experience declines due to climate change.

3.2 Methods

Sample collection

A challenge in studying clonal organisms is designing sampling schemes that will not bias estimates of clonal diversity. Sampling clones along transects or in small patches can lead to overestimates of clonal diversity, as these methods may capture only one or two members of much larger clones (Arnaud-Haound et al. 2007). Similarly, grid-based sampling designs, which are very effective for clone mapping, can overestimate clonal diversity as well if an inappropriate grid size is chosen (Arnaud-Haound et al. 2007). An ideal sampling density would cover several replicates of any particular lineage and many different clonal lineages. Given the limited prior information about clonal structure throughout the range of coast redwood, we chose to comprehensively map and sample all redwood trees over 10 cm diameter in square, one-hectare plots.

Plots were located in areas classified as "old-growth" on Save the Redwoods League maps, where coast redwood was the dominant species on the site. Three pairs of one-hectare plots were established within the natural range of coast redwood on the California coast (Figure 3.1). Pairs were installed at Big Basin State Park, Humboldt Redwoods State Park, and within the Redwood National and State Parks complex. Study plots within pairs were separated by 1.7 to 7.2 km. One of the Big Basin plots (hereafter "BBA") was located at a mid-slope position in an area that was treated with prescribed fire in 2000-2001 (Tim Hyland, personal communication). The second Big Basin plot ("BBB") was located at a lower slope position, approximately 380 m from Opal Creek. At Humboldt Redwoods State Park, one plot ("HRWA") was located in Founders Grove, with the entire plot located in the alluvial flat. A second plot at Humboldt ("HRWB") was located on a lower slope position, with the plot spanning both a north-facing slope and alluvial flat adjacent to Bull Creek. Within the Redwood National and State Parks complex, one plot ("RNP") was located at a

mid-slope position close to Lady Bird Johnson Grove in Redwood National Park. The farthest north plot was located at Prairie Creek Redwoods State Park ("PC"), approximately 230 m east of Godwood Creek in a lower slope position. Elevations and geographic coordinates for all plots are given in Table 3.1.

All trees over ten centimeters in diameter at breast height (1.37 m) were mapped, measured for diameter, classified by canopy position and strata, and identified to species. All coast redwood trees were cored for cambium/sapwood samples using an increment borer. Cambium/sapwood samples were preserved in bags of silica gel. Wherever foliage, epicormic sprouts, or basal sprouts were accessible, they were collected in a re-sealable plastic bag with a few drops of water. Both wood and leaf samples were stored in a 4 degree C freezer within two weeks of collection.

DNA extraction and analysis

DNA extraction followed a modified CTAB method (Cullings, 1992). Fragments for microsatellite analysis were amplified using six microsatellite primers. We used primers "seq8e8" (dinucleotide repeats) and "seq18d73" (trinucleotide repeats) from Bruno and Brinegar (2004) and "rw28" and "rw39" (tetranucleotide repeats) from Douhovnikoff and Dodd (2011). Additionally, we developed two new primers, "rw56" (tetranucleotide repeats) and "rwdi11"(dinucleotide repeats) (Narayan et al. 2015). Microsatellite data were analyzed using GeneMapper v4.0 software. Laboratory methods are described fully in Narayan et al. (2015).

Clonal identification

Clones were identified by calculating the Bruvo genetic distance between all pairs of trees and assigning any pairs with a genetic distance less than 0.2 into the same clonal lineage (Bruvo et al. 2004). This genetic distance was determined by Narayan et al. (2015) to be a suitable cutoff between clonal and sexually reproduced individuals. Genetic distance and clonal assignment were performed using *polysat* (version 1.3-2 – 1.3-3; Clark and Jasieniuk, 2011) in R (version 3.1.1; www.R-project.org). In addition to the probability of identity testing described in Narayan et al. (2015), we did two other checks of our genotyping protocol. The first check was to incrementally increase the genetic distance threshold for clonal assignment and visualize the results from different thresholds on our mapped plots. We were concerned that trees in close physical proximity would also tend to be genetically similar, and that increasing the genetic distance threshold for clonal assignment might result in a situation where increasing the genetic distance threshold gradually increased the size of clonal clusters. Instead, increasing the genetic distance threshold past our chosen cutoff tended to result in the assignment of trees in random spatial locations within the plots to the same genotype, and that increasing the cutoff caused more and more trees to be assigned to a single genotype, rather than forming larger groups around several distinct genotypes.

The second check of our clonal assignment protocol was to screen any sample pairs with genetic distances between 0.1 and 0.3. We compared the genotypes of these sample pairs to determine whether genetic distances between 0.1 and 0.3 were due to missing alleles at a few some loci, or whether certain loci had largely different alleles present. Generally, samples with genetic distances between 0.1 and 0.2 had a few alleles missing from one of the

samples. Samples with genetic distances between 0.2 and 0.3 typically had one or more loci with distinct alleles. For samples with distinct alleles, PCRs were re-run for the loci with distinct alleles to confirm that difference at a single locus were not due to laboratory error.

Clonal diversity

To compare levels of clonality and clonal diversity between study plots, indices to describe clonal richness, evenness, and heterogeneity suggested by Arnaud-Haond et al. (2007) were calculated. In all equations, *G* represents the number of genotypes in a plot and *N* is the number of redwoods genotyped at a plot. Clonal richness was described with *R* as proposed by Dorken and Eckert (2001) where

$$R = (G - 1)/(N - 1).$$

Clonal evenness was described using a scaled Simpson's complement index (*V*), (Hurlbert 1971), which approaches a value of 1 as evenness between genotypes is high, and zero if evenness between genotypes is low (Fager 1972). *V* was calculated as:

$$V = (D - D_{min}) / (D_{max} - D_{min})$$

where *D* is Simpson's complement, the probability of randomly sampling two different genotypes at a plot. It was calculated as 1 – the probability of randomly sampling two identical genotypes at a plot. D_{min} and D_{max} are the minimum and maximum values of Simpson's complement index, estimated as:

$$D_{min} = (((2N - G) \times (G - 1))/N^2) \times (N / (N - 1)) \text{ and}$$

$$D_{max} = ((G - 1) / G) \times (N / (N - 1))$$

Clonal heterogeneity was quantified using the slope of Pareto distribution (Pareto 1987, Arnaud-Haond et al. 2007). For each plot, "clone size" was defined as the number of ramets of each genotype. The cumulative frequency of each clone size was calculated as the proportion of the total number of clones with equal or greater clone size to the point of interest. To fit the Pareto distribution, logarithm of cumulative frequency of clone size was regressed on the logarithm of clone size. The magnitude of the slope parameter of this regression describes clonal evenness, as different clone sizes having similar frequencies will result in a steep slope in the cumulative distribution function. In the case where a few clonal lineages have a very large number of ramets, the regression slope will be much more shallow.

Dataset reduction

To compare genetic diversity, spatial autocorrelation, and population genetic structure among plots, the datasets for each plot were reduced to include only one individual per clone. There were small differences in genotype within clones due to null alleles or scoring errors (see Narayan et al. 2015). Ramets within a clone were selected on the basis of 1) having the fewest missing loci and 2) having the highest number of alleles (i.e., fewest missing alleles). If ramets of the same genotype were missing the same number of loci and had an equal number of alleles, we randomly selected a ramet to include.

Genetic diversity

To compare genetic diversity in different plots, allelic richness, heterozygosity, and number of private alleles were calculated for each study plot. Allelic richness for each study plot was calculated in two separate ways. First, allelic richness was calculated in each study plot including all ramets of all clones. Second, In order to control for the differing number of clones at each plot, 24 clones (the smallest number of genotypes found in any plot) were subsampled from each plot and included in the allelic richness calculations. Subsampling was repeated 1000 times to calculate average allelic richness and 95 percent confidence envelopes for allelic richness at all study plots except the plot with the lowest clonal diversity.

Within-study plot heterozygosity (Nei 1987) was calculated using population genetics software GenoDive (Miermans and Van Tienderen 2004). Heterozygosity was calculated from the reduced datasets from each plot, where one ramet per genotype was included. We specified that all samples were hexaploid, and used GenoDive's function to correct allele frequencies for unknown dosage of alleles. GenoDive's correction is a modified version of the algorithm for allele frequency calculations developed by De Silva et al. (2005), described in the GenoDive manual. The algorithm initially calculates the likelihood of observed phenotypes given the uncorrected allele frequencies. Next, allele frequencies are slightly modified and likelihood of observed phenotypes was recalculated. The changed allele frequencies are accepted if the likelihood is higher, and rejected otherwise. This process is repeated until a stable maximum likelihood is achieved.

Finally, we counted the number of private alleles in each plot, defined as alleles occurring exclusively in a given plot. For the private allele calculations, we included all individuals rather than reducing the data set to one individual per genotype.

Spatial arrangement of clones

To quantify the spatial aggregation of clones in each plot, an aggregation index was calculated following Arnaud-Haond et al. (2007). This metric describes the degree of spatial aggregation of clones by comparing the observed proportion of trees with a clone-mate as their nearest neighbor to the theoretical probability of having a clone-mate as a nearest neighbor if genotypes were randomly located within the study plot. An aggregation index value close to 1 signifies that clones are highly aggregated, while values close to zero would result from either clones being randomly dispersed throughout the plot or from having few trees in the plot belonging to the same clonal lineage. The significance of aggregation was tested by randomly shuffling the clonal identities of trees in each plot among the sampling coordinates. Nine hundred and ninety-nine shuffled data sets were created and the aggregation index value of each was calculated to generate a null distribution with no spatial aggregation of clones. Aggregation indices from the original, unmanipulated datasets were compared to the null distributions to determine whether there was significant aggregation at each study plot.

Clonal subrange, defined here as the physical range at which clonal spread occurs, was also visualized to describe spatial aggregation of clones. For each study plot, the distance between each pair of trees was calculated. These distances were binned into five-meter

distance classes. Within each distance class, the percentage of pairs that were clonally identical was calculated, giving the probability of clonal identity within each distance class. In addition to distance-binned probability of clonal identity calculations, the maximum distance between clones was calculated for each plot.

Spatial genetic structure

To investigate spatial genetic structure within plots we performed both a Mantel test and spatial autocorrelation analysis on the reduced dataset from each plot. For both analyses, the genetic data were reduced down to one individual per clone as previously described. Sample locations for multi-stemmed clones were calculated as follows. For clones with three or more ramets, the centroid of the convex hull containing the coordinates of all individuals of a given clone was used as the sample location. Both convex hulls and their centroids were calculated in the R package "spatstat" (Baddeley and Turner 2005). For clones with two ramets, we averaged the x and y coordinates of the ramets as the clone's location.

Using this reduced dataset, we performed standard Mantel tests (Mantel 1967) for each study plot in GenoDive. This tested for correlation between matrices of the Euclidean distances between sample pairs and genetic distances between sample pairs. The genetic distance matrices were calculated in GenoDive, using a Bruvo distance calculation that did not count missing data as mutation. We ran the Mantel test using the Mantel's r statistic and 999 permutations.

Spatial autocorrelation was also investigated using the software package SPAGeDi (Hardy and Vekemans 2002). We ran autocorrelation analyses using both Loiselle et al.'s kinship coefficient, F_{ij}, and R_{ij}, a kinship analogue based on allele size (Streiff et al. 1998) on data from each study plot separately. Pairs of trees were divided evenly into twenty distance classes and 999 permutations were used to test for significant autocorrelation between kinship and both distance and the natural logarithm of distance. *Sp* statistics, a metric for quantifying fine-scale spatial autocorrelation (Vekemans and Hardy 2004) were also calculated in SPAGeDi.

Population structure

To test for population genetic structure, a nested Analysis of Molecular Variance (AMOVA, Excoffier 1992) was performed in GenoDive. The data were reduced to one individual per genotype as previously described, and missing data were filled in using the "Restore dosage of polyploids" option. For samples with fewer than six alleles at a locus, GenoDive fills in missing alleles for that locus by sampling from alleles that are present based on their frequency in the population. Allele frequencies were calculated using GenoDive's modified De Silva et al. (2005) method as previously described. Clones were nested within study plots which were nested within "Sites". The "Sites" variable represented the three different geographic regions that were sampled (Figure 3.1). Genetic distances were estimated by the ploidy independent infinite allele model (Rho) developed by Ronfort et al. (1998). To check whether results were sensitive to how missing data were filled in by GenoDive, we created three replicate data sets using the filling function and checked the AMOVA results from each replicate data set. We also ran the nested AMOVA on a dataset

where the microsatellite data were coded as AFLP data, where every allele was treated as a separate locus coded as present or absent.

The program STRUCTURE version 2.3.2 (Pritchard et al. 2000, Falush et al. 2007) was also used to test for population genetic structure. To choose K, the most likely number of clusters, we attempted to follow the method suggested by Evanno et al. (2005). We ran the STRUCTURE 20 times at values of K between two and ten with a burnin of 10,000 runs and MCMC length of 50,000 runs. Evanno et al. advise selecting the number of clusters that maximizes delta K, defined as the second order rate of change of the posterior probability of the data for a given number of clusters. Delta K was highest at K=8, but the difference between delta K values at different values of K was not very pronounced. There was less than a two-fold difference between delta K(K=8) and delta K(K=5) in our data, whereas in Evanno et al, their maximum delta K was over five times greater than the next largest value. Given that this methodology did not give a clear result, we decided to do longer runs with K=3 and K=6. We chose to try K=6 because we had six study plots, and K=3 because these plots were grouped into three geographic locations. For both values of K, we did one set of runs with no prior and one set of runs with sample collection location included as an informative prior (Hubisz et al. 2009). For the runs with sample location included as a prior, the number of collection locations used in the prior matched the value of K. All runs in the final set had a burnin of 100,000 and 500,000 MCMC repetitions.

3.3 Results

Plot description

The number of trees in our one-hectare plots ranged from 111 to 282 (Table 3.1). Coast redwood trees made up 83% to 100% of basal area in each plot. The number of redwood trees genotyped ranged from 86 to 182. Species that were intermixed with coast redwood at each plot are listed in Table 3.1.

Clonal diversity

Clonal diversity statistics for each plot are presented in Table 3.2. The number of clones in a one-hectare plot ranged from 24 to 129, resulting in clonal richness values from 0.15 to 0.82. The lowest richness value was from the upland plot at Big Basin (BBA) and the highest richness value was from the alluvial flat at Humboldt (HRWA). Clonal evenness values ranged from 0.62 to 0.93, with the lowest value at Prairie Creek (PC). The slope of the Pareto distribution, which describes heterogeneity, ranged from 0.92 to 2.88. BBA had the shallowest slope, indicating that it had a few clones with many ramets. HRWA had the steepest estimated slope, suggesting that clonal lineages were of roughly the same size. Slope coefficients for the Pareto distribution were statistically significant ($\alpha<0.01$) at all plots except at PC and r^2 values always exceeded 0.9. The p-value for PC was p=0.133, suggesting that the slope from the Pareto distribution was extremely flat. This indicates that there were a few clones with many ramets in this plot, or that the Pareto distribution did not fit the PC data well. This plot consisted of clones that fell into three size categories: one-ramet clones, two-ramet clones, and five-ramet clones. The small number and gap in the distribution of size classes in this plot likely led to the nonsignificant slope value for the Pareto distribution

due to poor fit. A histogram showing the number of clones with each number of ramets is shown in Figure 3.2.

Genetic diversity

Results for allelic richness comparisons are presented in Table 3.2. Our comparison of allelic richness including all ramets in each plot gave comparable values for each plot, ranging from 100 to 131 total alleles for all six loci. When we subsampled 24 genets per plot, we found that the Big Basin plots had the highest allelic richness. Within-population expected heterozygosity values were very similar between plots, ranging from 0.82 to 0.86. There were few private alleles in any plot out of the 172 total alleles. Number of private alleles per plot ranged from 2 to 11.

Spatial arrangement of clones

Aggregation index ranged from 0.29 to 0.96 over the six plots, and was inversely correlated with clonal richness (Table 3.2). BBA had the highest aggregation index while HRWA had the lowest aggregation index. There was significant aggregation of clones in all plots (p<0.001).

Clonal subrange for each study plot is shown in Figure 3.3. At every study plot except BBA, trees separated by less than 15 m had a non-zero probability of being clonal, but trees separated by more than 15 m were almost always different clones. At BBA a substantial proportion of trees up to 25 m apart were found to be clonal. In the non-BBA plots, there were a few pairs of clonal trees with inter-ramet distances greater than 15 m. The highest inter-ramet distance was 60 m at the alluvial flat plot at Big Basin (BBB). Plots varied in the average probability that trees within five meters of each other were clones. At BBA, 97% of trees within five meters of each other were clones, whereas at HRWA, only 35% of trees within five meters of each other were clones.

Spatial genetic structure

Mantel tests showed a weak positive correlation between physical distance and genetic distance between trees at both Big Basin plots and the hill-slope Humboldt plot (HRWB) (Table 3.3). Spatial autocorrelation results using either F_{ij} and R_{ij} were very similar, as were results using either distance or the natural logarithm of distance. We chose to present results on the relationship between F_{ij} and the natural logarithm of distance (Table 3.3). Spatial autocorrelation analysis showed a negative correlation between F_{ij} and log distance only at BBA. *Sp* statistics from the study plots ranged from -1.7 x 10^{-3} to 3.5 x 10^{-2}. *Sp* statistics were positive at all study plots except PC, where the correlation between kinship and distance was not significantly different from zero.

Population structure

AMOVA results suggested that most of the genetic variation in coast redwood occurred within study plots (Table 3.4). There was significant variation between plots as well, but the ratio of variance partitioning of within to between plot variance was three to one. The variance component for Site was negative, but can be interpreted as zero, as

permutation tests showed no significant variation due to Site. AMOVA results from all replicate data sets generated with GenoDive's allele-filling procedure and the dataset recoded as AFLP data were similar, suggesting that the GenoDive's procedure for filling in missing alleles did not bias results.

STRUCTURE analysis showed weak evidence of population genetic structure in runs with both K=6 and K=3. With either number of groups, there was a great deal of admixture within individuals, and individuals within a study plot or site were not consistently assigned into a single population. However, there was some evidence of assignment of samples into groups that corresponded with their site. In the STRUCTURE run with K=3, 61% of samples collected at Big Basin had a majority of their assignment into the same group. The same was true for 53% of samples collected at Humboldt and 60% of the samples collected at the two farthest north populations, Redwood National Park and Prairie Creek. The results from the STRUCURE runs with K=6 were less clear, with only 25-49% of samples from a given plot being assigned into a consistent group.

3.4 Discussion

Clonal diversity and structure

Our results showed a great deal of variation in clonal structure between study plots. Clonal richness varied more than five-fold between plots. There was less variation in clonal evenness and heterogeneity but low clonal richness values tended to correspond with high clonal evenness values and low clonal heterogeneity values. Differences in clonal structure between plots are best described by looking at histograms of the frequency of clones with different numbers of ramets (Figure 3.2). The amount of clonal reproduction did not seem to vary with geographic location: BBA and HRWB had the lowest clonal richness values and clones with the highest numbers of ramets. Given our limited number of study plots, we cannot statistically test whether variation in clonality is due to site history or broad scale geographic patterns. However, our data suggest that variation in clonal diversity and structure is primarily determined by local site history rather than geographic location. Further study is needed to determine how local disturbances or environmental conditions impact the amount of clonal reproduction in coast redwood stands.

While aggregation index values varied three-fold between plots, all plots had highly significant aggregation of clones. Estimates of clonal subrange, the range of physical proximity at which neighboring trees have a non-zero probability of clonal identity, was generally consistent across five out of six plots. Probability of clonal identity was high between 0 and 5 m but dropped off steeply at greater distances. Trees more than 15 m apart were very seldom members of the same clone, although there were a few instances of spatially dispersed clones with ramets located up to 60 m apart.

Excluding the upland Big Basin plot, our findings on patterns of clone size and diversity were consistent with Rogers' (2000) description of clonal patterns at Humboldt Redwoods State Park. Percent of clones that consisted of a single-ramet and distribution of clone sizes in our plots were similar to those reported by Rogers. We also confirmed her finding that ramets of a given clone could be spatially disjunct, and that different clones were often present in close physical proximity.

We found clonal diversity and structure of BBA to be more similar to second-growth redwood stands at Jackson Demonstration State Forest (Douhovnikoff et al. 2004), where multi-ramet clones had an average size of 6.7 ramets per clone. BBA had much larger clone sizes than the other plots, with one clone consisting of 27 ramets. It also had only three single-ramet clones (13% of total clones/plot) compared to the 68 – 85% of clones in other plots being single-ramet clones. Clonal subrange showed different trends in BBA as well. Probability of clonal identity was close to one for trees that were zero to five meters apart, and pairs of trees had a nonzero probability of clonal identity until they were more than 25 meters apart. This difference could be due to the fire history of this plot, which had a prescribed burn in 2001 (Tim Hyland, personal communication). Another possibility is that the difference in clonal structure could be due to a past history of selective logging in this plot, prior to the acquisition of this piece of land by Big Basin Redwoods State Park in 1906. Stumps were observed within the plot, indicating that a light selection harvest likely removed individuals in this stand (O'Hara 2014).

Our protocol of intensively sampling one-hectare plots provides useful information for designing sampling protocols for future studies of clonal reproduction in coast redwood. To include repetitions of the same lineage and many different clonal lineages (Arnaud-Haound et al. 2007), plot size would vary between study areas due to the great variation in clonality between plots. At the upland plot at Big Basin, which had the most spatially extensive clones, having a plot with a minimum 25 m radius would be necessary for capturing multiple clonal lineages in a plot. At the alluvial Humboldt plot, which was the most clonally diverse, a 12 m radius plot would be sufficient for capturing multiple clonal lineages. Future studies of clonal reproduction in coast redwood should utilize circular plots with 10 – 25 m radii, with the exact size based on the tradeoff between plot size and number of replicates needed to answer different study questions.

Consistent with previous studies of coast redwood (Rogers 2000, Douhovnikoff et al. 2004), it was not possible to make inference with certainty about genotypic identity based on proximity or even physical connectedness between trees. Although trees within 0 to 5 m of each other were much more likely to be clonal than trees with greater inter-tree distances, trees in close physical proximity were not always clones. In the most diverse plot, trees within five meters of each other had a probability of clonal identity of 35%. Information on clonal subrange can be used to design future studies of spatial autocorrelation or parentage analyses, where researchers may want to sample many individuals in close physical proximity but avoid re-sampling the same clone multiple times.

Genetic diversity

We found that levels of genetic diversity as measured by allelic richness, heterozygosity, and number of private alleles did not vary greatly between study plots or sites. This finding was consistent with Douhovnikoff and Dodd's 2011 paper looking at lineage divergence in coast redwood, but contradicted Brinegar's (2011) finding that southern populations tended to have lower allelic diversity at a chloroplast microsatellite locus. Although we found high levels of genetic diversity in all of our study plots, the fitness consequences of low diversity could potentially be more of a threat to populations further south in the range of coast redwood, where this species is confined to canyons and is present

at lower densities. It is important to emphasize that we only sampled one geographic locality (Big Basin) in the southern portion of redwood's geographic distribution.

The high levels of diversity found in all study plots can be explained by coast redwood's hexaploid condition, ancient lineage, and historically greater distribution in Western North America. Early ancestors of coast redwood were likely present in the Northern Hemisphere over 100 million years ago. Coast redwood's ancient lineage may be part of the reason for its great diversity, as neutral mutations should accumulate in a species over time. As recently as 5,500 years ago, coast redwood was present in coastal California, and likely had a broader, more contiguous distribution (Sawyer et al. 2000). The diversity that we sample in current redwood populations may be residual from times when coast redwood population sizes were much greater. High levels of genetic diversity coast redwood may be preserved by clonal reproduction: while small populations are susceptible to the loss of neutral alleles through genetic drift during sexual reproduction, alleles cannot be lost during clonal reproduction. Although clonal reproduction precludes the generation of novel genotypes, it may be contributing to the retention of high levels of genetic diversity in present redwood populations.

Spatial autocorrelation

Our tests for within-plot spatial genetic structure showed slightly different results based on the methodology used, but neither Mantel tests nor spatial autocorrelation analyses showed evidence of strong spatial genetic structure. Mantel tests showed significant positive correlation between physical and genetic distance at BBA, BBB, and HRWB, but the coefficients of these correlations were extremely small in all cases. Results from spatial autocorrelation analysis showed even less evidence of spatial genetic structure than Mantel tests, with only BBA showing significant spatial autocorrelation in permutation tests. Excluding BBA, *Sp* statistics from our study plots were lower than 45 out of 47 of statistics from studies analyzed by Vekemans and Hardy (2004). Extremely low values of *Sp* are characteristic of outcrossing, wind-pollinated trees, and our estimates seem consistent with what is known about the reproductive biology of coast redwood. Coast redwood's unusually high levels of diversity, likely due in part to its hexaploid lineage, may have also led to the low *Sp* values we observed.

One limitation of our spatial autocorrelation results is that our study only tested for spatial genetic structure on a small scale. To better understand gene flow and spatial genetic structure in coast redwood populations, sampling across broader spatial scales is needed. Rather than comprehensively sampling all trees in a plot or sampling along a grid, Vekemans and Hardy (2004) recommend sampling along multiple intersecting transects or devising a system of subplots that allows both small and large scale spatial genetic structure to be captured. Given that some of the gene flow in coast redwood likely occurs through wind-dispersed pollen, it is possible that spatial genetic structure in coast redwood occurs at a larger spatial scale than could be captured in our one-hectare plots.

Population Structure

Previous studies of coast redwood based on common garden experiments (Anekonda 1992, Kuser et al. 1995), foliar monoterpene profiles (Hall and Langenheim

1987), and microsatellite markers (Douhovnikoff and Dodd 2011, Brinegar 2012) have suggested genetic differentiation between northern and southern populations of coast redwood, although the location of the inferred break between northern and southern populations varied between studies. Common garden experiments and monoterpene profiles suggested a break at the San Francisco Bay (37.7833 °N, 122.4167 °W) (Hall and Langenheim 1987, Anekonda 1992, Kuser et al. 1995), while recent microsatellite work has suggested a break close to the border between Sonoma and Mendocino counties (38.7992 °N, 123.0172 °W) (Douhovnikoff and Dodd 2011). A break in either of these locations would lead us to expect that trees from the Big Basin plots would be genetically differentiated from the trees in Humboldt, Redwood National Park, and Prairie Creek plots. Contrary to this, our AMOVA results did not show significant variation between geographic locations. Our results were consistent with Rogers' (2000) discovery of high levels of genetic diversity within stands (Rogers 2000) and also showed significant variation between study plots. The results of our STRUCTURE analyses were consistent with high levels of diversity within plots. Many individuals were admixed and within a given plot or site individuals were not consistently assigned into the same group. However, the STRUCTURE results did seem to suggest some population subdivision between the three sites, and divisions were more pronounced when the model was run with three rather than six groups.

While the results of our AMOVA and STRUCTURE analysis may indicate a real lack of strong population genetic structure in coast redwood, several other factors may have inhibited our ability to detect population genetic structure. As our AMOVA only included six study plots nested within three sites, lack of replication may have inhibited our ability to detect population structure. We also found tremendous allelic diversity within our study plots, some of which is likely due to coast redwood's hexaploid lineage. Additionally, nuclear microsatellite markers may not be ideal for detecting population genetic structure in wind-pollinated conifers, as there is a great deal of gene flow through pollen in these systems. Future studies focused on detecting population genetic structure in coast redwood should consider using chloroplast DNA (cpDNA). Although these markers do not offer the same advantage for conifers as they do for angiosperms where cpDNA is maternally inherited, the reduction in effective population size for these uniparentally inherited markers should make it easier to detect a signal of population structure (Provan et al. 2001, Weising et al. 2005).

Broader implications

Our finding of variation in clonal structure between study plots demonstrates the need for further study on how the local disturbance history and environmental factors impact the reproductive ecology of coast redwood. The high levels of clonal diversity and lack of strong spatial genetic structure found at most of our plots supports Rogers' (2000) theory of episodic sexual recruitment in coast redwood stands. If the redwood stands in our study plots had been established by a few genotypes, the diversity of current clones found in the plot would have resulted from the gradual accumulation of enough mutations to result in unique genotypes. In this case, we would still expect to see a signature of spatial autocorrelation due to the gradual spatial spread and mutation of clonal lineages. Contrary to this, spatial autocorrelation was weak or absent in our study plots. Moreover, when we tested our clonal assignment protocol by gradually raising the clonal assignment threshold, we did not observe patterns of nearby trees converging into the same clone. Instead, as the threshold went up, randomly located trees were increasingly assigned into a single clone,

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which is not what would be expected if trees in the study plot had descended from a few original genotypes. Our results suggest that sexual reproduction plays an important role in the reproductive ecology of coast redwood. Incorporating age measurements with genotype data and finding a way to age clonal lineages would be extremely useful for understanding regeneration in old coast redwood forests.

Our study also points to a potential need to understand the long-term effects of timber harvest on coast redwood forests. The upland Big Basin plot, BBA, where there was evidence of selection harvesting, had lower levels of clonal diversity and larger clone sizes than any of the other plots. As this was only documented at one plot, further study is needed to determine the long term effects of timber harvesting on clonal structure. Although resprouting plays a role in the coast redwood's response to fire (Lorimer et al 2009), it is possible that the amount of sprouting stimulated by harvesting exceeds the amount of sprouting stimulated by other disturbances. Another possibility is that fire suppression has decreased opportunities for sexual recruitment of coast redwood, increasing the role of sprouting in coast redwood forests. Prior to European settlement, fire return intervals in the areas where our study plots were located have been estimated 8-500 years, with most studies giving estimates in the 10-50 year range (Lorimer et al 2009). Long term studies of post-logging stand development will be critical for understanding how to restore clonal structure of coast redwood forests throughout the species' range. Finally, we found little ecological justification for the extensive planting of a single clone that occurs in some commercial redwood forests in northern California. In our study plots, clonal spread occurred primarily at a scale of less than 15 m, and instances of ramets of the same clone separated by more than 15 m were rare. We found no cases where a single clone dominated even a quarter of a hectare, and generally found high levels of genotypic diversity within stands. Land managers should maintain high levels of genotypic and genetic diversity within stands to emulate natural patterns of clonal diversity and to maximize the adaptive potential of forests in the future.

Plot	Latitude	Longitude	Elevation (m)	No. redwoods genotyped	Species	No. stems	% Basal area
BBA	37.18056	122.23278	529	155	SeSe	157	83.2
					PsMe	42	12.5
					NoDe	81	4.3
					QuCh	2	<0.1
					Total	282	
BBB	37.18528	122.21444	493	100	SeSe	100	88.7
					PsMe	12	7.3
					NoDe	145	3.7
					ArMe	2	0.3
					UmCa	3	<0.1
					Total	262	
HRWA	40.34833	123.9244	111	158	SeSe	162	100
					NoDe	1	<0.1
					Total	163	
HRWB	40.34028	123.94833	249	182	SeSe	183	99.6
					NoDe	9	0.1
					UmCa	9	0.3
					TaBr	1	<0.1
					Total	202	
RNP	41.30750	124.02667	443	89	SeSe	98	89.0
					PsMe	15	8.9
					AbGr	37	1.0
					NoDe	74	1.0
					TsHe	4	0.1
					Total	228	
PC	41.37250	124.02528	106	86	SeSe	86	97.4
					PsMe	3	2.1
					TsHe	7	0.5
					RhPu	15	<0.1
					Total	111	

Table 3.1. Plot descriptions. SeSe = *Sequoia sempervirens*, PsMe = *Pseudotsuga menziesii*; NoDe = *Notholithocarpus densiflorus*; QuCh = *Quercus chrysolepis*; ArMe = *Arbutus menziesii*; UmCa = *Umbellularia californica*; TaBr = *Taxus brevifolia*; AbGr = *Abies grandis*; TsHe = *Tsuga heterophylla*; RhPu = *Rhamnus Purshiana*.

	BBA	BBB	HRWA	HRWB	RNP	PC
Clonal Richness (R)	0.15	0.73	0.82	0.53	0.65	0.78
Clonal Evenness (V)	0.92	0.81	0.76	0.93	0.82	0.62
Pareto Slope (β)	0.92	2.7	2.88	1.83	1.95	0.62
Pareto P-value	<0.001	<0.001	0.009	<0.001	0.01	0.13
Aggregation Index	0.97	0.43	0.29	0.59	0.47	0.30
Aggregation Index P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Allelic Richness	103	131	123	120	100	116
Avg Allelic Richness[1]	NA	101	87	89	82	88
95% CI Allelic Richness[1]	NA	93-110	79-95	82-97	76-89	81-95
H_S[2]	0.84	0.86	0.85	0.84	0.85	0.82
Private Alleles	4	11	4	3	2	4

Table 3.2. Clonal diversity, genetic diversity, and clonal structure statistics. [1]Average allelic richness and 95% CI were generated by subsampling 24 genets from each plot. BBA was not sampled because this plot had only 24 genets present total.

	BBA	BBB	HRWA	HRWB	RNP	PC
Mantel Tests						
Coefficient[1]	<0.001	<0.001	0	<0.001	0	0
P-value	0.008	0.007	0.25	0.019	0.091	0.472
Spatial Autocorrelation						
Regression Coefficient	-0.033	-0.004	-0.002	-0.004	-0.003	-0.002
P-value	0.003	0.081	0.087	0.057	0.311	0.561
Sp	0.036	0.004	0.002	0.004	0.003	-0.002

Table 3.3. Spatial genetic structure test results. [1]Mantel test coefficients <0.001 were all positive.

Source of variation	df	SS	MS	Est. var.	% var.	P-val
Within plot	443	5851.55	13.21	39.20	97.6	-
Among plot	3	152.33	50.78	13.20	32.9	0.001
Among site	2	237.21	118.60	-12.23	-30.4	0.28

Table 3.4. Analysis of Molecular Variance Results. df = degrees of freedom, SS = sum of squares, MS = mean squares, Est. var. = estimated variance, % var. = percent of total variation, P-val = P-value.
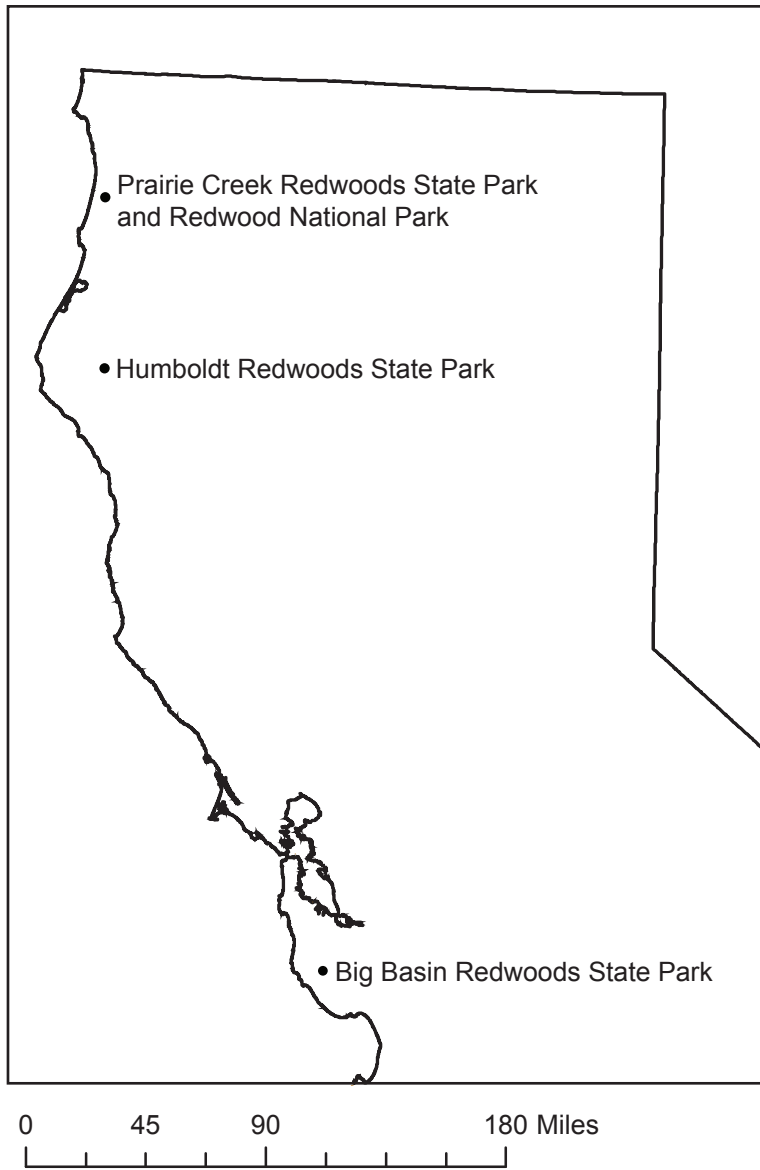
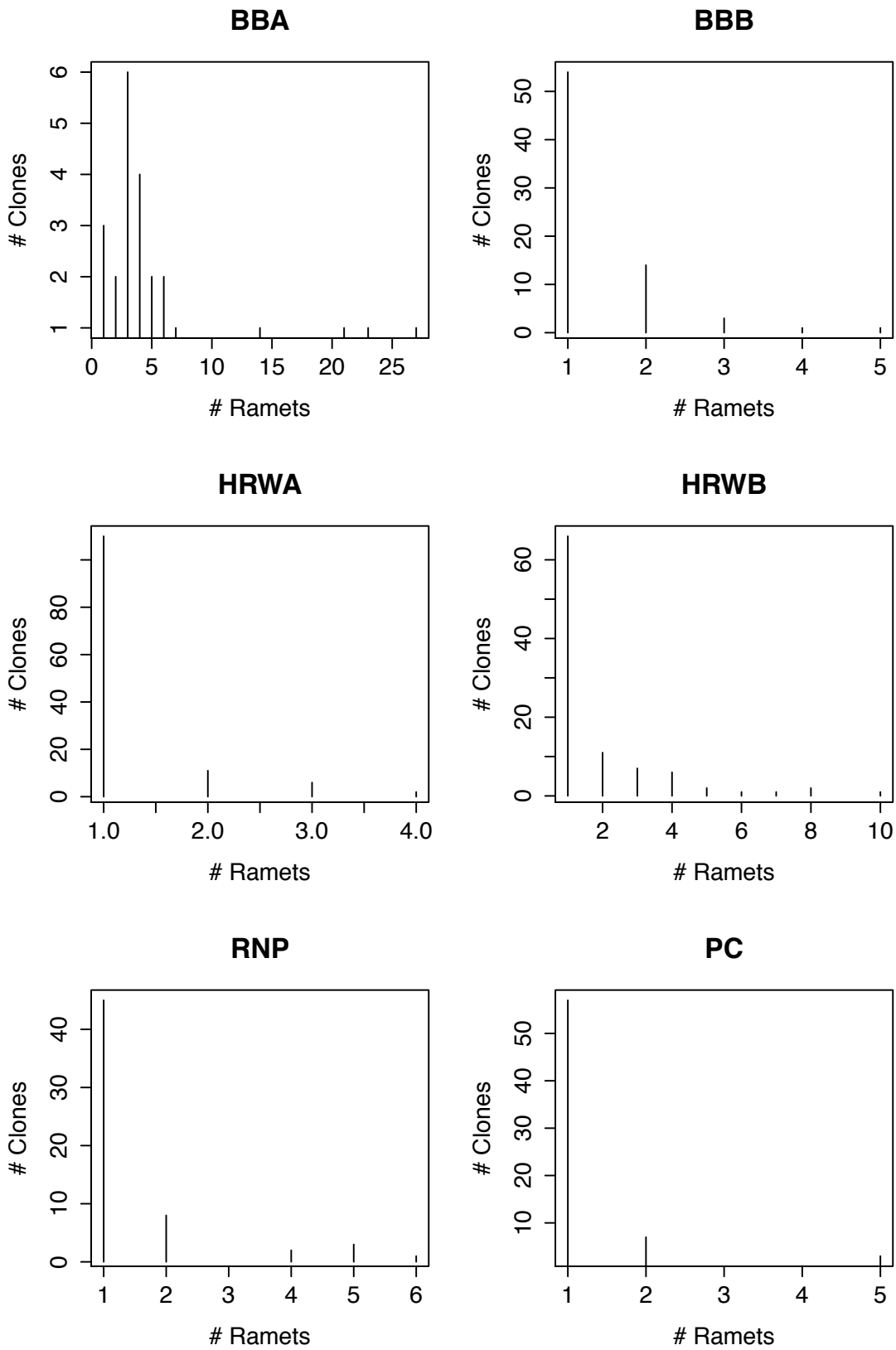Figure 3.1. Map of study plot locations.

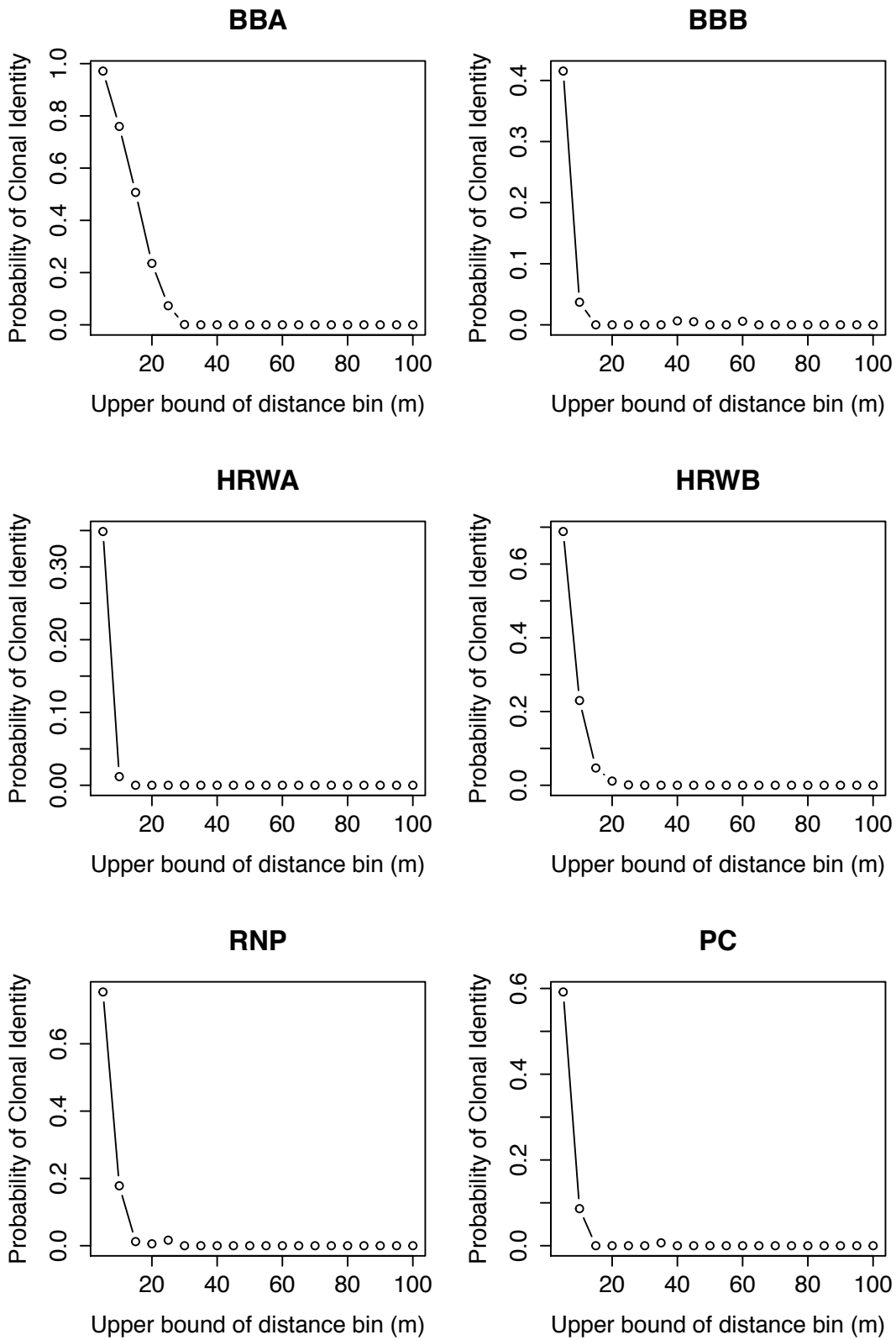Figure 3.2. Histogram of frequencies of clone sizes at each study plot.

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Figure 3.3. Clonal subrange at each study plot.

Chapter 4: Spatial Clonal Patterns and Forest Structure

4.1 Introduction

Over the past few decades, one important research area in applied forest ecology has been the definition and quantification of structural complexity in forested ecosystems. With the growing recognition that structural complexity is closely tied to ecosystem function and habitat value (Messier et al. 2013), forestry researchers are increasingly interested in quantifying spatial patterns of trees in old forests. Identifying spatial patterns and understanding the ecological processes that generate these patterns in old forests (i.e. old growth forests, (O'Hara et al. 1996)) is extremely useful in generating guidelines for the management and restoration of human-influenced forests.

In California, remaining old forests dominated by coast redwoods (*Sequoia sempervirens* (D. Don) Endl.) are unique ecosystems, hosting many wildlife species and storing great amounts of carbon due to their high productivity and the longevity of individual redwood trees (Noss 2000). However, approximately 95% of the historic range of coast redwood has been harvested, and second-growth forests have become an important source of timber in northern California (Noss 2000). Being able to restore and manage second-growth coast redwood forest to resemble old forest reference conditions may allow for improved ecosystem function and resilience on these landscapes.

Recent research has suggested that spatial heterogeneity is an important component of ecosystem resilience (Levin 1998, Churchill et al. 2013). One attribute of coast redwood's reproductive biology that may impact spatial patterns is its ability to reproduce clonally through sprouting. Sprouts may originate from dormant buds around the base, the stem, or branches, or from roots. Basal sprouting is stimulated by fire and harvesting (Lorimer et al. 2009, Ramage et al. 2010). Sprouts generally grow very quickly, easily outgrowing redwood seedling regeneration (Jameson and Robards 2007), but have reduced growth and survival probability under low light conditions (O'Hara and Berrill 2010).

Spatial patterns in old redwood forests have been a topic for study in recent decades with advances in spatial statistics. A previous study of spatial patterns of coast redwoods in three old redwood forests located on alluvial flats at Armstrong and Humboldt Redwoods State Parks found that understory regeneration (stems < 15 cm diameter at 1.37 m: dbh) tended to be clumped around larger trees (stems > 15 cm dbh) and posited that this regeneration was primarily of sprout origin (Dagley 2008). Although regeneration was clumped, the study found trees in the largest size class at each plot to be randomly dispersed. Similar findings were reported for one-hectare plots in an upland old redwood forest at Redwood National Park (van Mantgem and Stuart 2012). Using methods that controlled for differences in the underlying densities of coast redwoods, van Mantgem and Stuart found that coast redwood trees >20 cm dbh in three out of six one-hectare plots were clumped at small spatial scales (0-5m). At larger spatial scales, coast redwood trees were randomly distributed in all six plots.

The assumption that clumped regeneration results solely from clonal sprouts may not be correct. A previous study of clonal patterns of an old coast redwood stands at

Humboldt Redwoods State Park found that clumps of coast redwood trees were not always composed of a single clonal lineage (Rogers 2000). Instead, clumps sometimes contained more than one clone. This study also documented extremely high clonal diversity. Fifteen to thirty-four percent of redwood clones sampled were multi-stemmed, and multi-stemmed clones had averages of 1.17-1.53 stems per clone. This study also found some instances of spatially disjunct clones, with a maximum between-ramet distance of 340 m. Results from second growth stands at Jackson Demonstration State Forest in Mendocino County confirmed Roger's finding of clonally-mixed clusters and spatially disjunct clones (Douhovnikoff et al. 2004). However, clone sizes in Douhovnikoff's second-growth stands were much greater than those reported for old growth stands, and fewer single-ramet clones were present.

While past work has documented spatial and clonal patterns separately, our research integrates spatial sampling and genotyping to determine how clonal reproduction affects spatial patterns in coast redwood forests. By mapping and genotyping coast redwoods in six one-hectare plots in old forests, we are able to investigate variation in spatial patterns of coast redwood forests and determine how much of that variability is due to differences in clonality. This information has implications for redwood ecology and may inform appropriate planting and thinning practices and guide redwood restoration practices. For example, study results may also inform how laboratory-propagated clones are planted in second-growth timber forests by providing a frame of reference for the spatial patterns of clones, and clonal spread in old redwood forests.

4.2 Methods

Sample collection and stem mapping

Three pairs of one-hectare plots were established at sites within the natural range of coast redwood on the California coast (Figure 3.1). Study plots within pairs were separated by 1.7 to 7.2 km. One of the Big Basin plots (hereafter "BBA") was located at a mid-slope position in an area that was treated with prescribed fire in 2000-2001 (Tim Hyland, personal communication). A few trees were harvested in this plot before this area was added to Big Basin State Park, likely more than 100 years before sampling. The second Big Basin plot ("BBB") was located at a lower slope position, approximately 380 m from Opal Creek. At Humboldt Redwoods State Park, one plot ("HRWA") was located in Founders Grove, with the entire plot located in the alluvial flat. A second plot at Humboldt ("HRWB") was located on a lower slope position, with the plot spanning both a north-facing slope and alluvial flat adjacent to Bull Creek. Within the Redwood National and State Parks complex, one plot ("RNP") was located at a mid-slope position close to Lady Bird Johnson Grove in Redwood National Park. The farthest north plot was located at Prairie Creek Redwoods State Park ("PC"), approximately 230 m east of Godwood Creek in a lower slope position.

At each plot, all trees over ten cm dbh were mapped, measured for dbh, classified by canopy position and strata, and identified to species. All coast redwood trees were cored for cambium/sapwood samples using an increment borer. Cambium samples were preserved in bags of silica gel. Wherever foliage could be accessed—including epicormic sprouts or basal sprouts—it was collected in a ziplock bag with a few drops of water. Both wood and leaf samples were stored in a 4 degree C freezer within two weeks of collection.

DNA extraction and analysis

DNA extraction followed a modified CTAB method (Cullings, 1992). Fragments for microsatellite analysis were amplified using six microsatellite primers. We used primers "seq8e8" (dinucleotide repeats) and "seq18d73" (trinucleotide repeats) from Bruno and Brinegar (2004) and "rw28" and "rw39" (tetranucleotide repeats) from Douhovnikoff and Dodd (2011). Additionally, we developed two new primers, "rw56" (tetranucleotide repeats) and "rwdi11"(dinucleotide repeats) (Narayan et al. 2015). Microsatellite data were analyzed using GeneMapper v4.0 software. Laboratory methods are described fully in Narayan et al. (2015).

Clonal identification

Clones were identified by calculating the Bruvo genetic distance between all pairs of trees and assigning any pairs with a genetic distance less than 0.2 into the same clonal lineage (Bruvo et al. 2004). Genetic distance and clonal assignment calculations were performed using *polysat* (version 1.3-2 – 1.3-3; Clark and Jasieniuk, 2011) in R (R Core Team 2014, version 3.1.1; www.R-project.org). For details on the methodologies used to confirm the accuracy of clonal assignment, see Narayan et al. (2015).

Plot description

Histograms were used to visualize diameter distributions of trees in each of the six study plots. All species were included in the diameter differentiation histograms. Coefficients of variation in diameter and the diameter differentiation index were also calculated for redwood trees in each plot. Diameter differentiation index (*T*) was calculated only for first nearest neighbors as described in Pommerening (2002):

$$T_{ij} = 1 - (min(DBH_i, DBH_j) \; / \; max(DBH_i, DBH_j))$$

T_{ij} values for each tree and its nearest neighbor were averaged to calculate the diameter differentiation index for each plot.

As is typically found in coast redwood, there were some trees that were separate stems at breast height in each study plot, but physically connected lower down on the bole. The proportion of pairs of connected trees that were not from the same lineage was calculated.

Clonal pattern descriptors

Several different methods were used to describe clonal patterns at each plot. To quantify spatial aggregation of clones in each plot, aggregation indices were calculated as described by Arnaud-Haond et al. (2007). This metric describes the degree of spatial aggregation of clones by comparing the observed proportion of trees with a clone-mate as their nearest neighbor to the theoretical probability of having a clone-mate as a nearest neighbor if clones were randomly located within the study plot. An aggregation index value close to one signifies that clones are highly aggregated, whereas values close to zero would result from either clones being randomly dispersed throughout the plot or from having few

trees in the plot belonging to a common clonal lineage. The significance of aggregation in each plot was tested by randomly shuffling the clonal identities of trees among the sampling coordinates. Nine hundred and ninety-nine shuffled data sets were created and the aggregation index value of each was calculated to generate a null distribution with no spatial aggregation of clones. Aggregation indices from the original, unmanipulated datasets were compared to the null distributions to determine whether there was significant aggregation at each plot.

Diagrams were also created to visualize clonal subrange, defined here as the physical range at which clonal spread occurs. For each plot, the distance between each pair of trees was calculated. These distances were binned into five m distance classes. Within each distance class, we calculated the percentage of pairs that were clonally identical, giving the probability of clonal identity within each distance class. To complement clonal subrange analysis, pairwise distances between clonal trees were calculated for each plot. Intraclonal distances were visualized using histograms, and median, mean, and maximum intraclonal distances were calculated for each plot.

Spatial pattern analysis

To identify non-random spatial patterns of coast redwood trees, three different techniques were used: the aggregation index of Clark and Evans (1954), Ripley's K statistic (1976), and continuum percolation cluster analysis (Plotkin et al. 2002). The Clark-Evans index (R) is calculated by dividing the observed average distances between each tree and its nearest neighbor to the theoretical value that would be expected in a stand of randomly located trees (Clark and Evans 1954). Values of R less than one indicate spatial clustering whereas values greater than one indicate regularity. To evaluate the significance of clustering or regularity of coast redwood trees in our plots, we used a function in the R package spatstat (version 1.41-1; Baddeley and Turner 2005) that simulates random point patterns with the same intensity as the observed spatial pattern to generate a null distribution against which to compare the value of R from the observed data. We specified that the function should simulate 999 random patterns of trees for each plot, and used the "Donnelly" edge correction for both the observed and simulated data (Donnelly 1978).

For plots that deviated from spatial randomness in the Clark-Evans test when all coast redwood trees were included, we also performed the tests on two reduced datasets for each plot, where only one point per clone was included. Tests on reduced datasets were intended to assess whether deviations from spatial randomness were due to clonal reproduction. For the first reduction sample locations for multi-stemmed clones were calculated as follows: for clones with three or more ramets, we used the centroid of the convex hull containing the coordinates of all individuals of a clone as its spatial location. Both convex hulls and their centroids were calculated in the R package "spatstat" (Baddeley and Turner 2005). For clones with two ramets, we averaged the x and y coordinates of the clonal individuals as its location, and for one-ramet clones the original x and y coordinates were preserved. For the second reduction, we used the x and y coordinates of the largest-diameter ramet of each clone.

In addition to the Clark-Evans index, which only tests for departures from spatial randomness by looking at nearest neighbor differences, we tested for deviations from spatial

randomness at broader spatial scales using Loosmore and Ford's goodness-of-fit (GoF) test based on Ripley's K statistic (Loosmore and Ford 2006). Ripley's K statistics are calculated as the averaged number of points within a given radius of each point in a spatial point data set. Deviations from spatial randomness are assessed by comparing Ripley's K values from the observed data to Ripley's K values from simulated random point patterns of the same intensity. To correct for the inflated type I error rate that results from comparing the observed and null patterns at multiple spatial scales, the Loosmore and Ford method reduces the differences between observed and averaged null patterns at all distances at which Ripley's K is calculated to a single summary statistic (u_i). The value of this summary statistic from the observed point pattern is compared to values generated by comparing each simulated data set to the averaged null values to determine a p-value to test the hypothesis that the observed spatial pattern is non-random. The R code provided in the supplement of Loosmore and Ford (2006) was used to implement this test. The number of simulations to use for this test was selected using Loosmore and Ford's (2006) formulas for the desired precision in estimating the p-value. We chose a p-value of 0.04 and confidence interval width of 0.02, such that even if noise in p was equal to the extremes of the confidence interval, the p-value would not exceed 0.05. This methodology gave us 1535 as the number of simulated data sets to use. For plots that deviated from spatial randomness, Loosmore and Ford's test was also run on the two datasets that were reduced to one point per clone as previously described.

To investigate the scale of potential spatial clustering within plots, we used the continuum percolation cluster analysis method developed by Plotkin et al. (2002). In this method, trees within a threshold distance (d) of each other are assigned into the same cluster. For clusters consisting of more than two members, not all trees in a given cluster must be within d of each other, but only need to be within d of one other member of the cluster. For example, if tree A is within d of tree B, and tree C is within d of tree B, all three trees are assigned to the same cluster regardless of the distance between tree A and tree C. The number and size of clusters will vary with different values of d: when d = 0, the number of clusters will equal the number of trees, and each cluster will have a size = 1. As d becomes large, trees will gradually be assigned into a single cluster, with a size equal to the number of trees in the study area. To make results comparable between plots with different tree densities, Plotkin et al. suggest calculating normalized mean cluster size (c_{norm}) and normalized distance parameter (d_{norm}). Normalized mean cluster size (c_{norm}) is calculated as:

$$c_{norm} = c_{avg} \, / \, n$$

where c_{norm} is the mean cluster size at a given value of d and n is the number of trees in the study area. The normalized distance parameter (d_{norm}) is calculated as:

$$d_{norm} = 2d\sqrt{(n/A)}$$

where A is the sample area. To visualize how mean cluster size changes with different threshold distances, normalized mean cluster size was plotted against the normalized distance parameter for each plot. Generally, regions of the continuum percolation plots with a steep slope indicate regions where normalized cluster size increases rapidly in response to slight increases in d. Plateaus within the plot show regions where normalized cluster size remains constant within a range of d values. We were most interested in identifying plateaus

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in continuum percolation plots from our study areas to determine whether these scales of clustering corresponded with intraclonal distances and whether scales of clustering were consistent between plots.

In addition to inspecting continuum percolation plots for scales of clustering, we also developed a method for finding the value of *d* for each study plot that would lead to the assignment of trees into clusters that best matched clonal lineages. To do this, we compared clonal identities of each pair of trees to cluster identities assigned to pairs of trees at a range of d values. For each value of d, we counted the number of non-clonal tree pairs that were assigned into the same cluster and the number of clonal tree pairs that were assigned into different clusters. We divided the number of mis-assignments for a given value of *d* by the number of pairwise comparisons to calculate the error rate for a given value of *d*. The value of *d* that minimized error rate was recorded for each study plot. This optimal value of *d* that minimized the error rate of clonal assignment was used to assign trees into clusters, and the frequencies of cluster sizes for each plot were visualized as histograms.

4.3 Results

Plot description

Diameter distributions for all species are shown in Figure 4.1A – Figure 4.1.F. All six plots show negative exponential or "reverse-J" diameter distributions with large numbers of relatively small trees and fewer trees in the largest size classes. At all plots, the larger size classes were dominated by coast redwood trees whereas smaller trees were generally a mixture of redwood, tanoak (*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & Oh syn. *Lithocarpus densiflorus*), coast Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), grand fir (*Abies grandis* (Dougl. Ex D. Don) Lindl.), and California bay (*Umbellularia californica* (Hook. & Arn.) Nutt.).

Coefficient of variation (*CV*) and diameter differentiation index (Pommerening 2002) for each plot are presented in Table 4.1. The highest *CV* values were at the two Humboldt Redwoods State Park plots. At these plots, coast redwood was the most common species in all diameter classes. Upland Big Basin plot BBA had the next highest *CV* in coast redwood diameters, followed by the Prairie Creek (PC) plot and the Redwood National Park plot (RNP). The Big Basin plot BBB had the lowest *CV* in coast redwood diameters. This metric seemed to be strongly affected by the number of coast redwood trees in the smallest size classes at a given plot. Diameter differentiation index was generally similar between plots, ranging from 0.50 to 0.64. This range falls within the category of "big differentiation", on Pommerening's (2002) classification scale, suggesting that small and large trees were intermixed at all plots.

A total of 111 pairs of connected trees were found in the six plots. Eleven out of the 111 pairs were non-clonal. Connected non-clonal trees occurred in four out of six study plots.

Clonal pattern descriptors

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Aggregation of clones was significant at all six plots (p<0.001). Aggregation index was highest at BBA, where there were only 24 distinct clones detected in the 155 trees sampled. HRWB had the next highest aggregation index values, most likely due to the presence of a few clones with seven to ten ramets. HRWA and PC had the lowest aggregation index values.

Clonal subrange plots are shown in Figure 3.3. Clonal subrange at BBA differed from the other plots: in BBA, a considerable proportion of tree pairs up to 25 m apart were clonal and trees within 0-5 m of each other were almost always from the same clone. In the other five plots, probability of clonal identity generally tapered off around 15 m. Plots differed in the probability of clonal identity of trees within five m of each other. At HRWB, RNP, and PC roughly 60-80% of tree pairs separated by less than 5 m were from the same clonal lineage, whereas at BBB and HRWA only 35-45% of tree pairs separated by less than 5 m were clones.

Distributions of intraclonal distances for each plot showed a consistent pattern of numerous small intraclonal distances and considerable right skew (Figure 4.3, Table 4.2). Median intraclonal distance was highest by a considerable margin at BBA, and the distribution of intraclonal distances in BBA was continuous up to 25 m. The other five plots showed a majority of intraclonal distances less than 5 m, with median intraclonal distances ranging from 1.86 to 4.84. However, larger intraclonal distances were consistently present as well. Maximum intraclonal distances were greater than 20 m at all plots except HRWA, and the BBB plot contained one tree that was separated from other individuals in its clone by almost 60 m. The right skew in the distribution of intraclonal distances led to mean intraclonal distances that were considerably higher than the median values at each plot (Table 4.2).

Spatial pattern analysis

Clark-Evans tests performed on spatial patterns of all redwoods trees showed significant clustering in all plots except PC (Table 4.1). BBA had the lowest *R* value, indicating the greatest degree of clustering. When the datasets for each plot were reduced down to one point per clone, the patterns of clustering previously detected in all five plots were no longer present. The Clark-Evans test performed on the centroids of convex hulls containing the members of each clone showed nearest neighbors to be overdispersed at HRWB, but other than this test, none of the reduced datasets deviated from spatial randomness.

Deviations from spatial randomness detected by Loosmoore and Ford's (2006) GoF tests differed slightly from Clark-Evans test results. Clumping was detected by GoF tests at BBA, BBB and PC. When the datasets for these plots were reduced to one point per clone, deviation from spatial randomness was no longer detected at BBA, but remained at BBB and PC. For BBB and PC, we visualized the results for the observed Ripley's K value and the simulation envelope created by taking the minimum and maximum K values from 1535 random simulated point patterns to determine at what spatial scales clustering was occurring. At BBB, observed K values exceeded the simulation envelope at scales from roughly 8 – 25 m when the dataset was reduced to only the largest member of each clone. When the dataset was reduced by taking the centroid of the convex hull, the clustering appeared at the 20-25

m spatial scale. At PC, clustering appeared only at larger spatial scales, with observed K values exceeding the simulation envelopes at 21-25 m for the convex hull reduction and 23-25 m for the biggest tree reduction.

Continuum percolation plots showing normalized mean cluster size as a function of normalized distance did not show signs of spatial clustering at scales that reflected the influence of clonal patterns (Figure 4.4). Normalized values of *d* that led to the most accurate assignment of trees into clusters that corresponded with their clonal identity are marked onto the continuum percolation plots. Optimal *d* values were small, ranging from 2 to 5.75 (Table 4.3).

Plots BBA, BBB, and PC, where clustering was detected by GoF tests, appeared to have plateaus that could potentially indicate scales of spatial clustering. However, the range of *d* values spanned by plateau areas in these plots was much larger than optimal *d* values. Using the optimal value of *d* at each plot led to the assignment of the majority of trees into one-tree clusters (Figure 4.5). Plots showing the frequency of different cluster sizes showed highest frequencies of one and two-tree clusters, with the length of right-skewed tail varying between plots.

4.4 Discussion

Overall, our results suggest that clonal reproduction causes small-scale clustering in coast redwood forests, generally at scales of less than 10 m. The Clark-Evans tests for five out of six plots showed that coast redwoods were closer to their nearest neighbors than would be expected for randomly dispersed trees, but these trends disappeared when the data was reduced to one point per clone. This suggests that the clustering detected by this test was not due to factors such as topography or microsite quality. This is consistent with the low intraclonal distances found at each plot; although there were a few instances of clones being separated by larger distances (20-60 m), median intraclonal distances were low, and clones were spatially aggregated at every plot.

The Goodness of Fit test used to detect clustering at multiple spatial scales showed significant clustering in only three out of six plots (BBA, BBB, and PC). When the dataset was reduced to eliminate multiple points per clone, the GoF test results still showed significant clustering at BBB and PC, suggesting that clustering was not due to clonal patterns at these sites. Additionally, comparing observed values of Ripley's K from the reduced datasets to simulation envelopes showed clustering at a larger scale (8 - 25 m) than the majority of intraclone distances. Looking at stem maps for these plots (Figures 4.2.B and 4.2.F), there seems to be some variation in the underlying density of coast redwood trees in these plots, potentially due to topographic factors, which may explain some of the clustering detected by the GoF tests. In future studies, sampling environmental covariates could allow for a better understanding of factors that lead to aggregation at broader spatial scales.

Coast redwoods in all plots fell into a wide range of size classes, and diameter differentiation values were high. Diameter distributions from our plots were consistent with those previously documented in old redwood forests. Similar to the diameter distributions originally measured by Veirs (1982) and summarized in Lorimer et al. (2009), some of our diameter distribution plots displayed a negative exponential shape (BBA, HRWA, HRWB),

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but the others had somewhat irregular distributions. In plots where small coast redwood trees were not numerous, the smaller size classes were dominated by other species. These results are consistent with previous work in northern hardwoods that showed diameter distributions were sensitive to scale (Janowiak et al. 2008). At BBB the understory was dominated by tanoak. Tanoak dominated the understory of "RNP", along with grand fir. At PC, many small cascara buckthorn (*Frangula purshiana* (DC.)Cooper) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) trees were present. At all plots, coast redwoods were present in the smallest diameter classes along with other species, even when they were not the most abundant.

Results from our spatial pattern analysis were generally consistent with those reported in Dagley 2008 and van Mantgem and Stuart 2012, although differences in minimum tree size and methodology somewhat limit comparison. Consistent with results from Dagley's regeneration sampling and van Mantgem and Stuart's inhomogeneous K analyses, we found patterns of clumping at small spatial scales due to clonal reproduction. When we reduced our dataset to one point per clone, we found that coast redwoods appeared to be randomly dispersed at four out of six plots, which is consistent with Dagley's findings for trees in the largest size class at each of her plots. As previously described, two of our plots, BBB and PC, showed clustering in the reduced datasets, at scales >8 m. This differs from Dagley's findings for alluvial flats, but is consistent with van Mantgem and Stuart's finding that underlying densities of coast redwood trees varied within one-hectare plots. Results from these two studies and our findings demonstrate the variability in stand structure in old coast redwood forests, which may vary due to environmental factors, interspecific interactions, disturbance history, and site quality, in addition to varying in patterns of clonal reproduction.

Continuum percolation analysis provided an interesting complement to the Clark-Evans and GoF tests. The optimal values of *d* for clonal assignment for each plot were very similar to the median intraclonal distance at that plot (Tables 4.2 and 4.3), except for BBA. Using the optimal value of *d* at each plot led to the assignment of a majority of trees into one-tree clusters, which was consistent with high levels of clonal diversity and presence of many single-tree clones. Plots varied in their maximum cluster size, but aside from the BBA plot, clusters assigned using the optimal *d* were generally small (1-8 trees).

The BBA plot was an outlier in terms of clonal pattern. Coast redwoods were more highly aggregated at BBA than at the other plots, and fewer clones were represented at this site. A few clones in BBA were represented by numerous stems (up to 27) and larger clonal clumps led to greater mean and median interclonal distances at BBA compared to the other plots. The distinctiveness of BBA could be due to its site history. Prescribed fire took place in the stand within the last two decades (Tim Hyland, personal communication), which was most likely more recent than fires at other plots. There was also evidence of past selection harvesting at this plot. Land ownership records showed that the area of the park in which BBA was located was acquired from Big Basin Lumber Company in 1906, so this harvesting likely took place prior to that date. Tree removals would be expected to stimulate the development of new sprouts (O'Hara 2014) thereby developing many sprouts or individual stems in highly aggregated patterns.

Results from BBA suggest that selection harvesting may have long-lasting effects on clonal patterns in coast redwood forests, although inference can not be made with certainty due to lack of replication. Our finding of larger clone sizes at BBA is consistent with Douhovnikoff et al's 2004 study, which reported a mean clone size of 6.7 ramets/clone in 50-70 year old second-growth redwood forest sites at Jackson Demonstration State Forest in Mendocino County (Douhovnikoff et al. 2004). Further study is needed to determine whether clonal patterns in stands with a history of timber harvest gradually return to old-forest structure, or whether cutting fundamentally changes the regeneration ecology and spatial structure of coast redwood forests.

In terms of providing guidance for restoration, our results suggest that managers seeking to restore coast redwood forests to old forest structure should retain a mix of both small-scale clustering that mimics the spatial and clonal patterns of uncut old redwood forests and single-stem clones. In our five plots with no history of timber harvest, $68 - 85\%$ of clones were represented by a single ramet, and the optimal continuum percolation algorithm assigned the majority of trees into single-clone clusters. Spatial clonal clusters identified by this algorithm included 1-8 stems. We recommend that managers designing planting or thinning regimes consider the distribution of cluster sizes identified by the continuum percolation algorithm. For example, variable-density thinning protocols (Carey 2003, O'Hara et al. 2010) in dense second-growth coast redwood stands could be modified so the number of trees retained in a given area is randomly drawn from a distribution that matches the distribution of different clonal cluster sizes documented in old coast redwood forests. Additionally, given our finding of many single-clone clusters, planting seed-origin coast redwood seedlings in spatially random patterns in areas where clonal regeneration is present but patchy may be a very effective way to re-create the spatial and clonal structure of old redwood forests.

Regeneration in coast redwood forests is a mixture of clonal and sexual reproduction. The dominance of asexual regeneration in the regeneration of cutover stands is well-documented (Neal 1967, Noss 2000, Douhovnikoff et al 2004, O'Hara et al. 2010) and recruitment through sexual reproduction is believed to be infrequent due to low seed viability (Davidson 1970, Becking 1996, Ornduff 1998) and high seedling mortality (Jacobs 1987, Olson et al. 1990). The diversity of clones in the one-hectare plots sampled in this study demonstrates suggests sexual reproduction does occur, but probably on a very infrequent basis. Redwood stems may live 1000 years or longer (Fritz 1929, Veirs 1982) and clonal lineages may persist for many millennia. Hence, the addition of a very small number of seedling-origin redwoods per century may be more than adequate as a replacement rate in established stands, and the genotypes of original seedlings in newly colonized areas may persist long into the future.

Two other processes demonstrate the distinctive and complex role of asexual reproduction in the regeneration ecology of coast redwood. First, disjunct clones separated by as far as 60 m in this study and as far as 340 m in Rogers (2000), indicate that redwood clones can spread over very large distances but at unknown temporal scales. Second, we found fused trees that included multiple clones on 4 of 6 plots. Rogers (2000) and Douhovnikoff (2004) found similar mixtures of clones in old forest and second-growth stands, respectively. Long-term redwood regeneration ecology apparently involves migrating clones through possible mechanisms including 1) tree falls and branch falls that become

rooted to form disjunct clones; and 2) migration of clones through basal sprouting near the base of large-diameter trees. The result is that ramets may migrate and become separated, and clones may migrate towards other clones forming connected but non-clonal clumps. Over time, slow migration of clones may contribute to the patterns of diverse and intermixed genotypes we observe in coast redwood forests.

In summary, our work supports the idea that clonal reproduction plays an important role in determining small-scale spatial patterns in coast redwood-dominated old forests. Models of stand development that assume competitive exclusion will lead to reduced tree densities and greater inter-tree distances may not apply to coast redwood due to its unique reproductive ecology and the ability of distinct genotypes to fuse together. To re-create stand structures approximating old forest reference conditions, land managers should incorporate a mix of seedling and sprout-origin regeneration planted in both single and multi-tree clusters.

	BBA	BBB	HRWA	HRWB	RNP	PC
Coefficient of variation[1]	0.93	0.57	1.08	1.04	0.70	0.79
Diameter differentiation index[1]	0.60	0.50	0.64	0.63	0.53	0.63
Clark-Evans test						
All redwoods	0.54	0.71	0.88	0.80	0.72	0.95
P-value	0.002	0.002	0.008	0.002	0.002	0.378
Clonal centroid	1.15	0.90	1.07	1.12	0.96	-
P-value	0.19	0.16	0.16	0.04	0.58	-
Largest tree in clone	1.08	0.92	1.06	1.10	0.95	-
P-value	0.56	0.20	0.16	0.07	0.42	-
K test[2]						
All redwoods	0.0007	0.02	0.2	0.48	0.09	0.0007
Clonal centroid	0.07	0.02	-	0.04	0.69	0.0006
Largest tree in clone	0.19	0.03	-	0.04	0.69	0.0006

Table 4.1. Plot description and spatial pattern analysis metrics. [1]Coefficient and variation and diameter differentiation index were calculated for coast redwood trees in each plot. [2]P-values from Loosmore and Ford's method for statistical inference from Ripley's K values.

	BBA	BBB	HRWA	HRWB	RNP	PC
Aggregation index	0.97	0.43	0.29	0.59	0.47	0.30
Median intraclonal distance	9.88	2.55	1.86	4.84	3.93	3.03
Mean intraclonal distance	10.17	9.63	2.28	5.93	5.31	4.29
Maximum intraclonal distance	24.15	59.62	7.92	20.02	22.96	32.52

Table 4.2. Clonal pattern descriptors.

	BBA	BBB	HRWA	HRWB	RNP	PC
Optimal d for continuum percolation	5.75	2.25	2	3.5	4.5	3
Error rate with optimal d	0.03	0.005	0.002	0.009	0.007	0.005
Number of clusters identified with optimal d	23	79	127	108	51	69

Table 4.3. Results from continuum percolation optimization.
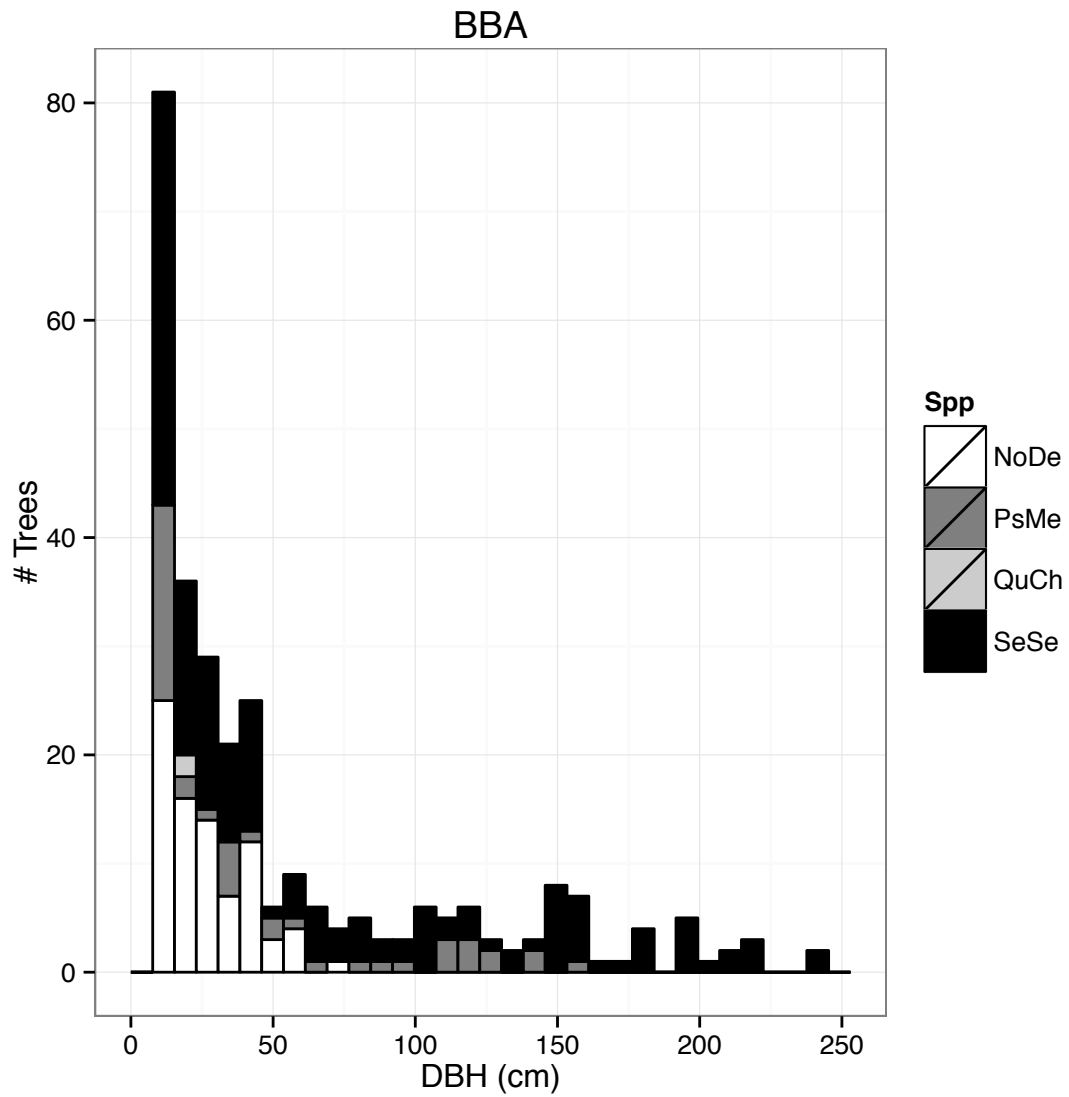
Figure 4.1.A. Diameter distribution for Big Basin A plot. NoDe = *Notholithocarpus densiflorus*; PsMe = *Pseudotsuga menziesii*; QuCh = *Quercus chrysolepis*; SeSe = *Sequoia sempervirens*.
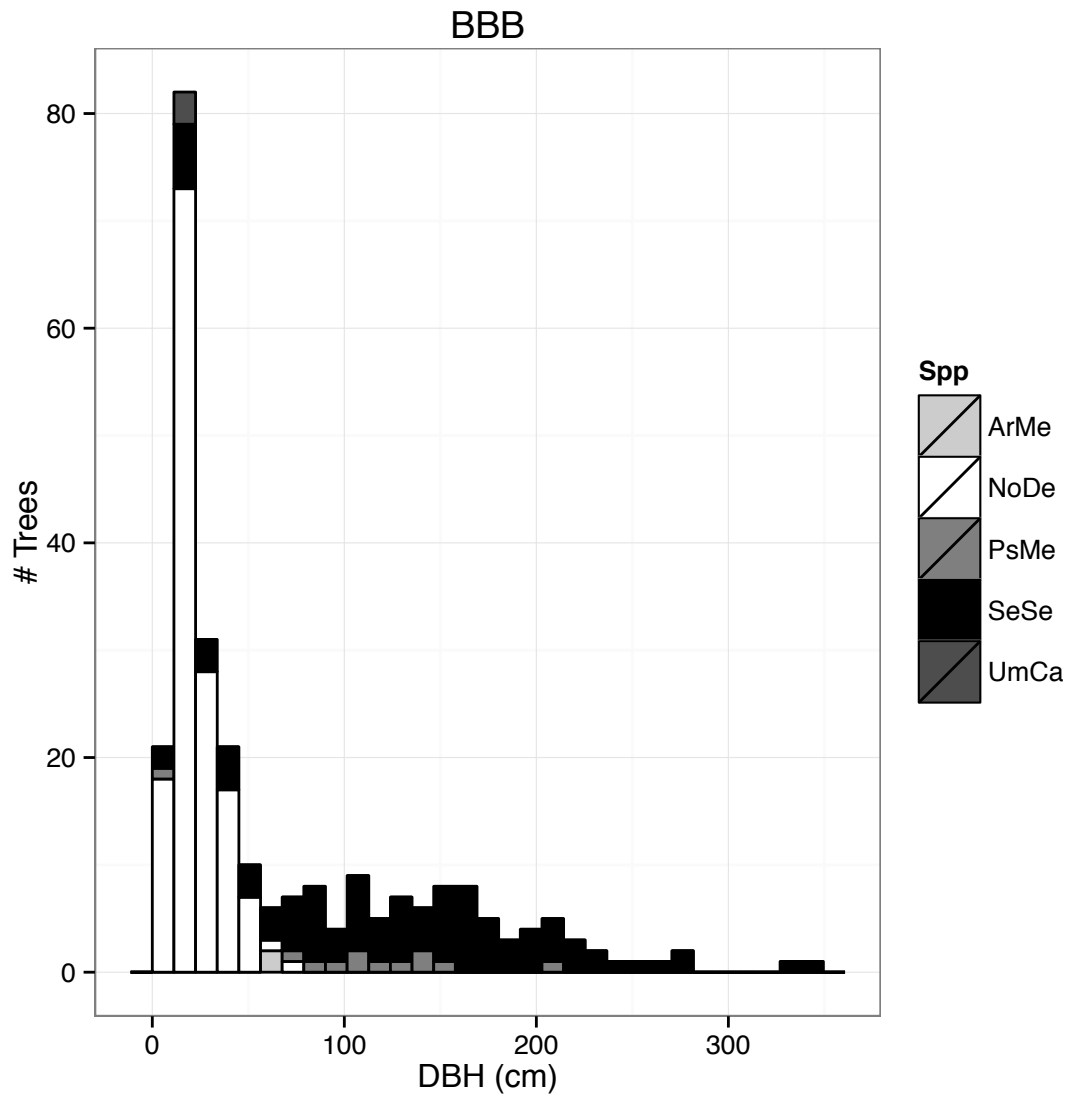
Figure 4.1.B. Diameter distribution for Big Basin B plot. ArMe = *Arbutus menziesii*, NoDe = *Notholithocarpus densiflorus*; PsMe = *Pseudotsuga menziesii*; SeSe = *Sequoia sempervirens*; UmCa = *Umbellularia californica*.
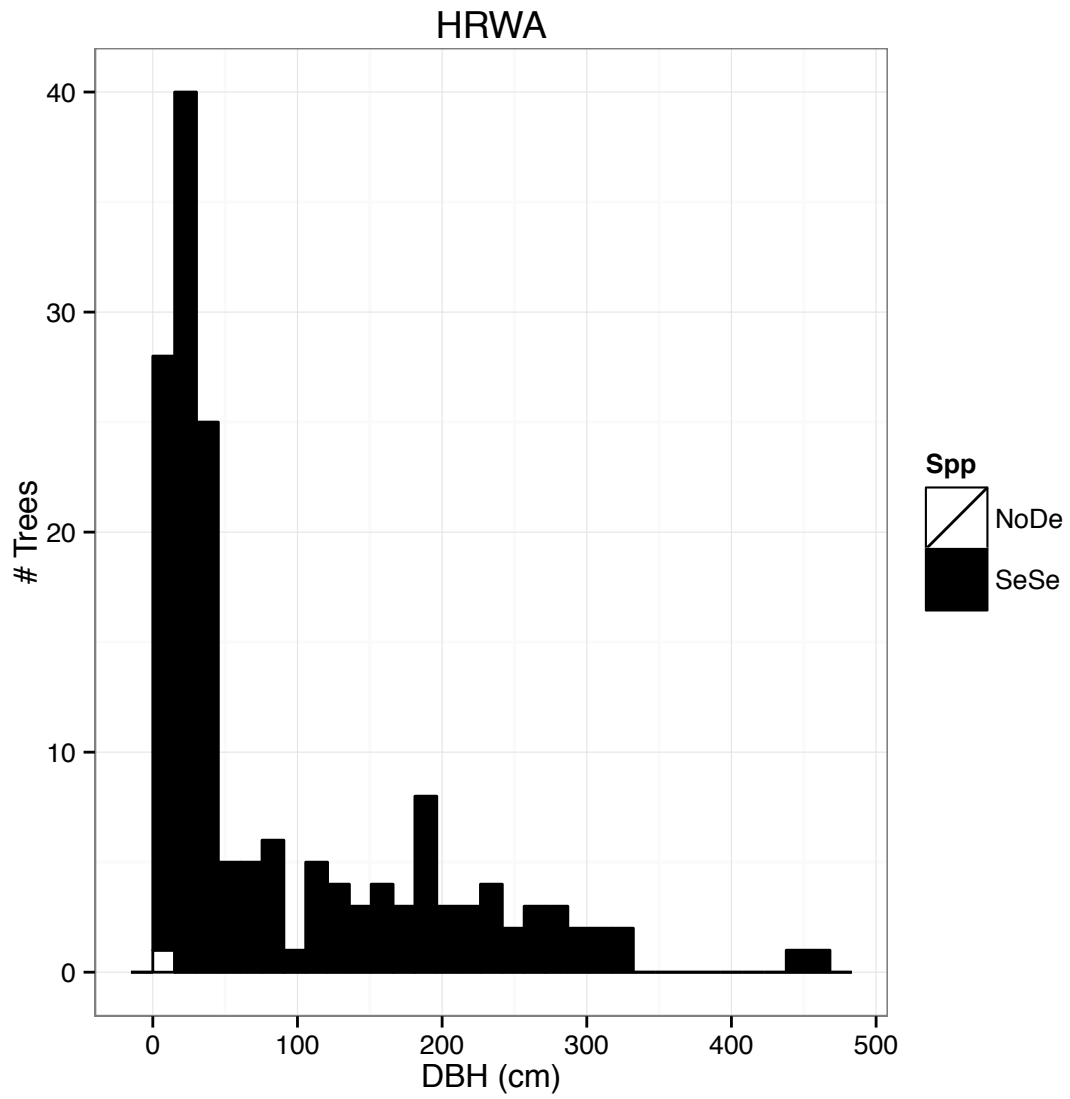
Figure 4.1.C. Diameter distribution for Humboldt A plot. NoDe = *Notholithocarpus densiflorus*; SeSe = *Sequoia sempervirens*.
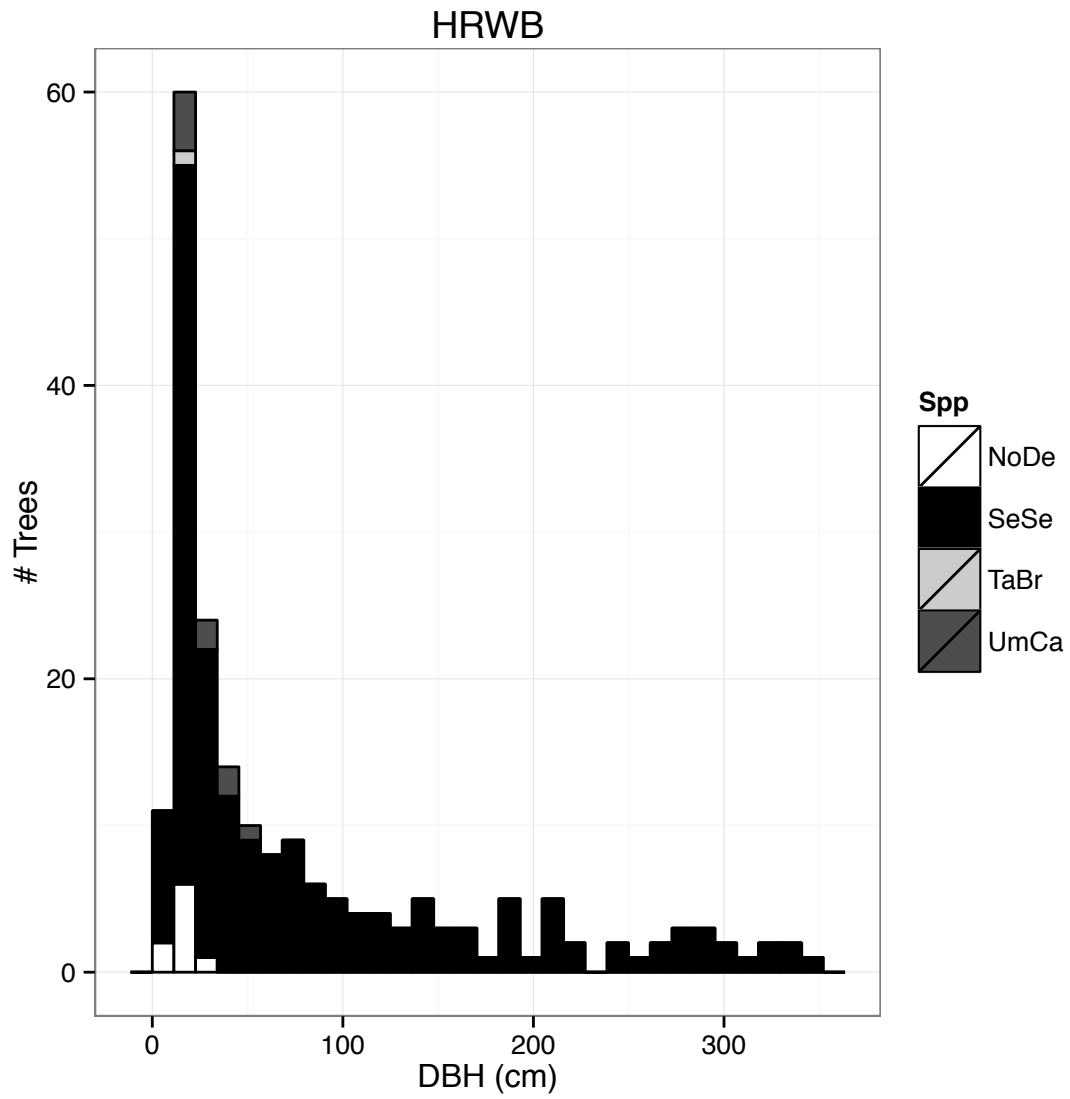
Figure 4.1.D. Diameter distribution for Humboldt B plot. NoDe = *Notholithocarpus densiflorus*; SeSe = *Sequoia sempervirens*; TaBr = *Taxus brevifolia*; UmCa = *Umbellularia californica*.
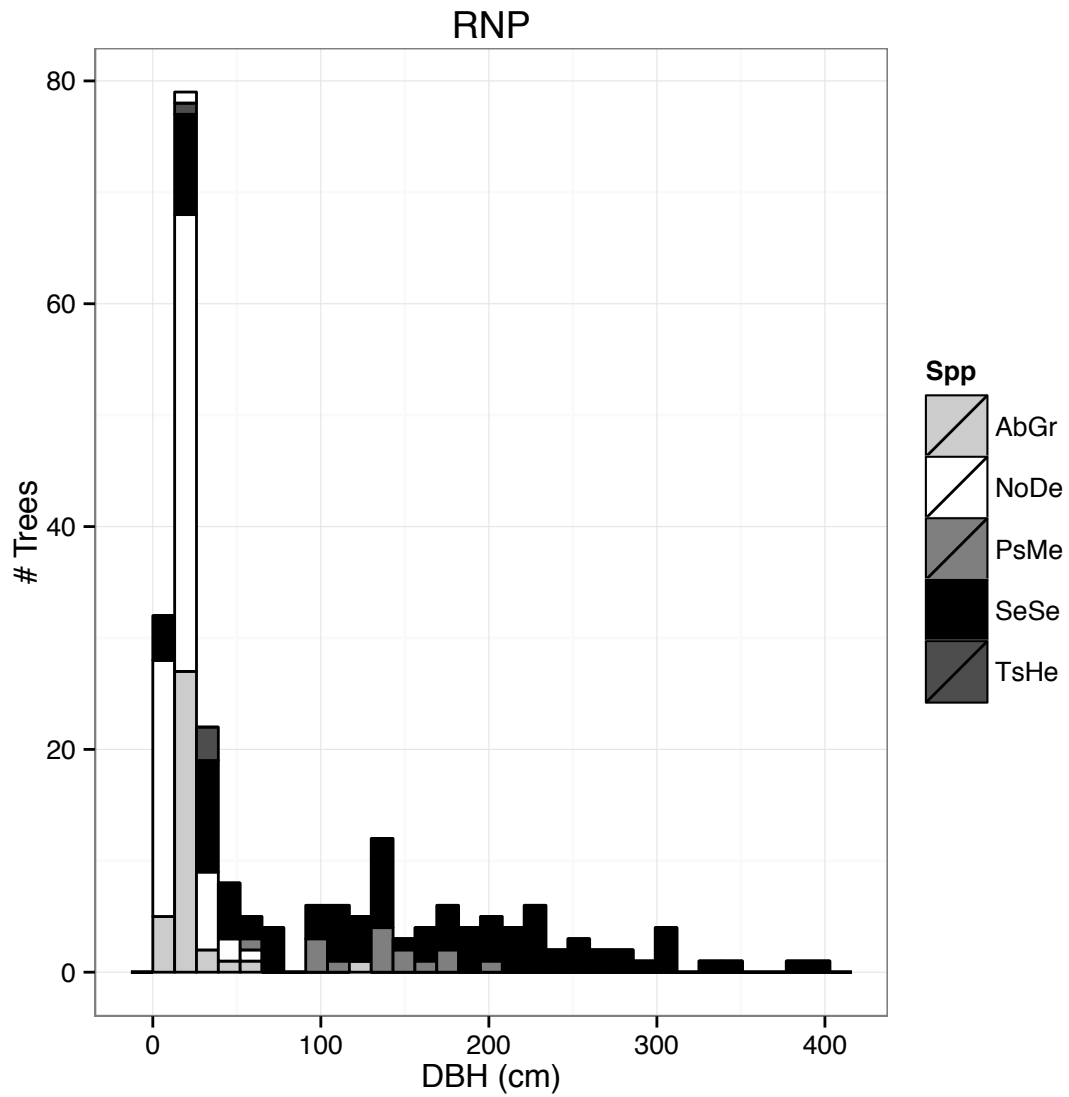
Figure 4.1.E. Diameter distribution for Redwood National Park plot. AbGr = *Abies grandis*; NoDe = *Notholithocarpus densiflorus*; PsMe = *Pseudotsuga menziesii*; SeSe = *Sequoia sempervirens*; TsHe = *Tsuga heterophylla*.
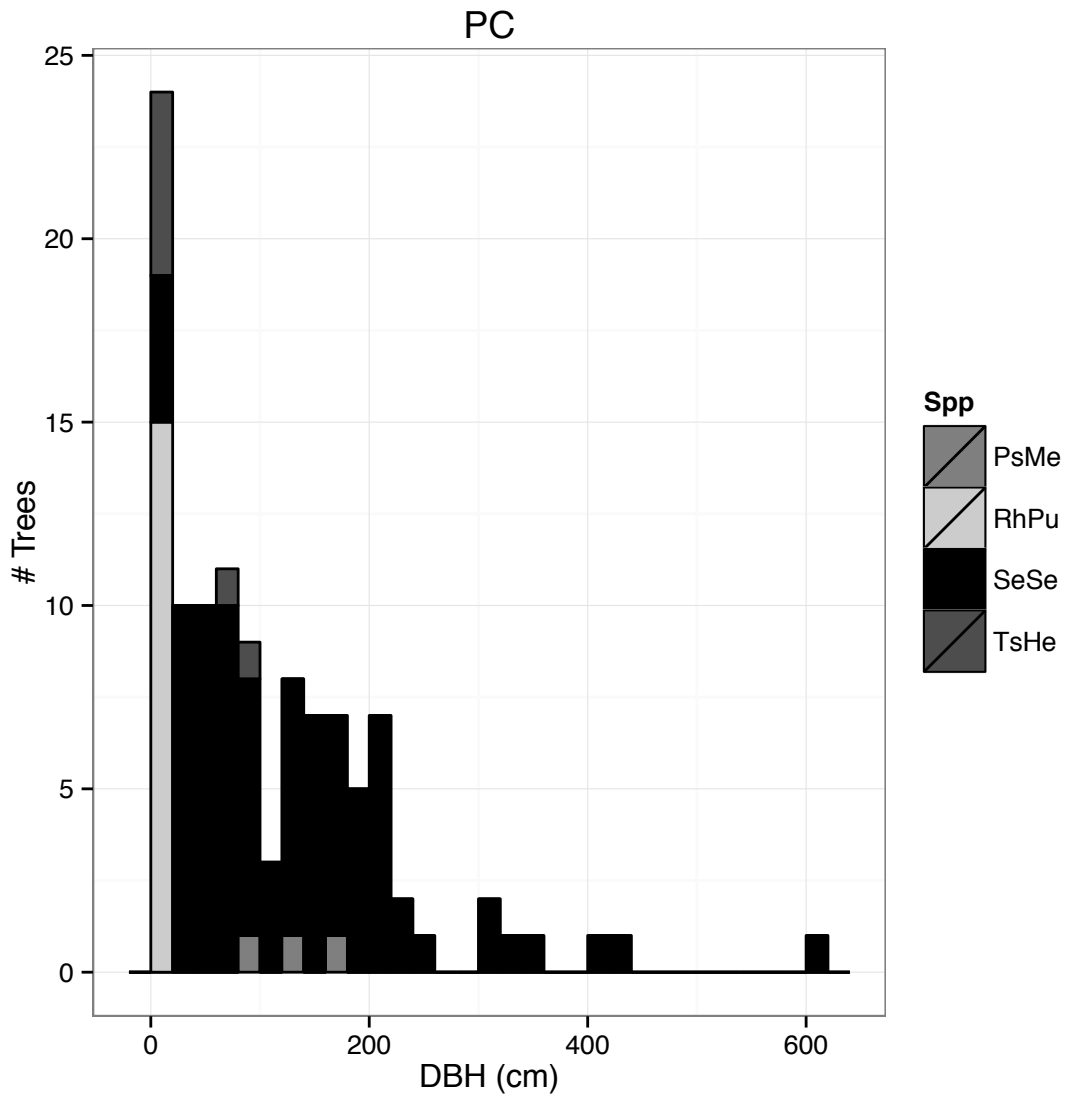
Figure 4.1.F. Diameter distribution for Prairie Creek plot. PsMe = *Pseudotsuga menziesii*; RhPu = *Rhamnus purshiana*; SeSe = *Sequoia sempervirens*; TsHe = *Tsuga heterophylla*.
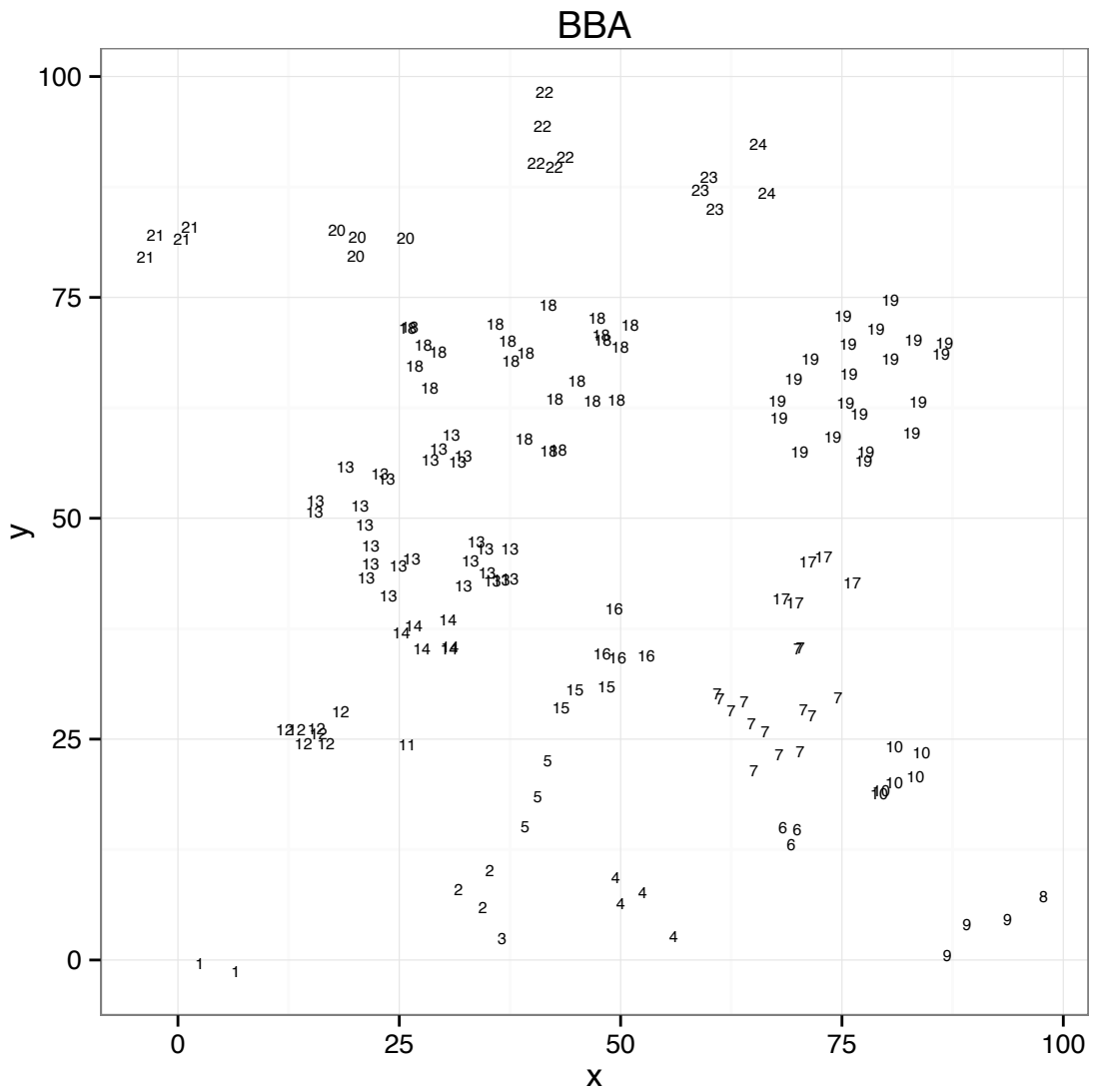
Figure 4.2.A. Clone map of Big Basin A plot. Maps are oriented to magnetic north. Numbers signify clonal identity. For example, all points labeled "1" are the same clone.
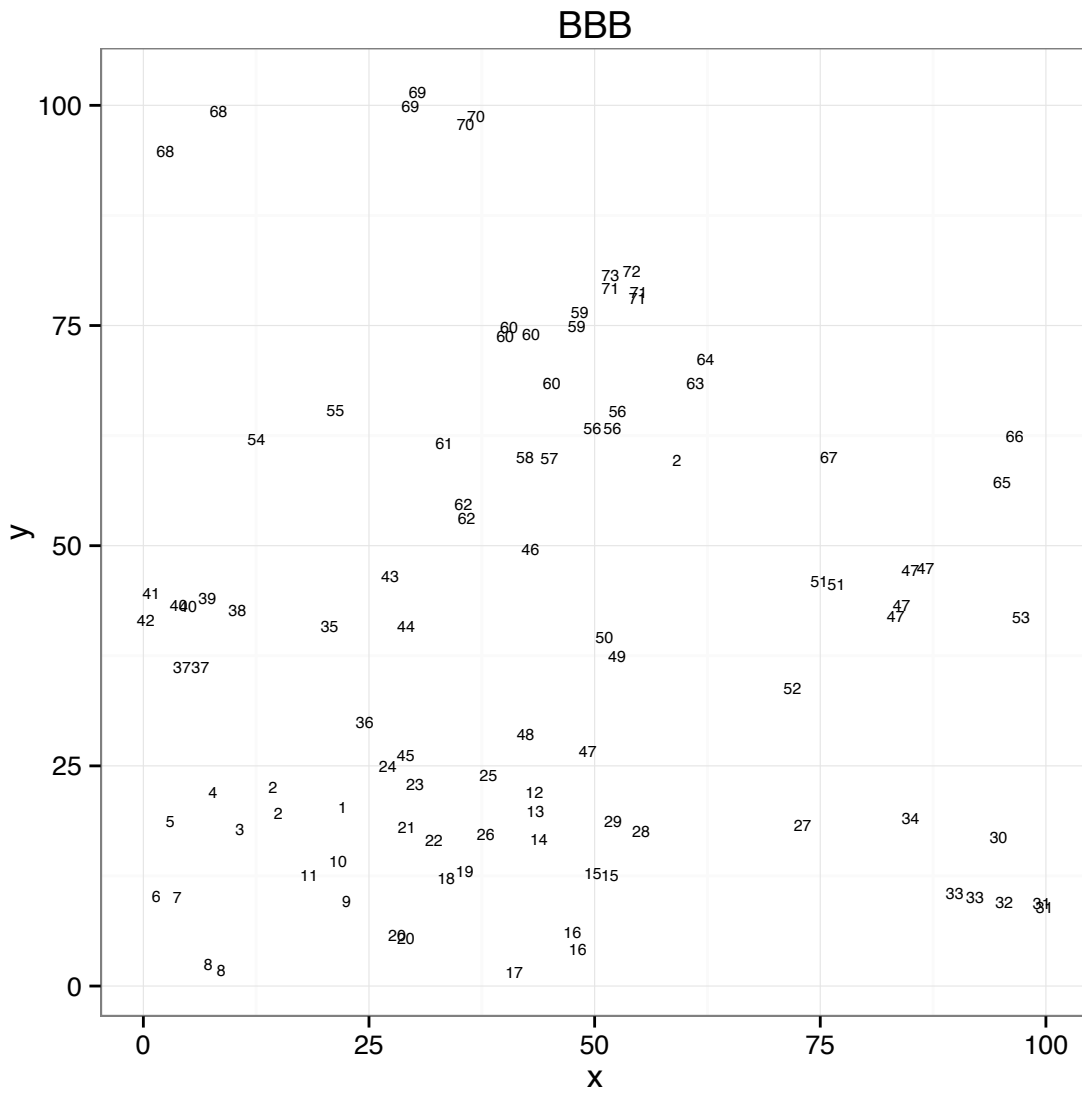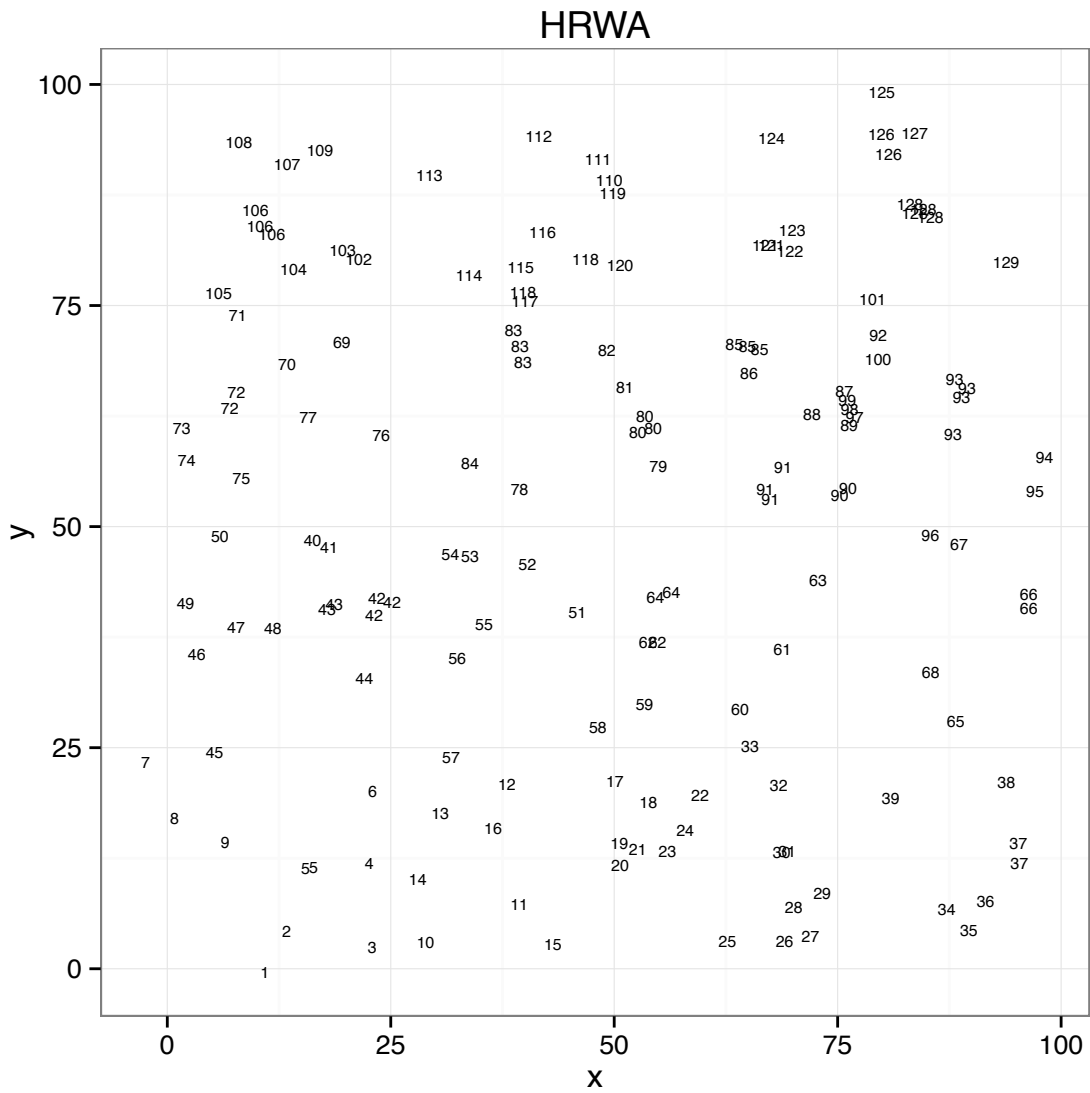
Figure 4.2.B. Clone map of Big Basin B plot. Maps are oriented to magnetic north. Numbers signify clonal identity. For example, all points labeled "1" are the same clone.

Figure 4.2.C. Clone map of Humboldt A plot. Maps are oriented to magnetic north. Numbers signify clonal identity. For example, all points labeled "1" are the same clone.
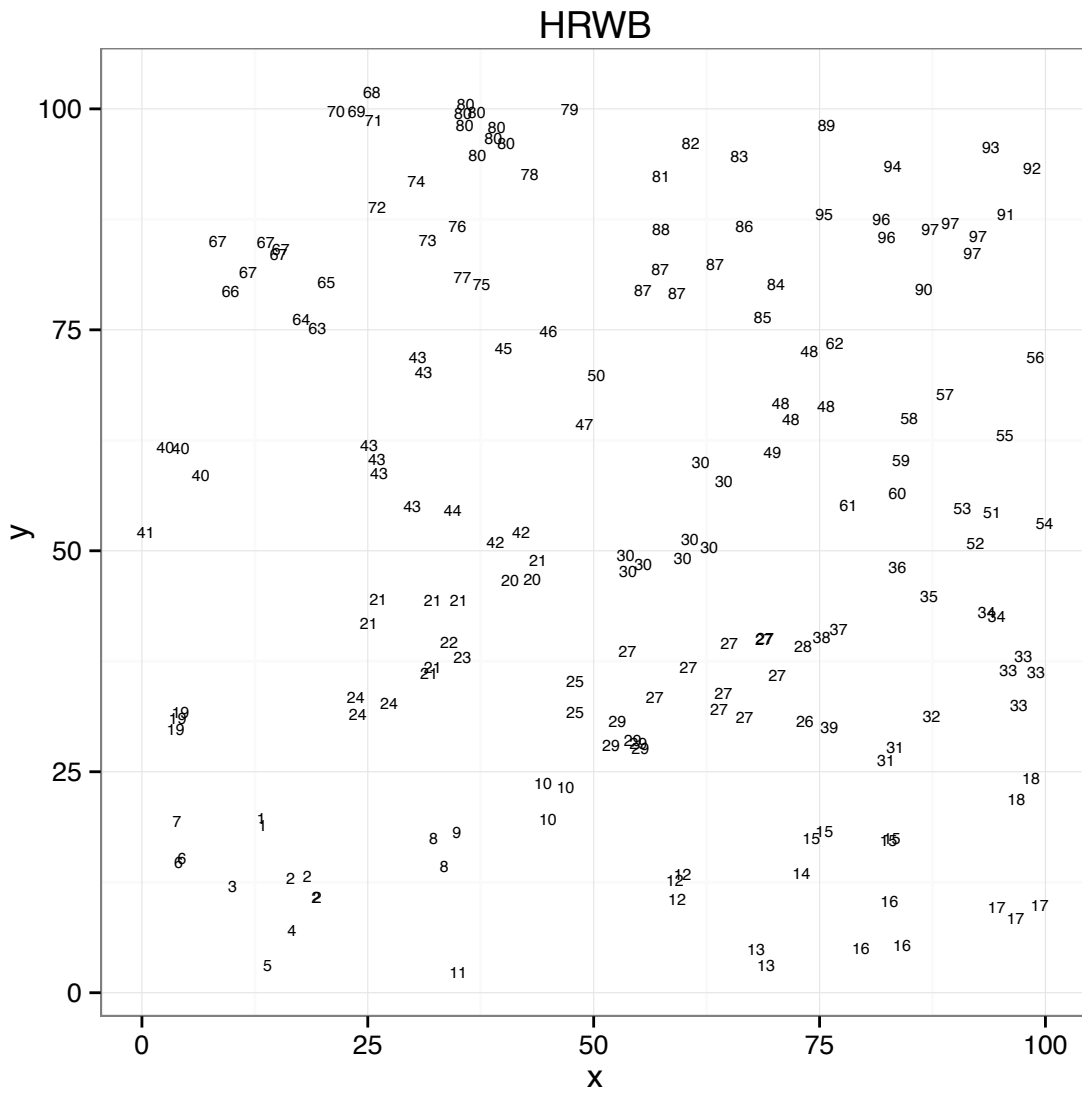
Figure 4.2.D. Clone map of Humboldt B plot. Maps are oriented to magnetic north.
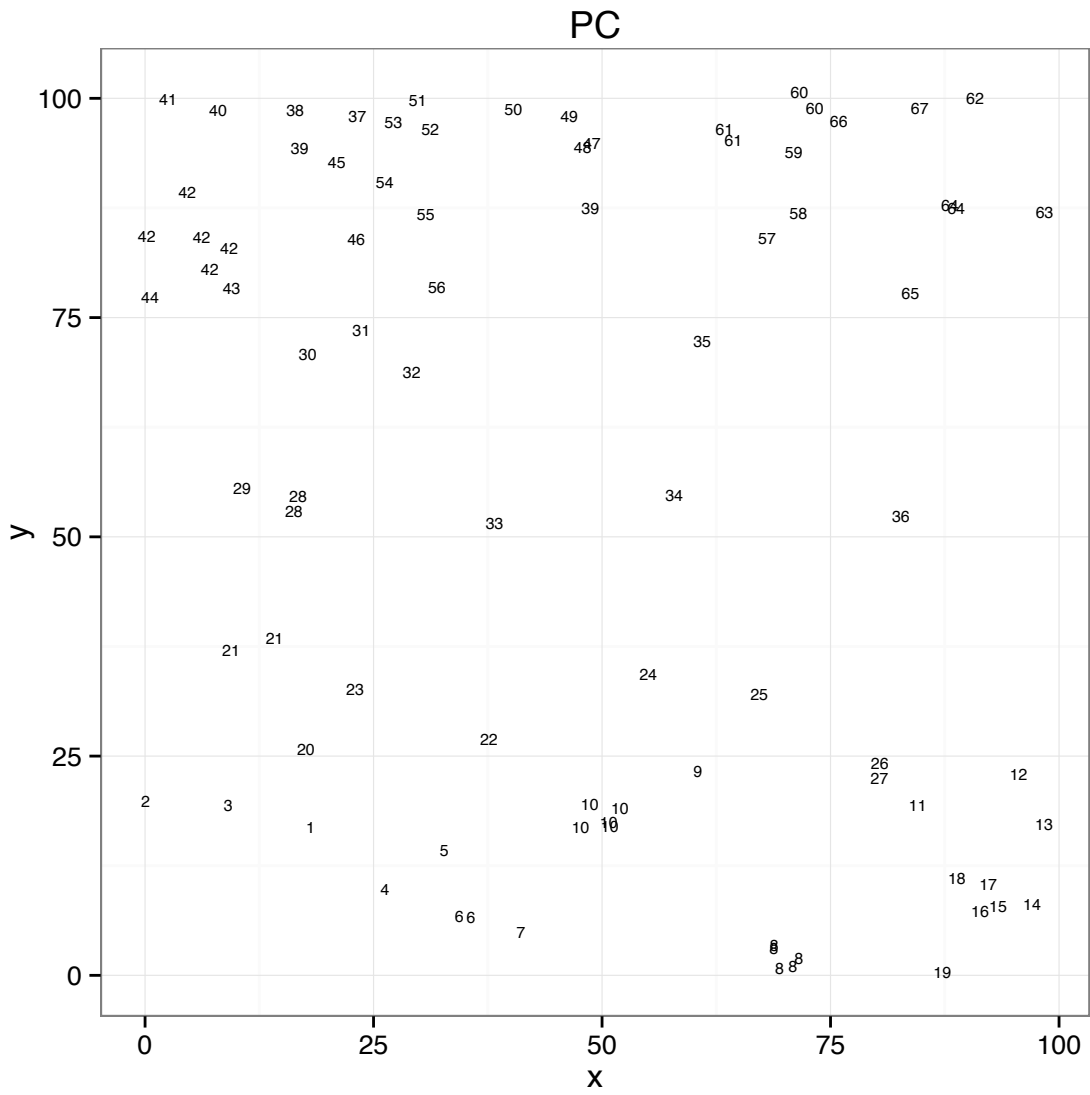Numbers signify clonal identity. For example, all points labeled "1" are the same clone.

Figure 4.2.E. Clone map of Redwood National Park plot. Maps are oriented to magnetic north. Numbers signify clonal identity. For example, all points labeled "1" are the same clone.

Figure 4.2.F. Clone map of Prairie Creek plot. Maps are oriented to magnetic north. Numbers signify clonal identity. For example, all points labeled "1" are the same clone.
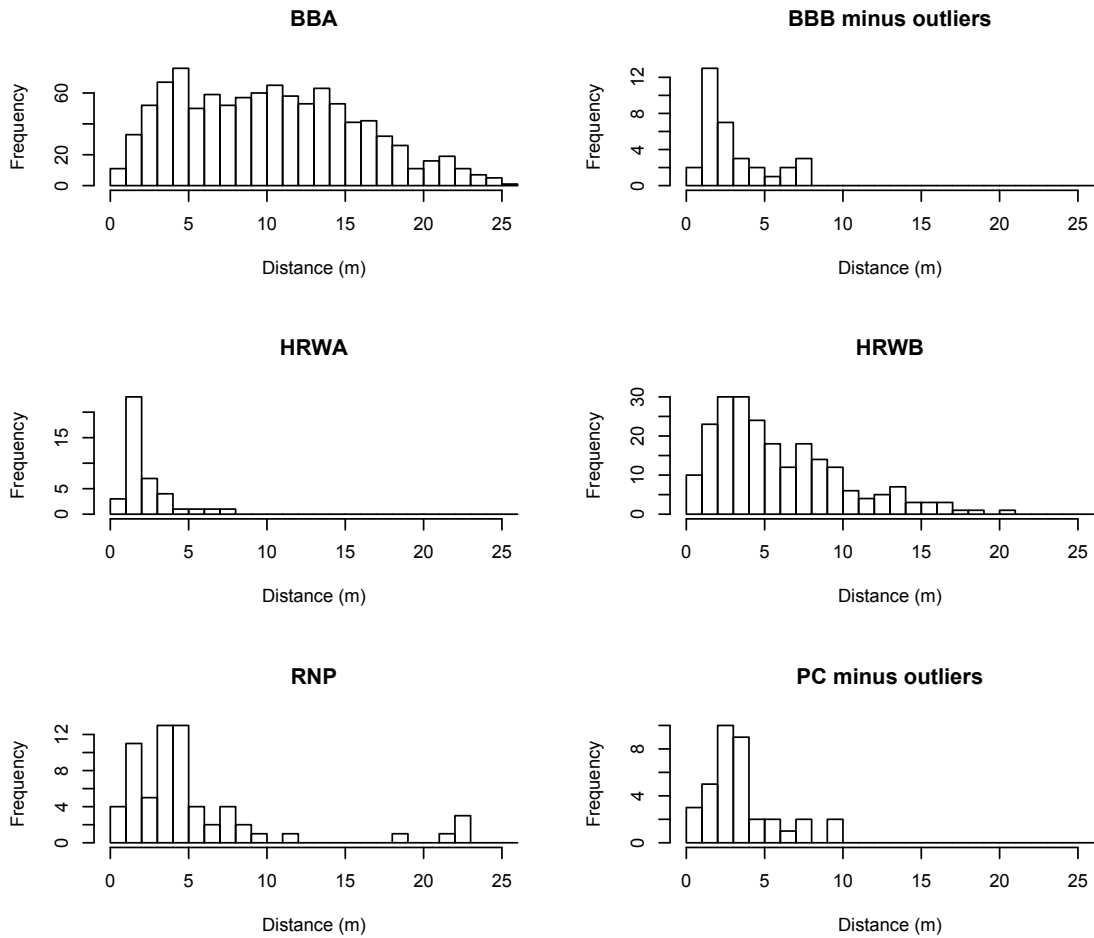
Figure 4.3. Frequency of intraclonal distances at each study plot. Outlier distances of greater than 26 meters were removed from the Big Basin B and Prairie Creek histograms.
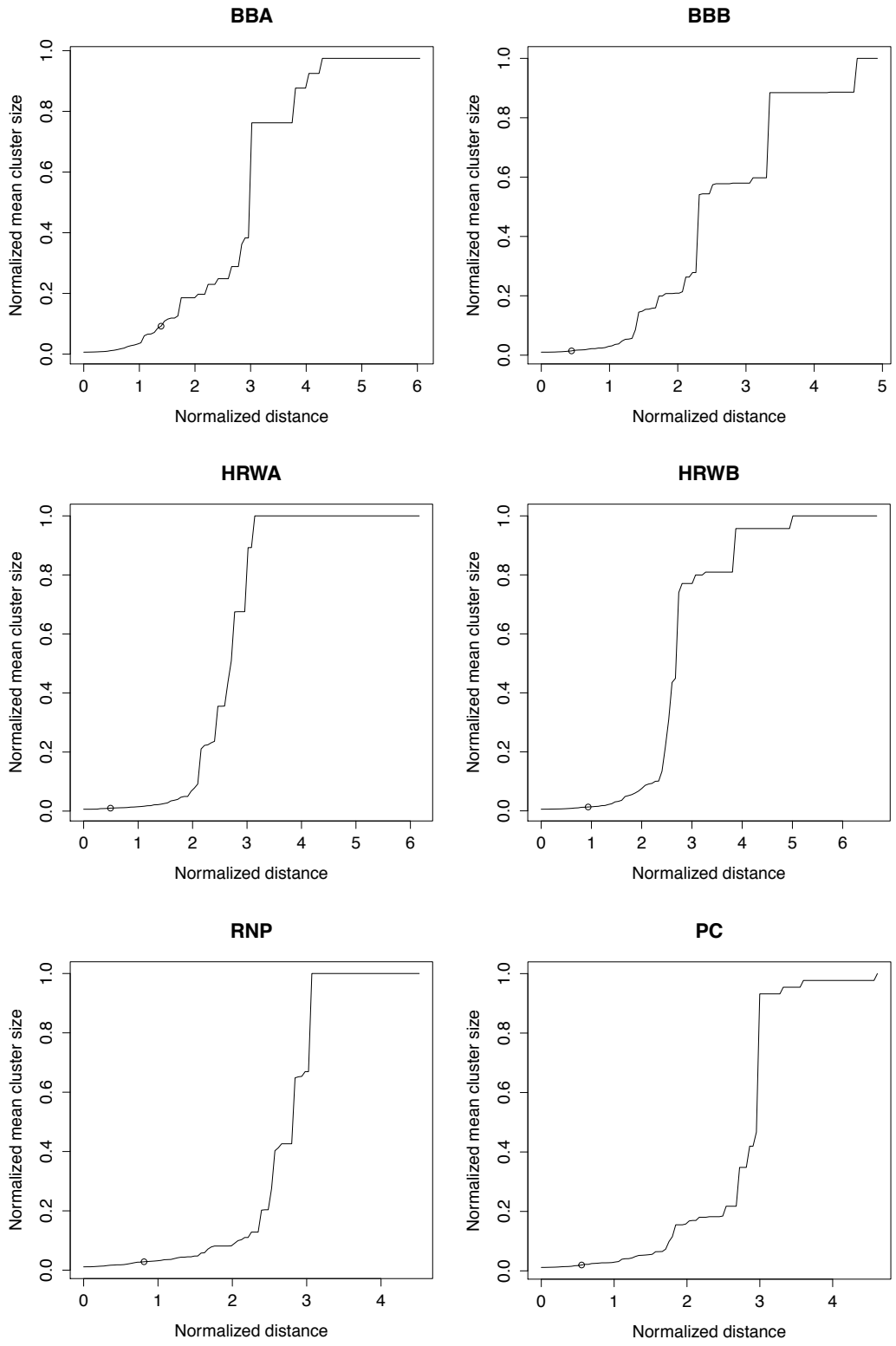
Figure 4.4. Continuum percolation plots showing normalized mean cluster size as a function of normalized distance. Normalized values of *d* that led to the most accurate assignment of trees into clusters that corresponded with their clonal identity are marked with a circle.
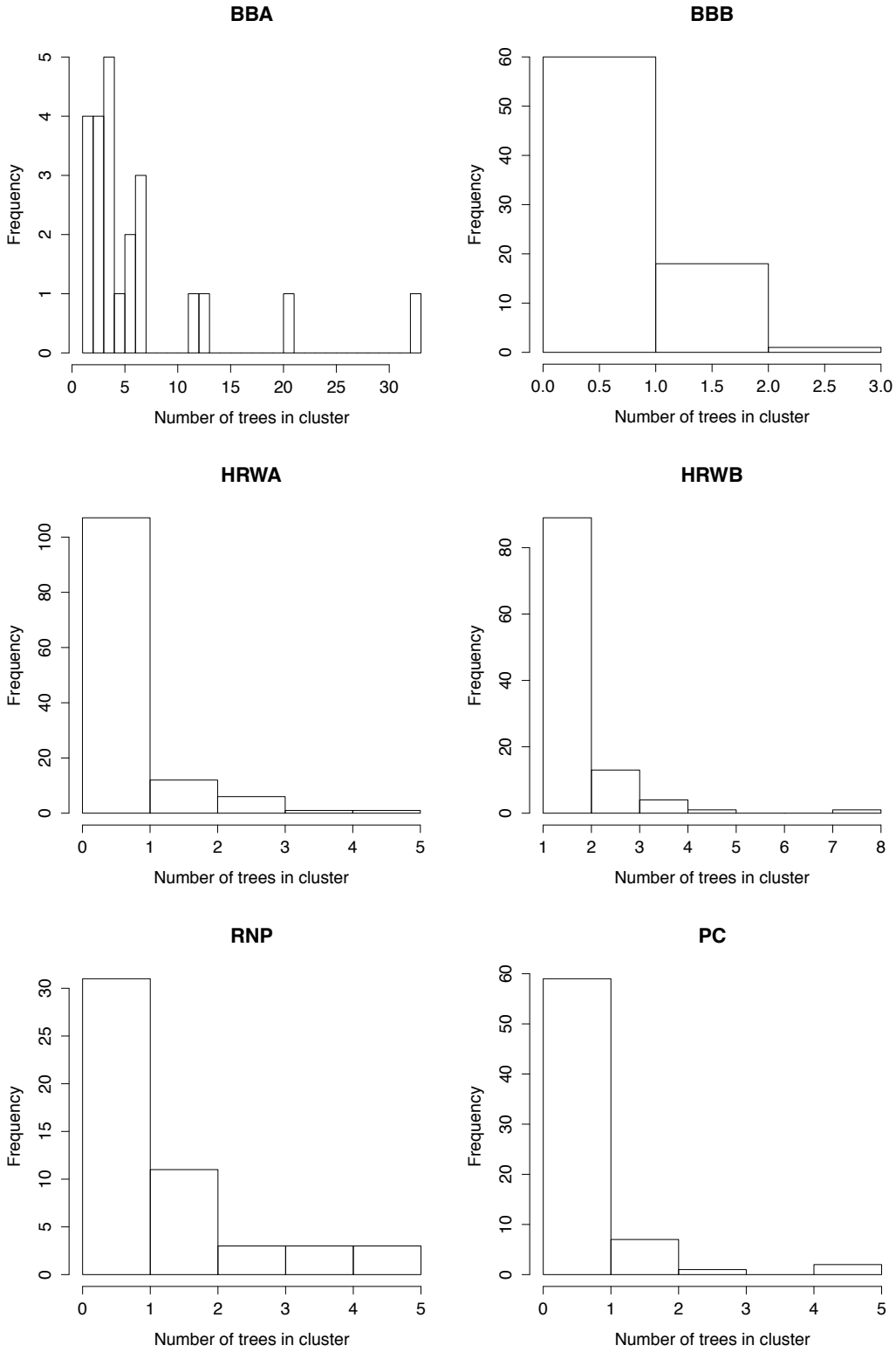
Figure 4.5. Frequency of cluster sizes assigned by continuum percolation clustering algorithm with *d* optimized for clonal assignment.

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