Population structure and gene flow in two rare, isolated Quercus species:

Q. hinckleyi and Q. pacifica

BY

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THESIS

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<tr>
<td>DAPC</td>
<td>Discriminant Analysis of Principal Components</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>GPS</td>
<td>Global Positioning System</td>
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<tr>
<td>GUMO</td>
<td>Guadalupe Mountains National Park</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy-Weinberg equilibrium</td>
</tr>
<tr>
<td>IUCN</td>
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<tr>
<td>PCoA</td>
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SUMMARY

My doctoral dissertation research uses DNA microsatellite analysis to determine population structure, genetic variability, and gene flow within and among areas in which each of two rare, isolated scrub oak species are found. *Quercus hinckleyi* C.H. Muller is a threatened species whose U.S. range is essentially limited to one county in West Texas. *Quercus pacifica* K. Nixon & C.H. Muller is an endemic species found on three of the eight Channel Islands off the coast of California. While one is isolated by land and the other by sea, both of these species exist on what are in effect islands: *Q. hinckleyi* has been identified at a handful of sites separated by Chihuahuan Desert terrain; *Q. pacifica* is found on three offshore islands, Santa Rosa, Santa Cruz and Santa Catalina, separated by open waters of the Pacific Ocean. Their present ranges were affected by climatic changes approximately 10,000 years ago and they are both located in areas shaped by human disturbance. Possible hybridization has been suggested for each species.

In the first chapter, I examine the rare *Quercus hinckleyi* C.H. Muller. Low levels of genetic variability, inbreeding, and limited gene flow are three possible threats to small, isolated plant populations as exemplified by this species. *Quercus hinckleyi* has survived over the past 10,000 years in a region in which the climate has become increasingly xeric. While more prevalent after the last ice age, its U.S. range is now limited to a few locations in one county in West Texas. This chapter looks at the
SUMMARY (CONTINUED)

genetic diversity of the relict metapopulation and resultant conservation implications. I used microsatellites to genotype a total of 204 ramets collected from three locations in Presidio County, Texas, that represent the known occurrences of *Q. hinckleyi*. Analyses of eight loci were used to determine levels of genetic variability, population structure and clonal growth. Genetic diversity for the sampled plants was high: for the total metapopulation, the mean number of alleles (Na) was 17.875; the mean observed heterozygosity (Ho) was 0.807 and the mean expected heterozygosity (He) was 0.853. There was no evidence of inbreeding as measured by the fixation index, FIS. Population structure analyses showed two distinct subpopulations with significant differentiation, as shown by G_{ST} (0.033, Bonferroni corrected *p* = 0.001) and D_{JOST} (0.451, Bonferroni corrected *p* = 0.001), unique alleles and genetic clustering. High clonality was discovered at the two smallest sites, with only seven unique genotypes among 58 ramets sampled. One clone was approximately 30 m across. Sexual reproduction appears to be present at the other sites, as indicated by less extensive cloning. Overall, I found that *Q. hinckleyi* is not genetically depauperate despite its rarity, although unique genets are reduced because of cloning. Asexual reproduction may in fact have allowed the small relict populations to survive extreme environmental change as their range has dwindled. The level of genetic diversity and differentiation among the remaining *Q. hinckleyi* sites warrants protection and preservation of them all.
In the second chapter, I characterize hybridization between Q. hinckleyi and two putative hybridizing species, Q. pungens Liebm. and Q. vaseyana Buckley. Hybridization among oaks is well-documented and is of special concern in conservation efforts directed toward threatened or endangered Quercus species. The two potential hybridizers in this study were sampled at Guadalupe Mountains National Park (GUMO), approximately 320 km from Q. hinckleyi. Quercus pungens and two possible hybrids located in near proximity to the relict populations of Q. hinckleyi were also sampled. Genetic variability was high in all three species, with mean number of alleles per locus ranging from 12.625 to 17.875, mean observed heterozygosity from 0.734 to 0.807, and mean expected heterozygosity from 0.851 to 0.869. Quercus hinckleyi is genetically differentiated from the putative hybridizers and has two distinct genetic clusters within its metapopulation. The two hybridizer species from GUMO, where they are sympatric, are not differentiated. The population of Q. pungens found near Q. hinckleyi is genetically distinct from the GUMO samples and has 5 of 8 genets with greater than 90% Q. hinckleyi introgression. Only two of the 14 Q. hinckleyi in close proximity to this population had Q. pungens introgression. Bayesian clustering analysis showed five percent of the samples identified as Q. hinckleyi in the field were hybrids and one putative hybrid was confirmed genetically. Overall, while there is some hybridization within the Q. hinckleyi population, there is no evidence of genetic swamping. This may
be explained by the spatial isolation of the *Q. hinckleyi* remnants relative to other oak species and by its common asexual (cloning) method of reproduction.

In the third chapter, I looked for evidence of founder events or limited gene flow in *Quercus pacifica* K. Nixon & C.H. Muller, an endemic oak found on three of the eight California Channel Islands, Santa Rosa, Santa Cruz, and Santa Catalina. I examined levels of genetic variability and genetic differentiation within and between islands among a total of 133 leaf samples genotyped at eight DNA microsatellite loci. There was no evidence of cloning or inbreeding in *Q. pacifica*. Levels of genetic variability were high and comparable to mainland oaks, with the mean number of alleles per locus per island ranging from 15.3 to 16.8, mean $H_o$ ranging from 0.795 to 0.828, and mean $H_e$ ranging from 0.832 to 0.862. Genetic differentiation measured by $G_{ST}$ and $D_{JOST}$ was small but significant. Principal Coordinates Analysis (PCoA) showed broadly overlapping distributions of samples from all three islands. Bayesian structural analysis indicated three genetic clusters with each individual composed of an admixture of the three. Principal component/discriminant analysis also showed three genetically related groups with individuals within them scattered across the different islands. While Bayesian spatial clustering analysis inferred barriers to gene flow between the islands, barrier permeability was also indicated. Individuals on both Santa Rosa and Santa Cruz shared genetic clusters with individuals on Santa Catalina and with each other. The
high levels of genetic variation and low levels of genetic differentiation found in this study suggests that *Q. pacifica* originated from a common, genetically diverse ancestral form and that gene flow among islands has maintained genetic continuity over great distances. While pollen movement in oaks has previously been shown to occur at long distances, these results suggest gene flow occurs over more than 100 km of open ocean, the distance between the northern islands and Santa Catalina. It is also possible that multiple colonization events between the islands have occurred, perhaps through seed dispersal by birds and humans. The results of this research suggest that the model of insular genetic isolation of species is not universal and that natural dynamics can result in a different picture.

Finally, chapter four looks at the genetic support for a recent reclassification of three California shrub oaks. Understanding the taxonomic structure and species boundaries among California oaks has proven challenging because of introgression and hybridization. The ‘*Quercus dumosa* shrub oak complex’ has been revised based on morphology, habitat, and associated plant communities, but no genetic evaluation had been completed prior to this research. I used DNA microsatellite analysis with eight loci to examine three of the taxa previously identified within the complex: *Q. berberidifolia* Liebm., *Q. dumosa* Nutt. *sensu stricto*, and *Q. pacifica* Nixon & C.H. Muller. Based on the revised taxonomy, *Q. berberidifolia* is a common, widespread species, *Q. dumosa*
sensu stricto is rare with a limited range, and Q. pacifica is an endemic found on only three of the Channel Islands. A total of 200 individuals were genotyped: 43 Q. berberidifolia and 24 Q. dumosa collected on mainland California and 133 Q. pacifica collected from the three Channel Islands that comprise its range. Allelic diversity was comparable to that found for other California oaks. All loci were polymorphic; mean expected heterozygosities ranged from 0.802 for Q. dumosa to 0.878 for Q. berberidifolia, and there was no evidence for inbreeding. Each species had private alleles not found in the other species. There was significant genetic differentiation among the species, with the greatest between the two mainland species, Q. dumosa and Q. berberidifolia (G_ST=0.029, D_JOSt = 0.341). Bayesian analysis identified three genetic clusters which corresponded to species delineations, with 187 individuals (93.5%) having >80% ancestry in the inferred cluster of their species. However, some gene flow, both from mainland to island and from island to mainland, appears to occur. In short, this study found that these three taxa, once considered a single species, show significant levels of genetic differentiation supporting the reclassification despite evidence of gene flow among them.
1. A STORY OF SURVIVAL OF A RARE, ISOLATED SPECIES: A TOUGH, LITTLE WEST TEXAS OAK, QUERCUS HINCKLEYI C.H. MULLER

1.1 Introduction

*Quercus hinckleyi* (Hinckley's oak) is a classic example of an extremely rare species with small populations in a fragmented landscape. Its U.S. range is now essentially limited to Presidio County\(^1\) in West Texas, although it was more prevalent across the Chihuahuan Desert region in the late Wisconsin/early Holocene period (approximately 18-9 ka) when the climate was more mesic (Nixon et al., 1997). Studies of packrat midden macrofossils, coupled with carbon dating, allow reconstruction of the paleo-plant assemblage communities in the Chihuahuan Desert from the Middle Wisconsin to the Late Holocene (approximately 40 ka to the present) (Van Devender & Spaulding, 1979). There is evidence of a pinyon-juniper-oak woodland complex in the area for at least 30,000 years, including macro-fossils of *Q. hinckleyi*. Although the North American ice sheets were far removed, glacial fluctuations drove weather patterns within its range and as climate changed the pinyon disappeared about 11 ka ago, while the juniper

\(^{1}\) The only known specimen from outside of Presidio County was collected on the eastern side of The Solitario in Big Bend Ranch State Park which is actually in Brewster County (University of Texas Herbarium Plant Resources Center, 2012).
continued until about 9 ka. *Quercus hinckleyi* survives to this day, although its numbers have precipitously declined and it is now a threatened endemic (Van Devender, 1990).

Cornelius H. Muller, who originally identified the species, described the scarcity and clonal habits of *Q. hinckleyi* (Muller, 1951). At that time only one population was located and no seedlings or young plants were found. This observation, along with the morphological homogeneity of the plants, led Muller to conclude that he had found a single clone of great age. In addition to stands NW of Solitario Peak, close to the location of the type specimen, two sites have been identified near Shafter (Powell, 1998) and a limited amount of sexual reproduction has been reported. Immediate threats to the relict populations in Texas are the low number of stands with few individuals, wildlife and insect predation, possible hybridization, poor regeneration from seed (Kennedy & Poole, 1992) and climate change.

Small, isolated plant populations and those reproducing clonally exhibit loss of genetic diversity and, in some cases, reduced fitness. A review of plant population studies published between 1987 and 2005 (Leimu et al., 2006) found significantly positive correlations between population size, fitness levels, and genetic diversity, suggesting negative consequences for small, fragmented populations. Based on these results, the relict populations of *Q. hinckleyi* may suffer loss of genetic variation, inbreeding, and reduced fitness across the metapopulation. In addition, the fragmented nature of the range of the remaining populations may limit gene flow. These factors, along with
abiotic restrictions of reduced habitat and environmental stochasticity, could then contribute to extirpation of the isolated populations or even species extinction.

The inevitable genetic decline of small plant populations has, however, been called into question. A review by Young et al. (1996) concludes that small populations are not necessarily lacking in genetic diversity and alleles lost may be those that were rare when the population was isolated; these populations may in fact be repositories for maintaining genetic diversity. A more complex dynamic may better characterize small, isolated populations, especially for plant species that can reproduce both sexually and clonally, experience gene flow by seed and pollen dispersal, are long-lived and have long generation times (Kramer et al., 2008). Clonal reproduction itself may be viewed as an adaptive response to habitat fragmentation and environmental disturbances that maximizes lifespans of individual genotypes (Jeník, 1994, May et al., 2009), reduces genetic drift (Hamrick et al., 1992), and limits introgression and swamping by other species (Muller, 1951). While in an obligate sexual species, small populations with few genotypes may experience reproductive failure and eventual extinction (Weekley et al., 2002), plants which can reproduce asexually appear to better withstand extinction events even in small, fragmented populations (Honnay & Bossuyt, 2005). This research, the first comprehensive genetic study of *Q. hinckleyi*, characterizes the neutral genetic diversity of *Q. hinckleyi*, the clonal and genetic structure of its remaining populations, and the subsequent implications for its conservation management. DNA microsatellite analysis was used to examine genetic variability, reproductive modes,
inbreeding, and population structure. Microsatellites generally result in a much greater level of resolution for landscape and population genetic analysis than other markers and have been successfully applied to a number of oak species (Aldrich et al., 2002, Ashley et al., 2010, Dow & Ashley, 1998, Isagi & Suhandono, 1997, Steinkellner et al., 1997, Dow et al., 1995, Kampfer et al., 1998). They also allow accurate, non-invasive identification of unique genotypes among the ramets in a clonal stand (Brzyski, 2010, Ortego et al., 2010, Wilk et al., 2009, Parks & Werth, 1993), important for determining true population sizes (Tepedino, 2012) and selecting plants for conservation initiatives.

1.2 Materials and Methods

1.2.1 Study species

*Quercus hinckleyi* (Fagaceae) is listed as a threatened species under the U.S Endangered Species Act and by the state of Texas and is identified as ‘critically threatened’ on the IUCN Red List. The species grows in an arid sub-tropical environment on dry limestone and sandstone slopes at approximately 1000-1400 m elevation. It is recognizable by its shrub-like thicket growth pattern (maximum height 0.75 m) and grey-green thickened holly-like leaves with pink petioles (Fig. 1-1).
It reproduces both clonally and sexually (Poole et al., 2007). Based on Muller (1951), growth ring counts on individual aerial stems showed them to be relatively short-lived, from 7 to 9 years, although the age of clones could not be determined. Through excavation he found that rhizomes were 4 to 15 cm long. Clumps, in which stems grew from old rhizomes, were themselves small (1 to 3 dm in diameter). With time, rhizome connections between clumps are broken, giving the above-ground appearance of separate individuals (Muller, 1951). Staminate catkins are generally 3-5 mm long with few flowers; flowering is in the spring; acorns which are single and produced annually are ovoid and approximately 8-12 mm wide by 10-20 mm long (Nixon et al., 1997, Muller, 1970).
1.2.2 Study sites and sample collection

Leaf samples used in this study were collected from all shrub clumps at two small sites (S1 and S2) approximately 3 km apart and a third site (B1) approximately 60 km from them. Site B1 includes a stand (identified for this study as site B1a) that is in the vicinity of the original type location described by Muller in 1950 (Fig. 1-2). Leaves were taken around the perimeter of each clump. A total of 204 ramets were sampled overall and GPS coordinates taken to identify their locations. Samples were stored at room temperature with Drierite desiccant (W.A. Hammond Drierite Co., Ltd.) until DNA extraction.
1.2.3 DNA extraction and microsatellite analysis

Genomic DNA was extracted from 20-30 mg of dry sample homogenized to a fine powder using a DNeasy Plant Kit (QIAGEN). DNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific). Eight microsatellite loci and primers developed and utilized successfully with other oaks in the white oak group (Quercus subgenus Quercus) (Isagi & Suhandono, 1997, Craft & Ashley, 2007, Ashley et al., 2010, Dow et al., 1995) were used in this study. Primers QpZAG1/5, QpZAG15,
QpZAG110, and QpZAG9 were originally developed for *Q. petraea* (Steinkellner et al., 1997); QpZAG15 and QpZAG11 for *Q. robur* (Kampfer et al., 1998); and MSQ4 and MSQ13 for *Q. macrocarpa* (Dow et al., 1995), all in subgenus *Quercus*. M69–2M1 was developed for *Q. myrsinifolia* in subgenus *Cyclobalanopsis* (Isagi and Suhandono 1997). PCR amplification followed the protocol as previously described (Abraham et al., 2011) using labeled forward primers as presented in Schuelke (2000). PCR products (0.9 – 1.5 µL) were genotyped on an ABI 3730 DNA Analyzer using GeneScan™ - 500 LIZ500® Size Standard (Applied Biosystems). All genotypes were scored using Applied Biosystems GeneMapper, version 3.7.

**1.2.4 Data analysis**

To test for clones, the R-package ALLELEMATCH (Galpern et al., 2012) was used to identify unique multilocus genotypes (UMGs) and provide a match probability, $P_{\text{sib}}$, to verify unique individuals. $P_{\text{sib}}$ is the probability that a sample is a sibling, rather than a replicate, of a UMG. Based on these results, the individual ramet samples were collapsed into UMGs, which were then used in subsequent analyses.

Descriptive statistics, such as allele frequency, observed and expected heterozygosity, fixation index, and number of private alleles, were calculated using GenAlEx 6.501 (Peakall & Smouse, 2012, Peakall & Smouse, 2006). Genetic variation among populations was measured by number of alleles (Na) and observed ($H_o$) and expected heterozygosity ($H_e$). Levels of inbreeding were ascertained through the fixation index,
Private alleles (alleles unique to a given population), which can be indicative of reduced gene flow and drift, were identified.

To examine population structure, three analyses were used: STRUCTURE 2.3.4, PCoA (GenAlEx 6.501), and the R-package DEMEtics. STRUCTURE (Pritchard et al., 2000, Falush et al., 2003, Falush et al., 2007, Hubisz et al., 2009) infers population structure of multilocus polymorphic genotypes using Bayesian analysis. The following STRUCTURE protocol was used: 50K burnin with 100K MCMC for the Admixture Model using LOCPRIOR with sampling locations as prior for a potential K of 1 to 7 at 30 reps each. Best K was determined by calculating l(K) and delta K (Evanno et al., 2005) using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). PCoA, Principal Coordinates Analysis, provides a visual representation of patterns resulting from a multivariate analysis of multiple loci and multiple samples. Individuals are presented two-dimensionally based on genetic variance with genetically similar individuals clustering in the diagram. DEMEtics (Gerlach et al., 2010) calculates the differentiation indices $G_{ST}$ (Nei, 1973) and $D_{JOST}$ (Jost, 2008). Values can range from 0 to 1, with higher values indicating greater genetic differentiation between pairs of populations. $P$-values are obtained using bootstrap resampling. To reduce the possibility of Type I errors caused by multiple pairwise comparisons of a single data set, Bonferroni correction was made to the $p$-values. While $G_{ST}$ has been widely used to measure genetic differentiation, $D_{JOST}$ has been shown to be a better indicator for polymorphic loci with more than two alleles (Jost, 2009, Gerlach et al., 2010). DEMEtics checks for
Hardy-Weinberg Equilibrium (HWE) in the input populations and loci and uses alleles if all populations are in HWE for a given locus, or genotypes if not (Goudet et al., 1996).

1.3 Results

Out of the 204 ramets sampled, 123 unique genotypes were identified with $P_{sib} < 0.02$ for each (Table I). Ramet data was collapsed and all further analysis was done using the 123 unique genotypes.

**Table I**: *Q. hinckleyi* ramets and multilocus genotypes.

<table>
<thead>
<tr>
<th>Site</th>
<th>Ramets Sampled</th>
<th>Unique Genotypes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>S2</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>B1</td>
<td>129</td>
<td>102</td>
</tr>
<tr>
<td>B1a</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>123</td>
</tr>
</tbody>
</table>

* $P_{sib} < 0.02$

Based on the results of the population structure analysis described below, Sites S1, S2, and B1a were grouped as Population 1 and site B1 was identified as Population 2. Sample size, number of alleles, observed and expected heterozygosity, and fixation index are shown by locus and population in Table II. All loci used were polymorphic with alleles per locus ranging from nine to 18. Although number of alleles varies with
population size, as would be expected, genetic diversity was high at all sampling sites. For the total metapopulation, mean number of alleles (Na) was 17.875, mean observed heterozygosity (Ho) was 0.807, and mean expected heterozygosity (He) was 0.853. The mean fixation index (FIS) of 0.036 showed no evidence for inbreeding. While some individual loci violated Hardy-Weinberg expectations, there were no consistent patterns across loci or populations. There were 27 alleles found in Population 1 that were not present in Population 2, and 50 in Population 2 not found in Population 1. Among the three sites that make up Population 1, S1 and S2 together had 39 alleles not found in B1a, while B1a had 34 not found in S1/ S2.

**Table II:** *Q. hinckleyi* descriptive statistics.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Population 1 (Sites S1, S2, B1a)</th>
<th>Population 2 (Site B1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Na</td>
</tr>
<tr>
<td>Q1/5</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Q110</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Q11</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Q9</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>QM69</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>MSQ4</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>Q15</td>
<td>21</td>
<td>12</td>
</tr>
<tr>
<td>MSQ13</td>
<td>19</td>
<td>15</td>
</tr>
</tbody>
</table>

N = number of samples, Na = number of alleles, Ho = observed heterozygosity, He = expected heterozygosity, FIS = fixation index. (GenAlEx 6.501).
STRUCTURE results were input into STRUCTURE HARVESTER for computation of mean \( \ln P(K) \), and \( \Delta K \), which indicate the most appropriate K value (number of genetic clusters given the multilocus genotype data) (Evanno et al., 2005). The mean of estimated \( \ln \) probability increased sharply from \( K=1 \) to \( K=2 \), but then less sharply from \( K=3 \) to \( K=6 \). The value of \( \Delta K \) peaked at \( K = 2 \). These results point toward two distinct genetic clusters. Output from STRUCTURE HARVESTER was run through CLUMPP (Jakobsson & Rosenberg, 2007) which clusters individuals into genotypes and in turn those results were fed into DISTRUCT (Rosenberg, 2004) to produce a visual representation of the clusters (Fig. 1-3). The two small sampling sites, S1 and S2, and the site near where the species was originally identified, B1a, grouped into one genetically distinct cluster (Population 1). The larger sampling site, B1, was genetically a single cluster (Population 2). There was limited introgression between the two populations.
**Figure 1-3:** *Q. hinckleyi* inferred admixture proportions in each genetic cluster for individual plants (columns).

Admixture Model using LOCPRIOR (STRUCTURE 2.3.4), best K= 2 (per Evanno method).

This structure was supported by the PCoA analysis which shows differentiation between Population 1 (S1, S2, B1a) and Population 2 (B1) with some genetic overlap as well as high genotypic diversity within the Population 1 sites (Fig. 1-4). Significant differentiation between the two populations was also found with $G_{ST}$ at 0.033 (Bonferroni corrected $p = 0.001$) and $D_{JOST}$ at 0.451 (Bonferroni corrected $p = 0.001$).
Clonal structure was characterized at one of the sampling sites, S1, where ramets were collected from every clump of *Quercus hinckleyi* present. Figure 1-5 shows two ramet clusters from this site and is representative of the appearance of the growth pattern and the spatial isolation of the clusters. Figure 1-6 presents this site in more detail, showing waypoint (GPS coordinate) identifiers for 13 locations within the site. Microsatellite analysis confirmed that the ramets sampled at this site represented only four unique
genotypes, shown by circled areas. There were open areas between all individual clumps, with a maximum distance of approximately 30 m between ramets of the same clone.

**Figure 1-5:** Spatial isolation of *Q. hinckleyi* clumps of a single clone at site S1.

Photo by W. Backs
Figure 1-6: *Q. hinckleyi* clonal growth patterns at site S1.

Circles indicate unique genotypes. The clonal circle with dashed lines contains ramets separated by approximately 30 m (arrowed line).

1.4 Discussion

1.4.1 *Quercus hinckleyi* as a rare isolated species

The first objective of this research was to examine the genetic variability of the relict populations of *Q. hinckleyi*. Study findings contradict the conventional view that small, isolated populations will have low levels of allelic diversity, limited heterozygosity, and inbreeding. Instead, the observed patterns of genetic diversity reveal a genetically viable population and no evidence of inbreeding. While the existence of clones limits the number of unique individuals, allelic diversity and levels of heterozygosity across the
metapopulation (Population 1 and 2) are comparable to non-threatened oak species (Craft et al., 2002, Abraham et al., 2011, Dutech et al., 2005). In sum, although Q. hinckleyi is limited in numbers, substantial genetic diversity still exists.

The remnant populations of Q. hinckleyi exhibit strong population differentiation. As mentioned above, in more mesic times the range of Q. hinckleyi was much more extensive than it is today. The persistence of two differentiated populations, each maintaining genetic diversity, may best be understood in the context of a once more widespread species. Remnants of the historic population, now isolated, are persisting in limited numbers. These are not outliers or pioneers on the fringe exhibiting founder effects or genetic bottlenecks, but survivors which remained as the plant assemblages around them disappeared in a changing climate. As such, each represents a unique and variable collection of alleles.

Findings at the two small sites, S1 and S2, may help explain how asexual (clonal) reproduction protects allelic diversity. Though asexual reproduction exists across the metapopulation, these sites are predominately clonal. Ramets separated by as much as 30 m with no apparent connections are in fact clones sharing identical multi-locus genotypes. Although there are only seven individual genets here, they show relatively high levels of genetic variation and no evidence of inbreeding. Studies of packrat midden macrofossils found near Shafter and reaching to the Big Bend area (Powell, 1998, Van Devender, 1990) confirm that this relict population was within a much more extensive range of Q. hinckleyi. This suggests that while the species was disappearing
around it, this disjunct group of clones was able to survive. A similar type of relict
survival has been reported for a California scrub oak clone believed to be over 13,000
years old (May et al., 2009). While some feel that clonal reproduction with a lack of
sexual reproduction merely prolongs the time to extinction (Silvertown, 2008), the
identification of this clonal group suggests that clones can be a repository of genetic
diversity contributing to species survival. In this case, it appears that asexual
reproduction has served to protect against loss of alleles and inbreeding.

It remains unclear, however, why there is so little sexual reproduction at these sites.
Possible reasons include human-induced habitat degradation, poor regeneration from
seed coupled with limited number of genets, and wildlife and insect predation. Both of
the sites are on private land and may have experienced plant and seed loss to
introduced herbivores in the past (USFWS, 2009). In addition, oaks are predominantly
outcrossing, and there may be genetic barriers to seed production because of the
limited number of genotypes available for crossing. As clones get larger, flowers are
surrounded by more pollen from individuals of the same clone and may result in less
viable seed set (Handel, 1985). Finally, there were few signs of acorns as well as
evidence of herbivory when samples were collected, suggesting plant and seed
predation is a factor limiting recruitment of new genets.

The sites in Big Bend Ranch State Park (B1 and B1a) are more genetically diverse with
a greater number of unique individuals and higher genetic variability. There are two
distinct subpopulations, each containing unique alleles. Although a few clones were
identified within these two subpopulations, asexual reproduction was limited. Sexual reproduction, with resulting acorns, appears to be present. This is a positive factor for continued diversity because genetic mixing adds new genotypes to the population. In addition, while clones are literally rooted to their locations, acorns are movable and are the only natural means of dispersing *Q. hinckleyi* to new areas. Whether accelerated climate changes and unforeseen human activities will eventually lead to its extirpation in its restricted United States range remains to be seen, but the fact that genetic diversity and sexual reproduction persist in the remaining populations argues for continued conservation efforts for this species.

### 1.4.2 Conservation management

The second objective of the study was to examine the viability of remaining *Q. hinckleyi* populations and the implications for conservation management strategies. Documenting the genetic diversity of species at risk is one of the conservation criteria defined by the IUCN and others (McNeely et al., 1990). By providing a detailed characterization of the existing *Q. hinckleyi* metapopulation in West Texas, this study fulfills two priority one tasks in the Hinckley Oak Recovery Plan: #3212 to assess genetic viability and needs, and #3231 to determine types of reproduction and contribution to population (Kennedy & Poole, 1992, USFWS, 2009). The findings that show high levels of genetic variability and clonal as well as sexual reproduction, and that provide identification of unique genetic individuals can now be used to refine the recovery plan.
This leads to two specific conservation questions. First, are current levels of protection sufficient to preserve the genetic diversity that this study has identified? The answer, in short, is that there are some gaps. Conservation of *Q. hinckleyi* should begin with sustaining the known relict populations in order to preserve as much genetic variation as possible. While the significant differentiation among the sites argues for the preservation of them all, levels of protection vary. At this time, *Q. hinckleyi* within Big Bend Ranch State Park is protected because it is listed as threatened by the State of Texas, but concerns about climatic and anthropogenic effects remain. For example, a recent well-planned and researched conservation effort, the reintroduction of desert bighorn sheep (*Ovis canadensis*) (Brewer & Hernandez, 2011), may create a conservation conflict. While the sheep are an historic species, *Q. hinckleyi* is no longer a dominant shrub and the juxtaposition of threatened oak species and introduced herbivore could have unintended negative consequences for *Q. hinckleyi*. The sites near Shafter are on private land where *Q. hinckleyi* does not have protected status. While the rarity of *Q. hinckleyi* is appreciated by residents near these locations (personal observations), there is nothing that would legally prevent ecological degradation or collection of plant material from these sites. Finally, since the habitat in which *Q. hinckleyi* is found in Texas is comparable to the Chihuahuan Desert of Mexico, *Q. hinckleyi* may be found there as well, and in fact specimens of possible *Q. hinckleyi* hybrids have been collected (Nixon et al., 1997). The distribution of this rare species thus illustrates the need to coordinate conservation efforts across international borders.
Second, how can the results of this study inform potential reintroduction efforts? Although the limited number of genets is a conservation concern, the overall genetic variability found by this study supports continued conservation efforts for *Q. hinckleyi*. The importance of the ability to map clones and identify unique molecular genotypes cannot be overstated. This enables accurate population counts and selection of appropriate plants for conservation initiatives. Because of the limited numbers of *Q. hinckleyi*, conservation management strategies in addition to site protection may be necessary. These may include supplementing the numbers of unique genotypes by hand pollination and translocation of genets among remaining populations or to suitable *ex situ* locations. Long-term conservation of oaks provides its own challenges. Acorns do not remain viable in seed banks, and are inappropriate candidates for planting out to produce fresh acorns because of the long maturation time of oaks. Advances in micropropagation and cryopreservation may offer *ex situ* solutions (Kramer & Pence, 2012). *Quercus hinckleyi* conservation efforts will be challenging, but the inclusion of the genetic data provided by this study facilitates more informed management strategies and will benefit future planning.
1.5 References


2. HYBRIDIZATION AS A CONSERVATION CONCERN FOR THE THREATENED SPECIES, QUERCUS HINCKLEYI C.H. MULLER (FAGACEAE)

2.1. Introduction

Oaks as a genus have muddled and perhaps spurred efforts to delineate a theory of species going back to Darwin (Darwin, 2009). In the twentieth century, they were acknowledged as the ‘stimulus’ for the idea of the ecological species concept (Van Valen, 1976). The biological species concept, which focuses on reproductive isolation as the defining attribute of a unique species, has been confounded by their apparent ease of hybridization (Coyne & Orr, 2004, Mayr, 1996, Burger, 1975). Oak's frequent introgression continues to be a challenge when developing a taxonomic hierarchy of Quercus species (Nixon, 2002).

Hybridization has been described as both a threat and as a benefit. On the one hand it can be a risk to species identity (López-Pujol et al., 2012, Haig & Allendorf, 2006, Vila et al., 2000) because regional genetic diversity is diminished (Abbott, 1992). From this perspective, introduced non-native species can genetically overwhelm native plants and even non-viable hybrids can affect regional species by usurping reproductive material (and energy) that might be used in the sexual reproduction of the native plant (Zaya, 2013). In the case of oaks, the natural tendency to hybridize does not necessarily require introduction of non-natives; proximity to other species in the same Quercus...
subgenus is enough (Craft et al., 2002, Petit et al., 2004, Abraham et al., 2011, Burgarella et al., 2009). Alternatively it may be a means of introducing novel genetic material that can lead to new, stable, well-adapted hybrid types (Arnold, 2004). In this view, hybridization is a means of conserving the genetic variability of species that are not viable in the long-term because of changing environmental conditions (Briggs, 1997, Willis & McElwain, 2002, Anderson & Stebbins Jr, 1954), and a case can be made for protecting zones of hybridization because of the novel adaptations found there (Thompson et al., 2010). Both of these viewpoints play a part in conservation strategies.

This study assesses potential hybridization between *Q. hinckleyi*, listed as a threatened species (Department of the Interior, 1988), and other oaks. Although a number of oaks in the white oak subgenus (*Quercus*) have ranges that coincide with *Q. hinckleyi* (Powell, 1998), this study focuses on two that have been identified as possible hybridizers: *Q. pungens* (Kennedy & Poole, 1992, Nixon, 1997) and *Q. vaseyana* (Terry & Scoppa, 2010). The U.S. range of *Q. hinckleyi* is essentially limited to Presidio County in West Texas. *Quercus pungens* and *Q. vaseyana* have wider ranges that overlap with *Q. hinckleyi* (Powell, 1998) (Fig. 2-1). Macrofossils show that *Q. hinckleyi* and *Q. pungens* were both part of a once more extensive pinyon-juniper-oak woodland complex. As the climate became more xeric over the last 10,000 years, however, the plant assemblage gradually changed, ranges shifted, and *Q. hinckleyi* became the rare species it is today (Van Devender, 1990).
Figure 2-1: *Q. hinckleyi* collected at Shafter and Big Bend Ranch State Park. *Q. pungens* and *Q. vaseyana* collected at Guadalupe Mts. N.P. Additional *Q. pungens* collected at Big Bend Ranch State Park. Current ranges of *Q. pungens*, *Q. vaseyana*, and *Q. hinckleyi* shown below.


The initial Hinckley Oak Recovery Plan listed hybridization as a potential threat (Kennedy & Poole, 1992), and the follow up review continues to list assessment of hybridization as an important recovery action (USFWS, 2009). This is the first
application of genetic analysis focusing on hybridization of *Q. hinckleyi*. Microsatellite genotyping in oaks has been effective in resolving different species known to hybridize (Muir et al., 2000), determining levels of interspecies hybridization (Craft et al., 2002), investigating pollination patterns (Abraham et al., 2011), studying gene flow (Burgarella et al., 2009, Valbuena-Carabana et al., 2005), and looking into plant invasion mechanisms (Petit et al., 2004).

This research has three goals related to *Q. hinckleyi* and the two putative hybridizing species, *Q. pungens* and *Q. vaseyana*: first, to examine levels of introgression from the putative hybridizing species within the relict *Q. hinckleyi* populations; second, to determine the level of genetic differentiation between the two putative hybridizers; and third, to examine the genetic identity of morphologically identified hybrids between *Q. hinckleyi* and *Q. pungens* or *Q. vaseyana* at its relict sites.

### 2.2 Materials and Methods

#### 2.2.1 Study Species

The focal species in this study, *Q. hinckleyi* C.H. Muller (common name: Hinckley Oak), is listed under the U.S Endangered Species Act and by the state of Texas as a threatened species and is categorized as ‘critically threatened’ on the IUCN Red List. In the United States it is found in two genetically distinct populations (Chapter 1) in Presidio County, Texas (Powell, 1998) on predominately limestone substrates in Chihuahuan Desert habitat at elevations of approximately 1000–1400 m. Since this
region extends into Mexico, *Q. hinckleyi* might be found there as well (Nixon, 1997), although its presence and status is not known. Its small (1–1.5 cm diameter), grey-green thickened spiny leaves distinguish it from other species. In its native environment it grows as a shrub-like thicket with a maximum height of approximately 0.75 m. (Kennedy & Poole, 1992, Poole et al., 2007, Muller, 1951). While some acorn reproduction has been reported, *Q. hinckleyi* reproduces readily through underground rhizomes, forming clonal patches and clusters (Muller, 1970). The Hinckley Oak Recovery Plan issued by the U.S. Fish and Wildlife Service identifies a number of threats to the relict U.S. populations, including low population numbers and few individuals, wildlife and insect predation, possible hybridization with *Q. pungens* Liebm. (see below) and poor regeneration from seed (Kennedy & Poole, 1992).

*Quercus pungens* Liebm. (common name: Sandpaper Oak, Scrub Oak) and *Q. vaseyana* Buckl. (common name: Vasey Shin Oak; previously var. of *Q. pungens*) are both common species across the Trans-Pecos region (although *Q. pungens* is more widespread) and are found on limestone substrate on desert slopes; they form low shrubs and sometimes small trees (*Q. pungens*: 2–3 m; *Q. vaseyana*: 7 m). Both are found in the same region as *Q. hinckleyi* as well as in the Guadalupe Mountains. Leaves of *Q. pungens* are stiff, with coarsely toothed margins; the upper surface is lustrous while lower surfaces are densely pubescent. The common name reflects the feel of the leaves. *Quercus vaseyana* leaves are oblong, either entire or with 3–5
toothed or lobed margins; upper surfaces are lustrous and lower surfaces pubescent, but also somewhat lustrous green (Powell, 1998, Miller & Lamb, 2006, Nixon, 1997).

2.2.2 Study Sites and Sample Collection

In 2009 and 2012, 204 leaf samples from Q. hinckleyi ramets were collected from the remnant populations in Presidio County near Shafter and from the vicinity of the Solitario in Big Bend Ranch State Park (BBRSP). Two possible hybrids in close proximity to Q. hinckleyi at the Shafter sites were also sampled in 2009. In order to include putative hybridizers with no possibility of introgression by Q. hinckleyi, 20 Q. pungens and 15 Q. vaseyana individuals were sampled in Guadalupe Mountains National Park (GUMO) in 2010 approximately 320 km from the Q. hinckleyi populations (Fig. 2-1). Leaves from nine additional Q. pungens ramets were collected in 2012 from a small group in BBRSP near a stand of Q. hinckleyi, several of which were clustered along the drip line of the largest Q. pungens.

2.2.3 Microsatellite Genotyping

DNeasy Plant MiniKit (Qiagen) was used to extract DNA from approximately 20 mg of dry leaf material. DNA concentrations and quality were verified on NanoDrop spectrophotometer (Thermo Scientific). Genotyping was completed using eight primer pairs previously used with oaks in the Quercus (White Oak) subgroup of which the three species in this study are members: QpZAG1/5, QpZAG15, and QpZAG110 (Steinkellner et al., 1997), QpZAG15 and QpZAG11 (Kampfer et al., 1998), MSQ4 and MSQ13 (Dow et al., 1995), and M69–2M1 (Isagi and Suhandono 1997). A description
of the PCR amplification protocol has been published elsewhere (Abraham et al., 2011). PCR products (0.9 – 1.5 µL) were genotyped on the ABI 3730 DNA Analyzer using LIZ500 ladder (Applied Biosystems). Applied Biosystems GeneMapper, version 3.7 was used for genotype scoring. After genotyping, individuals were tested for cloning using ALLELEMATCH (Galpern et al., 2012) and clones were collapsed into unique genotypes that were used in the following analyses.

2.2.4 Genetic data analysis

Allele frequency, observed and expected heterozygosity, and fixation index were determined using GenAlEx 6.501 (Peakall & Smouse, 2012, Peakall & Smouse, 2006). Three methods were used to examine levels of hybridization and species differentiation: STRUCTURE 2.3.4 (Pritchard, Stephens et al. 2000; Falush, Stephens et al. 2003; Falush, Stephens et al. 2007; Hubisz, Falush et al. 2009), Principal Coordinates Analysis (PCoA) using GenAlEx 6.501, and the R-package DEMEtics (Gerlach et al., 2010). STRUCTURE performs Bayesian clustering analysis to infer genetic populations based on multi-locus genotypes and computes the proportion of the inferred clusters in each individual. PCoA employs multivariate analysis across multiple loci and samples and presents a visual representation of genetic structural patterns. DEMEtics calculates differentiation indexes $G_{ST}$ and $D_{JOST}$ using bootstrap resampling and provides Bonferroni corrected $p$-values.

To analyze genetic differentiation between the *Q. pungens* and *Q. vaseyana* individuals, the Admixture Model of STRUCTURE with LOCPRIOR (using sampling locations as
prior) for K 1 to 7, with 100,000 MCMC and 50,000 burnin for 10 reps each was used. Best K was determined by calculating I(K) and delta K (Evanno et al., 2005) using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The STRUCTURE procedure was then rerun using Best K with MCMC of 250,000 and burnin of 50,000 to get the proportions of membership of the sampling locations in the inferred clusters.

For purposes of hybrid analysis, STRUCTURE parameters were set to use a mixed-ancestry model with no prior population information (Thompson et al., 2010) and set K=3, corresponding to the three species in the study. Initial burnin was 50,000 iterations followed by an MCMC of 250,000 iterations. ANCESTDIST options were activated to capture 95% posterior probability intervals (Blair & Hufbauer, 2009). Individuals were identified as hybrid if the q-value (the posterior probability of an individual belonging to a single genetic cluster) was < 0.85 (Abraham et al., 2011).

2.3 Results

Out of 204 ramets, Q. hinckleyi had 123 unique genotypes. All Q. pungens and Q. vaseyana individuals sampled in GUMO were genetically unique. Of the nine Q. pungens collected in BBSRP eight were unique genets.

All loci were polymorphic and highly variable for each of the three species (Table III). Mean number of alleles, mean observed heterozygosity, and mean expected heterozygosity respectively for Q. hinckleyi were 17.875, 0.807, and 0.853, for Q. pungens 12.625, 0.734, and 0.851, and for Q. vaseyana 13.250, 0.789, and 0.869. The
mean $F_{IS}$ over all populations was 0.096, indicating no significant deviation from Hardy-Weinberg Equilibrium. Each of the species had alleles not observed in the other species; mean number of private alleles across loci was 7.125 for $Q.\ hinckleyi$, 1.500 for $Q.\ pungens$ and 1.875 for $Q.\ vaseyana$.

**Table III:** Descriptive statistics for $Q.\ hinckleyi$, $Q.\ pungens$, and $Q.\ vaseyana$.

<table>
<thead>
<tr>
<th>Locus</th>
<th>$N$</th>
<th>$Na$</th>
<th>$H_0$</th>
<th>$H_e$</th>
<th>$F_{IS}$</th>
<th>$N$</th>
<th>$Na$</th>
<th>$H_0$</th>
<th>$H_e$</th>
<th>$F_{IS}$</th>
<th>$N$</th>
<th>$Na$</th>
<th>$H_0$</th>
<th>$H_e$</th>
<th>$F_{IS}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1/5</td>
<td>121</td>
<td>16</td>
<td>0.901</td>
<td>0.897</td>
<td>-0.005</td>
<td>28</td>
<td>11</td>
<td>0.643</td>
<td>0.853</td>
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<td>112</td>
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<td>0.904</td>
<td>0.072</td>
<td>27</td>
<td>18</td>
<td>0.926</td>
<td>0.922</td>
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<td>1.000</td>
<td>0.911</td>
<td>-0.098</td>
<td>16</td>
<td>14</td>
<td>0.813</td>
<td>0.869</td>
<td>0.065</td>
</tr>
</tbody>
</table>

$N =$ number of samples, $Na =$ number of alleles, $H_0 =$ observed heterozygosity, $H_e =$ expected heterozygosity, $F_{IS}=$fixation index. (GenAlEx 6.501)

STRUCTURE results for the $Q.\ pungens$ and $Q.\ vaseyana$ analysis indicated they cluster into two genetic groups. $Quercus\ pungens$ and $Q.\ vaseyana$ from GUMO cluster into one group (hereafter referred to as the 'Q. pungens/Q. vaseyana' cluster), while the $Q.\ pungens$ genotypes from BBRSP are genetically distinct from them (Table IV).
Table IV: *Q. pungens* and *Q. vaseyana*: Proportion of membership of each pre-defined population in each of the 2 inferred clusters.

<table>
<thead>
<tr>
<th>Given Population</th>
<th>Inferred Cluster</th>
<th>Number of Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Q. pungens</em> (GUMO)</td>
<td>0.004</td>
<td>0.996</td>
</tr>
<tr>
<td><em>Q. pungens</em> (BBRSP)</td>
<td>0.710</td>
<td>0.290</td>
</tr>
<tr>
<td><em>Q. vaseyana</em> (GUMO)</td>
<td>0.042</td>
<td>0.958</td>
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</table>

(STRUCTURE 2.3.4)

Previous research (Chapter 1) found that *Q. hinckleyi* is differentiated into two well-defined subpopulations. One includes individuals from the Shafter sites and the stand in BBRSP closest to the *Q. pungens* collected there (B1a) and the other is comprised of the remaining individuals sampled in BBRSP (B1). Results of the STRUCTURE hybrid analysis showed six individuals identified in the field as *Q. hinckleyi* have < 85% *Q. hinckleyi* inferred ancestry and are likely hybrids. Six of the eight individuals identified as *Q. pungens* in BBRSP have < 85% of the inferred ancestry found in the ‘*Q. pungens/Q. vaseyana*’ cluster with the remaining percentage in the cluster of the *Q. hinckleyi* population geographically closest to it (Table V).
Table V: Individuals identified morphologically as *Q. hinckleyi* or *Q. pungens* with inferred levels of introgression.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Q. hinckleyi</th>
<th>Q. pungens/Q. vaseyana</th>
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<td></td>
</tr>
<tr>
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<tr>
<td>QUHI065 (S2)</td>
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<td>17%</td>
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<tr>
<td>QUHI190 (B1a)</td>
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<td>89%</td>
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<td>QUHI191 (B1a)</td>
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<tr>
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<td></td>
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<tr>
<td>QUHI093 (B1)</td>
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<td>72%</td>
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<tr>
<td>QUHI153 (B1)</td>
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<tr>
<td>QUHI162 (B1)</td>
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<td>90%</td>
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<td><strong>Q. pungens</strong></td>
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<td></td>
</tr>
<tr>
<td>BBRSP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QUPU021</td>
<td>21%</td>
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<tr>
<td>QUPU029</td>
<td>99%</td>
<td>1%</td>
</tr>
</tbody>
</table>

(Criteria for hybrid: posterior probability of the individual belonging to the genetic cluster associated with its species is < 0.85)

PCoA results confirm *Q. hinckleyi* is genetically differentiated from *Q. pungens* and *Q. vaseyana* and that, while *Q. pungens* and *Q. vaseyana* from GUMO are not genetically distinct, *Q. pungens* collected in BBRSP is differentiated from them (Fig. 2-2).
Figure 2-2: Principal Coordinates Analysis of *Q. hinckleyi*, *Q. pungens*, and *Q. vaseyana*.

Circles = *Q. hinckleyi*, squares = *Q. pungens*, triangles = *Q. vaseyana*, diamonds = potential hybrids. Principal coordinate 1 and 2 account for 6.79% and 5.44% of the variation, respectively (GenAlEx 6.501).
The limited differentiation between *Q. pungens* and *Q. vaseyana* from GUMO was confirmed with $G_{ST}$ at 0.007 (Bonferroni corrected $p = .003$) and $D_{JOST}$ at 0.104 (Bonferroni corrected $p = 0.039$). Genetic distance between *Q. hinckleyi* and the putative hybridizers is greater. For *Q. hinckleyi* and *Q. pungens*, $G_{ST}$ is 0.039 (Bonferroni corrected $p = 0.003$) and $D_{JOST}$ is 0.457 (Bonferroni corrected $p = 0.003$); for *Q. hinckleyi* and *Q. vaseyana* $G_{ST}$ is 0.029 (Bonferroni corrected $p = 0.003$) and $D_{JOST}$ is 0.415 (Bonferroni corrected $p = 0.003$).

Of the two individuals that were suspected of being hybrids, the one that exhibits features of both *Q. hinckleyi* and *Q. vaseyana* (Terry & Scoppa, 2010), was verified genetically to be a hybrid, with 50% *Q. hinckleyi* and 50% *Q. pungens/Q. vaseyana* inferred ancestry. The other individual, though in close proximity to *Q. hinckleyi*, was genetically 99% in the ‘*Q. pungens/Q. vaseyana*’ genetic cluster.

2.4 Discussion

This research examines three questions related to *Q. hinckleyi* and the putative hybridizing species, *Q. pungens* and *Q. vaseyana*. First, is there evidence for introgression from these other oak species within the relict *Q. hinckleyi* populations? Low levels of hybridization were found between *Q. hinckleyi* and the *Q. pungens/Q. vaseyana* GUMO cluster with no evidence of genetic swamping. Overall, approximately 95% of the samples identified as *Q. hinckleyi* in the field have predominantly *Q. hinckleyi* inferred ancestry. The relative isolation of the few remaining *Q. hinckleyi*
plants, along with the species’ propensity to form clones, may contribute to the low levels of genetic introgression. While these findings bode well for continued uniqueness of the *Q. hinckleyi* individuals, the fact remains that they are in a threatened position due to small numbers in their native U.S. range, potential natural and human threats, and a rapidly changing climate.

Second, is there genetic differentiation between the two potential hybridizers, *Q. pungens* and *Q. vaseyana*? *Quercus pungens* and *Q. vaseyana* from GUMO cluster together genetically in agreement with reports that they form hybrid swarms in areas where they are sympatric (Nixon, 1997). Further research, based on samples from allopatric populations, is needed to clarify the genetic differentiation between them. The *Q. pungens* stand from BBRSP is genetically distinct from the GUMO cluster, which may be explained by the high proportion of *Q. hinckleyi* introgression in these individuals.

Lastly, is there genetic confirmation for classification of morphologically identified hybrids between *Q. hinckleyi* and *Q. pungens* or *Q. vaseyana* at its relict sites? The two putative hybrids that were examined, both collected near *Q. hinckleyi* at the Shafter sites, were resolved. One sample fell into the *Q. pungens/Q. vaseyana* genetic cluster and did not show *Q. hinckleyi* introgression. The other, which exhibits physical characteristics of both *Q. hinckleyi* and *Q. vaseyana*, was verified to be a hybrid. It is genetically evenly split between *Q. hinckleyi* and *Q. pungens/Q. vaseyana* and therefore may be an F1 hybrid, although more loci would be needed to confirm this.
Overall, the study confirmed that hybrid plants do not necessarily exhibit external features of the introgressed species. Plants that are identifiable as one species or the other may in fact be admixtures of the two. While this was not the case for the vast majority of *Q. hinckleyi*, the plants identified morphologically as *Q. pungens* in BBRSP are highly admixed at the neutral loci examined in this study. While these plants do not exhibit *Q. hinckleyi* characteristics, over 90% of the inferred ancestry in five of the eight that were genotyped is within the cluster of *Q. hinckleyi*. This finding concurs with other *Quercus* hybridization studies which found individuals identified as morphologically ‘pure’ but with high levels of genetic introgression by other species (Ortego & Bonal, 2010, Lee & Choi, 2014). Several important conclusions can be drawn from these findings: genetic analysis is a crucial component for final determination of levels of introgression (Burgarella et al., 2009); and hybrids, even morphologically unidentifiable hybrids, may be repositories of genetic material of threatened species.

One of the conservation tasks in the ‘Hinckley Oak (*Quercus hinckleyi*) Recovery Plan’ is to examine hybridization as a potential threat. There is no evidence of genetic swamping from other oaks, lending support for continuation of the protected status of the remaining *Q. hinckleyi* as a unique species. Sustaining the remaining populations and surrounding habitat, both in protected areas and on private land, will be crucial to its continued survival.

Finally, a stand of *Q. pungens* is acting as a repository of *Q. hinckleyi* genetic material. How should these individuals be treated? The broad conservation question is whether
hybrids should be protected at a time of heightened threats to biodiversity. Some feel the answer is no if they may potentially overwhelm an endangered species. Others recognize that in peripheral zones the hybrid swarm may represent an area of increased biodiversity that should be preserved (Thompson et al., 2010). In an attempt to justify its acceptability, some ask if hybridization is the result of a ‘natural’ process, a part of evolution, or the result of human disturbance (Allendorf et al., 2001). Even in cases in which it is accepted, there is difficulty defining a clear-cut strategy for delimiting a suitable level. Official policy regarding hybrids as defined by the Endangered Species Act has been debated since its enactment in 1973. It has gone from no mention of hybrids in the initial Act, to a proposed new terminology change in 1996 shying away from the actual word ‘hybrid’ and referring to an ‘intercross’ policy which provided flexibility in the way that hybrids are treated. As of this writing, the policy was never adopted or formally withdrawn (Haig & Allendorf, 2006). As discussed above, hybrids do not necessarily exhibit recognizable parental characteristics, and they can act as storehouses for genetic variation found in threatened species. While it is true that endangered species can be genetically swamped by congener species, it is not always the case, and rather than focusing on hybridization, conservation management may be better served by protecting threatened habitat (Kothera et al., 2007) which may include hybrids. To preserve the *Q. hinckleyi* genetic variability that may be stored in the neighboring oak species, protection of the cryptic *Q. pungens* should be included as part of *Q. hinckleyi*’s conservation strategy.
2.5 References


3. GENETIC CLUSTERING AND GENE FLOW IN AN ENDEMIC CHANNEL ISLAND OAK: QUERCUS PACIFICA K. NIXON & C.H. MULLER (FAGACEAE)

3.1 Introduction

There are about 100 endemic plant species on the California Channel Islands, approximately 20 of which are shared between the Northern (Anacapa, San Miguel, Santa Rosa, and Santa Cruz) and Southern (San Clemente, San Nicolas, Santa Barbara, and Santa Catalina) Island groups (Raven, 1967, Philbrick, 1980, Moody, 2001). The origin of a given endemic, whether through speciation on the islands or as a relict population of a species that has disappeared from the mainland, is often not clear (Moody, 2001). *Quercus pacifica* is endemic to two of the northern islands, Santa Rosa and Santa Cruz, and the southern island of Santa Catalina. While there have been studies related to conservation efforts and diseases of *Q. pacifica* (Knapp, 2002, Stratton, 2001), this is the first study to examine its genetic diversity and population structure using microsatellite DNA analysis, a method that has been used extensively to answer population questions including variability, clone identification and gene flow in oaks (Dow & Ashley, 1998, Steinkellner et al., 1997b, Ashley et al., 2010, Ashley, 2010). The overall objective of this research was to examine the evolutionary history of *Q. pacifica* by evaluating evidence for genetic drift and/or genetic differentiation due either to separate colonization histories or limited gene flow among islands.
Interpreting this evidence requires an understanding of the geologic and settlement history of the islands and of oak reproductive biology. Since their emergence, the islands have been sculpted by continued uplift, volcanism, varying sea levels due to changes in climate, soil formation and plant settlement, and erosion events caused by herbivory. The uplift of the Channel Islands began about five million years ago (Schumann et al., 2012, Atwater, 1998). Evidence of geological and geophysical activity over the last 2.6 million years indicates that there were no land bridges between the islands and the mainland, or between the northern and southern islands (Vedder & Howell, 1980, Junger & Johnson, 1980), although during that time there were a series of glaciation events which resulted in changes in sea level. As recently as the last ice age (20–12,000 CYBP) Santa Rosa and Santa Cruz, located off the Santa Barbara coast, were joined with Anacapa and San Miguel islands into a larger landmass, known as Santarosae. Because of the lower sea levels, Santarosae had an area approximately 2.5 times that of the current separate islands and was within 9 km of the mainland. In this period Santa Catalina, located farther south off the coast from Los Angeles, was approximately 1.3 times its current area and was within 24 km of the mainland and over 100 km from Santarosae. Today Santa Cruz is approximately 15 km from Santa Rosa, 40 km from the mainland, and approximately 125 km from Santa Catalina, and Santa Catalina is approximately 40 km from the mainland (Porcasi et al., 1999, Moody, 2001).
While plant species were present on the islands well before humans arrived (Rick et al., 2014), settlers may have played a role in gene flow of Quercus species and certainly were responsible for habitat transformation on the islands. Native Americans and more recently non-native settlers have continuously occupied the California Channel Islands for over 10,000 years (Erlandson et al., 2008). The Chumash and the Gabrielino, sophisticated maritime cultures, made use of the resources of the islands for millennia (Collins, 1991). Marine travel followed established routes and facilitated exchange of goods, including acorns (Arnold, 1992). Humans likely affected the natural plant communities before European settlement, possibly transplanting oaks as well as other organisms (Erlandson et al., 2004). Acorns are a food source still used by mainland Native Americans (Anderson, 2005) and the palatability of acorns of Q. dumosa, the scrub oak group with which Q. pacifica was originally identified, has been documented (Bainbridge, 1987). Recent archaeological excavations conducted on Santa Cruz have found few acorn remains or in-ground grinding sites, as well as little isotopic evidence for an herbivorous diet in analysis of human remains, suggesting acorns may not have been a major trade item among the Chumash (Fauvelle, 2013). This does not preclude, however, limited exchange during ritual cycles such as mourning ceremonies which brought together diverse communities albeit for short periods of time (Hollimon, 2001).

When Europeans began settling the islands in the mid-19th century, they brought non-native herbivores to Santa Rosa, Santa Cruz, and Santa Catalina. Over the past 200 years, introduced sheep, cattle, pigs, and horses, as well as elk and mule deer on
Santa Rosa and goats and bison on Santa Catalina, have led to over-grazing and habitat destruction (Westman, 1983, Knowlton et al., 2007). The human footprint continues to be evident on the islands. Santa Catalina, for example, has been used as a resort area for over 100 years and annually attracts thousands of visitors (Sweitzer et al., 2005, Moody, 2001). Recently, however, efforts to restore and preserve native ecosystems through removal of non-indigenous herbivores have been successfully undertaken. For example, eradication of feral sheep and pigs on Santa Cruz has resulted in a rapid recovery of native tree and shrub communities (Klinger et al., 2003), and cattle, feral goats, feral pigs, and sheep have been eliminated from Santa Catalina (Catalina Island Conservancy, 2014).

Koenig & Ashley, 2003), including distances of more than 80 km (Buschbom et al., 2011). In addition to sexual reproduction, some oak species may also reproduce clonally, so that stands of trees which may appear to be multiple individuals have only one unique molecular genotype.

3.2 Materials and Methods

3.2.1 Study Species

Prior to 1994, *Q. pacifica* was identified as *Quercus dumosa* Nuttall var. *polycarpa* Greene. At that time, based on morphology, ecology and distribution, the scrub oak growing on the Channel Islands was renamed *Q. pacifica* Nixon and C.H. Muller and designated an endemic species. Leaves are leathery with either smooth or irregularly toothed margins. A key component of the scrub oak chaparral, it is found at elevations between 50 – 150 m on ridges and open slopes as well as in canyons and can cover relatively large areas. The species occurs in both a shrub and tree form up to 5 m in height. *Quercus pacifica* may be closely related phylogenetically to *Q. berberidifolia*, or possibly be a nothospecies resulting from hybridization between *Q. douglassii* and *Q. berberidifolia* or another species no longer on the islands (Nixon, 2002, Nixon & Muller, 1994). While not listed as a threatened or endangered species, as an insular endemic *Q. pacifica* is a conservation concern. Evolving for some time without native herbivores other than insects, it exhibits significantly reduced morphological defenses as exemplified by fewer and shorter spines on leaves compared with those of its closely related mainland counterparts (Bowen & Vuren, 2003). Current threats to the species...
include episodic oak die-back, root rot fungus, and herbivory by insects and non-native animals (Knapp, 2002). Although *Q. pacifica* has been negatively impacted by human disturbance, it benefits from conservation efforts to rid the islands of non-native herbivores and to monitor oak health (Knapp, 2002), as well as to restore the chaparral ecosystem (Stratton, 2001).

### 3.2.2 Genetic Analysis

Leaf samples from a total of 133 *Q. pacifica* trees were collected across the three islands on which it is found (Table VI). Forty-six trees were sampled on Santa Rosa, 39 on Santa Cruz, and 48 on Santa Catalina. Approximately 20 mg of dry leaf material was homogenized to a fine powder using Talboys High Throughput Homogenizer (Troemner). DNA was extracted with DNeasy Plant MiniKit (Qiagen). Microsatellite loci and primers used in this study were developed and applied successfully with other oaks in the white oak group (*Quercus* subgenus *Quercus*) (Dow et al., 1995, Isagi & Suhandono, 1997, Craft & Ashley, 2007, Ashley et al., 2010). Each tree was genotyped at eight neutral microsatellite loci: QpZAG1/5, QpZAG15, and QpZAG110, originally developed for *Q. petraea* (Steinkellner et al., 1997a); QpZAG15 and QpZAG11, developed for *Q. robur* (Kampfer et al., 1998); MSQ4 and MSQ13, developed for *Q. macrocarpa* (Dow et al., 1995); and QM69–2M1, developed for *Q. myrsinifolia* (Isagi and Suhandono 1997). PCR amplification followed the protocol described previously by Abraham et al. (2011) using labeled forward primers as described by Schuelke (2000). PCR products (0.9 – 1.5 µL) were genotyped on an ABI
3730 DNA Analyzer using GeneScan™ - 500 LIZ500® Size Standard (Applied Biosystems). All genotypes were scored using Applied Biosystems GeneMapper, version 3.7.
Table VI: *Quercus pacifica* sample (ID) locations.

<table>
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<th>ID</th>
<th>Santa Rosa latitude</th>
<th>Santa Rosa longitude</th>
<th>ID</th>
<th>Santa Cruz latitude</th>
<th>Santa Cruz longitude</th>
<th>ID</th>
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3.2.3 Data analysis

Statistical analyses such as allele frequency, observed and expected heterozygosity, and fixation index were analyzed using GenAlEx 6.501 (Peakall & Smouse, 2012, Peakall & Smouse, 2006). Testing for clones was done using ALLELEMATCH version 2.03 (Galpern et al., 2012), an R-package designed to identify unique multilocus genotypes where the number of individuals is not known a priori and where limited genotyping error and missing data may be present as is often the case in sampling and genotyping natural populations. The R-package DEMEtics (Gerlach et al., 2010) was used to examine population differentiation giving fixation indices $G_{ST}$ (Nei, 1973) and $D_{JOST}$ (Jost, 2008). To reduce the possibility of Type I errors caused by multiple pairwise comparisons of a single data set, this package uses bootstrapping to calculate Bonferroni corrected $p$-values. While $G_{ST}$ has been widely used to measure genetic differentiation, it has been shown that $D_{JOST}$ is a better indicator for polymorphic loci with more than two alleles (Jost, 2009, Gerlach et al., 2010). This R-package also checks for Hardy-Weinberg Equilibrium (HWE) in the input populations, and uses either alleles or genotypes as appropriate based on HWE for a given locus (Goudet 1996).

Population structure, both within and across islands, was analyzed using four tools: STRUCTURE (Pritchard et al., 2000, Falush et al., 2003, Falush et al., 2007, Hubisz et al., 2009), GENELAND (Guillot, 2008, Guillot et al., 2005a, Guillot et al., 2005b, Guillot et al., 2008), Principal Coordinates Analysis (GenAlEx 6.501), and DAPC (Jombart & Ahmed, 2011, Jombart et al., 2010). Both STRUCTURE and GENELAND use
Bayesian clustering algorithms based on genotypes of individual samples. GENELAND, however, includes geographic locations of samples to infer population structure and potential physical barriers to gene flow, even in populations with low genetic differentiation (Bech et al., 2014, Vernesi et al., 2012, Blair et al., 2012, Rieux et al., 2013). Principal Coordinates Analysis (PCoA) provides a visual representation of patterns resulting from a multivariate analysis of multiple loci and multiple samples. Discriminant Analysis of Principal Components (DAPC) is a non-model based method that uses a two-step PCA and Linear Discriminant Analysis multivariate process to infer genetic clusters. For STRUCTURE, the following procedure was used: 50K burnin with 100K for MCMC for potential K of 1 to 7 for 10 reps each, using the Admixture Model with NOLOCPRIOR. Best K was determined by calculating I(K) and Δ K (Evanno et al., 2005). With GENELAND, the GUI interface was used to run the correlated frequency, spatial model at 100K MCMC, thin rate 100, and burnin 200 for 10 runs at K of 1 to 10. This procedure was repeated five times. For each repetition, the run with the highest posterior probability was chosen, and from these five results best K was inferred from the modal value of K with the highest posterior probability. For DAPC analysis, 70 Principal Components (PCs) were retained to determine clusters and, based on the PC results, three clusters were indicated; in order to ensure stable results, 44 PCs were used for discriminant analysis of principal components and, based on results, two discriminant functions were retained.
3.3 Results

All loci used in this study were polymorphic with the mean Na (number of alleles) per locus across the islands varying from 15.3 to 16.8, mean Ho (observed heterozygosity) from 0.795 to 0.828, and mean He (expected heterozygosity) was 0.832 to 0.862 (Table VII).
Table VII: Descriptive statistics for *Quercus pacifica* by island on which it is found.

| Locus  | N   | Na  | Ho   | He   | FIS  | N   | Na  | Ho   | He   | FIS  |
|--------|-----|-----|------|------|------|-----|-----|------|------|------|------|
| MSQ13  | 46  | 19  | 0.826| 0.782| -0.056| 39  | 18  | 0.897| 0.826| -0.087|
| MSQ4   | 45  | 16  | 0.911| 0.900| -0.012| 38  | 19  | 0.763| 0.900| 0.152 |
| Q1/5   | 46  | 12  | 0.543| 0.821| 0.338 | 36  | 17  | 0.639| 0.877| 0.271 |
| Q11    | 46  | 17  | 0.761| 0.763| 0.003 | 38  | 15  | 0.684| 0.868| 0.212 |
| Q15    | 46  | 14  | 0.978| 0.883| -0.108| 38  | 12  | 0.737| 0.824| 0.106 |
| Q110   | 46  | 18  | 0.848| 0.844| -0.005| 38  | 15  | 0.921| 0.887| -0.038|
| Q9     | 46  | 18  | 0.913| 0.887| -0.030| 38  | 15  | 0.868| 0.884| 0.018 |
| QM69   | 44  | 9   | 0.841| 0.775| -0.086| 37  | 11  | 0.892| 0.827| -0.079|
| **Mean**| 45.6| 15.4| 0.828| 0.832| 0.006| 37.8| 15.3| 0.800| 0.862| 0.069|

| Locus  | N   | Na  | Ho   | He   | FIS  | N   | Na  | Ho   | He   | FIS  |
|--------|-----|-----|------|------|------|-----|-----|------|------|------|------|
| MSQ13  | 48  | 21  | 0.875| 0.869| -0.007| 44.3| 19.3| 0.866| 0.826| -0.050|
| MSQ4   | 44  | 19  | 0.750| 0.914| 0.179 | 42.3| 18.0| 0.808| 0.905| 0.106 |
| Q1/5   | 48  | 16  | 0.688| 0.817| 0.159 | 43.3| 15.0| 0.623| 0.839| 0.256 |
| Q11    | 48  | 14  | 0.729| 0.813| 0.104 | 44.0| 15.3| 0.725| 0.815| 0.106 |
| Q15    | 47  | 15  | 0.872| 0.889| 0.019 | 43.7| 13.7| 0.862| 0.865| 0.005 |
| Q110   | 48  | 18  | 0.896| 0.854| -0.050| 44.0| 17.0| 0.888| 0.861| -0.031|
| Q9     | 48  | 21  | 0.750| 0.902| 0.169 | 44.0| 18.0| 0.844| 0.891| 0.052 |
| QM69   | 45  | 10  | 0.800| 0.823| 0.028 | 42.0| 10.0| 0.844| 0.808| -0.046|
| **Mean**| 47.0| 16.8| 0.795| 0.860| 0.075| 43.5| 15.8| 0.808| 0.851| 0.050|

N = number of samples, Na = number of alleles, Ho = observed heterozygosity, He = expected heterozygosity, FIS = fixation index (GenAlEx 6.501).

This level of genetic diversity is high and comparable to values found for mainland California oaks (Craft et al., 2002, Dutech et al., 2005, Abraham et al., 2011). In spite of short distances between some of the samples, there was no evidence of cloning,
although scrub oak is given to asexual reproduction through underground rhizomes resulting in stands of genetically identical plants (AlleleMatch $p_{\text{null}} < .001$). This suggests that recruitment is primarily sexual rather than asexual. The fixation index ($F_{IS}$) was low for each island: 0.006 for Santa Rosa, 0.069 for Santa Cruz, and 0.075 for Santa Catalina. Mean $F_{IS}$ overall was 0.051. As suggested by the low fixation index, no significant deviation from HWE was found. Pairwise differentiation as measured using $G_{ST}$ and $D_{JOST}$ for *Q. pacifica* on Santa Rosa, Santa Cruz and Santa Catalina showed low but significant values with $p = .003$ (Bonferroni corrected) for each result (Table VIII).

### Table VIII:  Pairwise differentiation index using $G_{ST}$ and $D_{JOST}$ for *Q. pacifica* on Santa Rosa, Santa Cruz and Santa Catalina.

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$p = 0.003$ (Bonferroni corrected)

$G_{ST}$ results above diagonal; $D_{JOST}$ results below diagonal (DEMEtics).

STRUCTURE results were analyzed with STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to compute mean LnP(K), and $\Delta K$. $\Delta K$ peaked sharply at $K=3$, indicating three distinct genetic clusters. Output from STRUCTURE HARVESTER for $K=3$ was run
through CLUMPP (Jakobsson & Rosenberg, 2007) to group individuals into genotypes and those results were input into DISTRUCT (Rosenberg, 2004), producing a summary image of the runs (Fig. 3-1). Although three clusters were indicated, they did not coincide with the separate islands; rather, each individual had substantial percentages of each genetic cluster.
Discriminant Analysis of Principal Components (DAPC) also found three clusters of genetically related individuals (Fig. 3-2). However, samples from each genetic cluster were located on each of the islands: Santa Rosa had 13 individuals in Cluster 1, 7 in Cluster 2, and 26 in Cluster 3; Santa Cruz had eight individuals in Cluster 1, 12 in Cluster 2, and 19 in Cluster 3; and Santa Catalina had 15 individuals in Cluster 1, 29 in Cluster 2, and 4 in Cluster 3.
**Figure 3-2:** Scatterplot of individuals on the two principal components.

Ellipses indicate genetic clusters. Dots represent individuals. (DAPC)

Principal Coordinates Analysis (Fig.3- 3) showed individuals from each island intermixed across the coordinates, with no discernible grouping. Each of these models inferred three distinct genetic clusters among the samples with membership in the clusters across the islands.
Figure 3-3: *Q. pacifica* Principal Coordinates Analysis of variance.

Principal coordinate 1 and 2 account for 5.48% and 5.37% of the variation, respectively (GenAlEx 6.501).

GENELAND results indicated the individuals fell into five clusters (Table IX). Using the assumption that there is often spatial dependence among individuals and making use of the spatial input to infer origins of samples, GENELAND has the capability of detecting underlying population structure on a more granular level (Coulon et al., 2006). Although geographic barriers restricted gene flow among the sampling locations, there was still porosity in the barriers. Members of the same cluster were found on Santa Rosa, Santa Cruz and Santa Catalina, and two clusters were found on both Santa Cruz and Santa
Catalina, suggesting gene flow between the northern islands and Santa Catalina. Both Santa Rosa and Santa Cruz had clusters found on no other island (Fig. 3-4).

Table IX: *Q. pacifica* inferred cluster membership based on spatial clustering analysis.

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(GENELAND)
3.4 Discussion

There is no geological or geophysical evidence that land bridges between the California Channel Islands and the mainland, or between the northern and southern island groups, were present over the last 2.6 million years (Vedder & Howell, 1980, Junger & Johnson, 1980). The islands have been wooded since well before humans arrived, and
pollen from Santa Rosa shows that Quercus species grew there at least 10,000 years ago (Rick et al., 2014). Given these findings, this research examined the evolutionary history of Q. pacifica by evaluating evidence for genetic drift and/or genetic differentiation due either to separate colonization histories or limited gene flow within or among islands. The study found levels of genetic diversity comparable to that observed in mainland oaks and little genetic differentiation in Q. pacifica among the islands on which it is found, Santa Rosa, Santa Cruz, and Santa Catalina. Structure analyses revealed overlap of genetic clusters across the three islands. This high level of genetic variability and lack of genetic differentiation among the islands precludes a history of population bottlenecks or founder effects. Founder effects have often been suggested as the underlying factor for low genetic diversity in island populations and especially island endemics (Frankham, 1997, Templeton, 2008, DeJoode & Wendel, 1992). Recent interpretation, however, points to the fact that endemics have existed on their islands through many generations during which time founder effects could be erased (Stuessy et al., 2014), and so lack of evidence of founder effects in existing Q. pacifica may not be surprising. The current genetic state of Q. pacifica indicates extensive gene flow has kept the diversity of the species rich, a finding in agreement with a study of another Channel Island endemic, Acmispon (Fabaceae), leading the authors to conclude that the islands are close enough to each other and to the mainland to facilitate repeated gene flow (McGlaughlin et al., 2014). Several interesting questions regarding the origins of Q. pacifica and the mechanisms contributing to the high degree of genetic sharing remain.
Gene flow in oaks is accomplished through movement of acorns and spread of pollen. Given the geologic history of the islands, initial oak colonization had to occur through overwater dispersal of acorns, probably by water currents or birds. This event or series of events occurred well before humans appeared on the islands. Genetic and ecological investigation indicates that a colonization event of species of *Acacia* has taken place between Pacific islands over 18,000 km apart (Le Roux et al., 2014), so it is reasonable to assume that the relatively close proximity of the Channel Islands to the mainland would not present a barrier to acorn colonization. Once oaks were established, how did the species *Q. pacifica* evolve on the islands? There are several possible explanations. One is that it arrived on the islands as a result of acorn colonization of a mainland species. Although another endemic, *Q. tomentella*, has been found in fossil remains on the mainland (Philbrick, 1980), there is no evidence of *Q. pacifica* on the mainland, so this hypothesis is not supported. The other explanation is that *Q. pacifica* is a true island species that evolved from another scrub oak species or emerged through hybridization. It has been suggested that *Q. pacifica* is actually a hybrid of *Q. douglussii* and another scrub oak, perhaps *Q. berberidifolia* (Nixon, 2002). Although this hypothesis has not been genetically verified, such interspecific gene exchange has been suggested as a mechanism for bypassing colonization as oaks establish new territories (Petit et al., 2003). If *Q. pacifica* is the product of hybridization, this study found no indication of locations at which it may have initially occurred.
There is evidence that long distance genetic continuity has been maintained within and among the islands through gene exchange. Two potential participants in acorn translocation among the islands are humans and birds. Given the sophisticated maritime cultures of the Native Americans who inhabited the islands, there was potentially a human role in moving acorns from island to island as part of trade or ceremonial exchanges. Birds can also act as acorn transporters (Scofield et al., 2010, Caldwell et al. 2013).

Pollen has been found to travel long distances and studies have found a significant percentage of paternal material comes from outside oak stands. Oak phenology combined with weather conditions determines the amount of pollen available for transport in any given year, but winds and fog banks along the coast both have the potential of carrying pollen. Spatial cluster analysis conducted as part of this study indicates genetic barriers between the islands, although these are not absolute. The presence of physical barriers is suggested by the existence of unique genetic clusters on both Santa Rosa and Santa Cruz, while the porosity of the barriers is implied by the clusters common to these two islands and by the clusters they share with Santa Catalina. Pollen flow carried by prevailing northwesterly winds appears to be indicated by the findings that three clusters from the northern islands are found on Santa Catalina and there are no clusters unique to Santa Catalina.
In summary, the genetic variability and lack of structure found in *Q. pacifica* on the California Channel Islands suggests a dynamic history for the species, affected by weather, climate, landscape, humans and other animals, and *Quercus* biology. This research contributes to the overall understanding of island endemics and suggests that geographical, climatic, and biological dynamics must be considered when attempting to characterize them.
3.5 References


4. UNRAVELLING THE TAXONOMIC PUZZLE OF THREE CALIFORNIA SHRUB OAK SPECIES: QUERCUS DUMOSA NUTT., Q. BERBERIDIFOLIA LIEBM. AND Q. PACIFICA NIXON & C.H. MULLER

4.1 Introduction

Species of oaks (Quercus L., Fagaceae) retain genetic and ecological boundaries (Gailing & Curtu, 2014, Muir et al., 2000, Cavender-Bares & Pahlich, 2009) in spite of well-documented interspecific gene flow (Burgarella et al., 2009, Petit et al., 2003, Craft et al., 2002, Abraham et al., 2011, Valbuena-Carabana et al., 2005, Lepais et al., 2009). This apparent disparity between genetic admixture and species integrity challenges those seeking to understand how species boundaries in oaks are formed and are maintained, as well as those investigating oak phylogeny.

Clarification of taxonomic designations of California oaks has been ongoing for over a century (Fryer, 2012). During this time the name ‘Quercus dumosa’ was applied to a number of shrub oaks and the ‘Q. dumosa complex’ included at least five species that are now recognized as separate taxa based on acorn morphology, leaf vestiture, and habitat (Nixon, 2002). Three of those species are the subject of this study. Quercus berberidifolia (common name: California scrub oak) is widespread in the Central and South Coast ranges of California at elevations of 100-1800 m. It grows on a variety of soils in chaparral plant communities. From Santa Barbara southward, it does not
descend to the low elevations where *Q. dumosa* is found. *Quercus dumosa* Nutt. *sensu stricto* (common name: coastal sage scrub oak) is found only in Southern California and northern Baja California, growing in open chaparral on coastal bluffs and hillsides near the sea, often on loose sandstone or granitics. Its habitat is dwindling due to human encroachment into the desirable real estate locations it occupies. Misclassification of other species as 'dumosa’ may have contributed to lack of appreciation of the scarcity of this species. *Quercus pacifica* (common name: island scrub oak) is endemic to three of the Channel Islands, Santa Rosa, Santa Cruz, and Santa Catalina, but is not found on the mainland. It is a key component of the island scrub oak chaparral and can cover relatively large areas, growing on ridges and open slopes as well as in canyons at elevations between 50-150 m. It occurs in both a shrub and tree form up to 5 m in height (Nixon, 1997, Nixon, 2002, Fryer, 2012) (Fig. 4-1).
To examine genetic structure across the three species this study used eight neutral nuclear microsatellite markers previously applied successfully in studies of white oak species (Craft et al., 2002, Dow et al., 1995, Abraham et al., 2011, Dow & Ashley, 2008, Isagi & Suhandono, 1997, Ashley et al., 2010). Microsatellite analysis continues to be appropriate for evaluating population differentiation and resolving ecological questions (Guichoux et al., 2011, Ashley, 2010, Selkoe & Toonen, 2006). While the work of Nixon and others (Tucker, 1993, Nixon & Muller, 1994, Nixon, 2002) was based on morphological and ecological characteristics, this is the first genetic analysis to examine the reclassification of these three recently identified species.
4.2 Materials and Methods

4.2.1 Genetic Analysis

Leaf samples were collected from individuals of each species: 60 *Q. berberidifolia* from the western Santa Monica Mountains and near Topanga; 24 *Q. dumosa* from the Point Loma Peninsula and Torrey Pines State Park; and 133 *Q. pacifica* from the three Channel Islands on which it is found (46 from Santa Rosa, 39 from Santa Cruz, and 48 from Santa Catalina) (Fig.4-2).
Samples were stored in desiccant for transport and prior to DNA extraction. DNA was extracted from approximately 20 mg of the dry leaf material using DNeasy Plant MiniKit (Qiagen). DNA concentrations and quality were verified on a NanoDrop spectrophotometer (Thermo Scientific). Genotyping was completed using eight primer pairs: QpZAG1/5, QpZAG15, and QpZAG110 which were developed for *Q. petraea* (Steinkellner et al., 1997), QpZAG15 and QpZAG11 developed for *Q. robur* (Kampfer et al., 1998), MSQ4 and MSQ13 developed for *Q. macrocarpa* (Dow et al., 1995), and
M69–2M1 developed for *Q. myrsinifolia* (Isagi and Suhandono 1997). PCR amplification was completed according to the protocol described in (Abraham et al., 2011). Genotyping of PCR products (0.9 – 1.5 µL) was done on the ABI 3730 DNA Analyzer using LI500 ladder (Applied Biosystems). Applied Biosystems GeneMapper version 3.7 was used for genotype scoring of the raw data.

4.2.2. Data analysis

All samples were tested for clones with ALLELEMATCH version 2.03 (Galpern et al., 2012), an R-package that identifies unique multilocus genotypes (UMGs). Clones were collapsed into UMGs for all additional analyses. Allele frequency (Na), observed (H_o) and expected (H_e) heterozygosity, and fixation index (F_{IS}) were determined with GenAlEx 6.501 (Peakall & Smouse, 2012, Peakall & Smouse, 2006).

Three methods of analysis were used to examine population differentiation and levels of introgression among the three species. Differentiation indices G_{ST} (Nei, 1973) and D_{JOST} (Jost, 2008) were examined with the R-package DEMEtics (Gerlach et al., 2010) which employs bootstrapping to provide Bonferroni corrected *p*-values to test significance of differentiation. G_{ST} is a widely employed measure of genetic differentiation, but D_{JOST} is reportedly a more accurate measure for polymorphic loci with more than two alleles (Jost, 2009, Gerlach et al., 2010). DEMEtics checks for Hardy-Weinberg Equilibrium (HWE) in the input populations, randomizing either alleles or genotypes depending on whether or not all populations are in HWE for a given locus (Goudet 1996). GenAlEx 6.501 was used for Principal Coordinates Analysis (PCoA).
This method provides a visual representation of patterns resulting from a multivariate analysis of multiple loci and multiple samples. Bayesian analysis was performed with STRUCTURE (Pritchard et al., 2000, Falush et al., 2003, Falush et al., 2007, Hubisz et al., 2009) using the Admixture Model with LOCPRIOR and a 50K burnin and 100K MCMC for potential K of 1 to 7 for 10 reps each. Optimum number of genetic clusters (K) was determined by calculating $I(K)$ and delta K (Evanno et al., 2005) using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Using the best K value, the STRUCTURE project was rerun with 50K burnin and 250K MCMC to determine $q$-values (posterior probability), representing the percentage of individual genotypes in each K cluster. These were utilized to infer the degree of admixture among the species. The $q$-value threshold selected to evaluate significant introgression, for example to identify F$_1$ hybrids or to substantiate conservation management decisions, varies according to the questions being asked (Burgarella et al., 2009, Vähä & Primmer, 2006). For purposes of this research, the goal of which is to detect genetic introgression among the three study species, the criteria of $q < 0.80$ was used to identify individuals with significant admixture.

4.3. Results

ALLELEMATCH ($p_{\text{sil}} < .001$) showed that the original set of 60 Q. berberidifolia samples contained 43 UMGs. No clones were found among the 24 Q. dumosa and 133 Q. pacifica samples. Clones were collapsed for Q. berberidifolia and from that point only unique genotypes were used for analysis. All loci were polymorphic in each species.
Allelic diversity is comparable to that found for other California oaks (Dutech et al., 2005, Craft et al., 2002, Abraham et al., 2011). For Q. berberidifolia, Na (number of alleles) varies from 14 to 24 per locus, H₀ (observed heterozygosity) varies from 0.558 to 0.977, and Hₑ (expected heterozygosity) from 0.812 to 0.928; for Q. dumosa, Nₐ varies from 5 to 16 per locus, H₀ from 0.542 to 0.917, and Hₑ from 0.597 to 0.903; and for Q. pacifica, Na varies from 15 to 31 per locus, H₀ from 0.623 to 0.886, Hₑ from 0.830 to 0.915 (Table X). Although some loci show homozygous excess, others show heterozygous excess; overall, there is no consistent pattern across species or loci.

Each species has alleles not found in other species: Q. berberidifolia has 26, Q. dumosa has five, and Q. pacifica has 49. Mean Fₛᵢₛ (fixation index) for Q. berberidifolia is 0.039, for Q. dumosa it is 0.029, and for Q. pacifica it is 0.072. These low values suggest an absence of inbreeding (Table X).

### Table X: Q. berberidifolia, Q. dumosa, and Q. pacifica descriptive statistics.

<table>
<thead>
<tr>
<th>Locus</th>
<th>N</th>
<th>Na</th>
<th>H₀</th>
<th>Hₑ</th>
<th>Fₛᵢₛ</th>
<th>N</th>
<th>Na</th>
<th>H₀</th>
<th>Hₑ</th>
<th>Fₛᵢₛ</th>
<th>N</th>
<th>Na</th>
<th>H₀</th>
<th>Hₑ</th>
<th>Fₛᵢₛ</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁₅</td>
<td>43</td>
<td>18</td>
<td>0.977</td>
<td>0.892</td>
<td>-0.095</td>
<td>24</td>
<td>16</td>
<td>0.917</td>
<td>0.903</td>
<td>-0.015</td>
<td>133</td>
<td>31</td>
<td>0.865</td>
<td>0.864</td>
<td>-0.001</td>
</tr>
<tr>
<td>S₁₆</td>
<td>43</td>
<td>18</td>
<td>0.860</td>
<td>0.868</td>
<td>0.009</td>
<td>24</td>
<td>14</td>
<td>0.792</td>
<td>0.858</td>
<td>0.077</td>
<td>127</td>
<td>23</td>
<td>0.811</td>
<td>0.915</td>
<td>0.114</td>
</tr>
<tr>
<td>Q₁/₅</td>
<td>43</td>
<td>20</td>
<td>0.744</td>
<td>0.883</td>
<td>0.157</td>
<td>24</td>
<td>10</td>
<td>0.542</td>
<td>0.794</td>
<td>0.318</td>
<td>130</td>
<td>21</td>
<td>0.623</td>
<td>0.851</td>
<td>0.267</td>
</tr>
<tr>
<td>Q₁₁</td>
<td>43</td>
<td>14</td>
<td>0.558</td>
<td>0.812</td>
<td>0.313</td>
<td>24</td>
<td>5</td>
<td>0.875</td>
<td>0.710</td>
<td>-0.232</td>
<td>132</td>
<td>24</td>
<td>0.727</td>
<td>0.831</td>
<td>0.124</td>
</tr>
<tr>
<td>Q₁₅</td>
<td>43</td>
<td>16</td>
<td>0.930</td>
<td>0.869</td>
<td>-0.071</td>
<td>24</td>
<td>10</td>
<td>0.750</td>
<td>0.835</td>
<td>0.102</td>
<td>131</td>
<td>16</td>
<td>0.870</td>
<td>0.892</td>
<td>0.025</td>
</tr>
<tr>
<td>Q₁₁₀</td>
<td>43</td>
<td>22</td>
<td>0.837</td>
<td>0.928</td>
<td>0.098</td>
<td>24</td>
<td>11</td>
<td>0.875</td>
<td>0.837</td>
<td>-0.046</td>
<td>132</td>
<td>23</td>
<td>0.886</td>
<td>0.880</td>
<td>-0.007</td>
</tr>
<tr>
<td>Q₉</td>
<td>42</td>
<td>24</td>
<td>0.905</td>
<td>0.914</td>
<td>0.011</td>
<td>24</td>
<td>13</td>
<td>0.875</td>
<td>0.883</td>
<td>0.009</td>
<td>132</td>
<td>26</td>
<td>0.841</td>
<td>0.906</td>
<td>0.072</td>
</tr>
<tr>
<td>Q₉₆₉</td>
<td>42</td>
<td>17</td>
<td>0.952</td>
<td>0.861</td>
<td>-0.106</td>
<td>24</td>
<td>10</td>
<td>0.583</td>
<td>0.597</td>
<td>0.023</td>
<td>127</td>
<td>15</td>
<td>0.843</td>
<td>0.830</td>
<td>-0.016</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>42.8</td>
<td>18.6</td>
<td>0.846</td>
<td>0.878</td>
<td>0.039</td>
<td>24.0</td>
<td>11.1</td>
<td>0.776</td>
<td>0.802</td>
<td>0.029</td>
<td>130.4</td>
<td>22.5</td>
<td>0.808</td>
<td>0.871</td>
<td>0.073</td>
</tr>
</tbody>
</table>

N = number of samples, Na = number of alleles, H₀ = observed heterozygosity, Hₑ = expected heterozygosity, Fₛᵢₛ = fixation index (GenAlEx 6.501).
The highest level of genetic differentiation is between the two mainland species, *Q. berberidifolia* and *Q. dumosa*, with $G_{ST} = 0.029$ and $D_{JOST} = 0.341$, Bonferroni corrected $p = 0.003$. Comparison between mainland and island species shows lower but significant levels of differentiation, with that between *Q. berberidifolia* and *Q. pacifica* ($G_{ST} = 0.014$, $D_{JOST} = 0.219$, Bonferroni corrected $p = 0.003$) less than that between *Q. dumosa* and *Q. pacifica* ($G_{ST} = 0.022$, $D_{JOST} = 0.274$, Bonferroni corrected $p = 0.003$).

Principal coordinates analysis using PCoA supports these patterns (Fig. 4-3). The greatest degree of separation is between *Q. dumosa* and *Q. berberidifolia*. *Quercus pacifica* genotypes overlap both *Q. berberidifolia* and *Q. dumosa*, although there is more overlap with *Q. berberidifolia*. 
Figure 4-3: *Q. berberidifolia*, *Q. dumosa*, and *Q. pacifica* Principal Coordinates Analysis of variance.

Principal coordinate 1 and 2 account for 4.84% and 4.50% of the variation, respectively (GenAlEx 6.501).

In STRUCTURE HARVESTER, $\Delta K$ peaked sharply at $K=3$, indicating three distinct genetic clusters which corresponded closely to the three named species (Fig. 4-4).
The proportion of inferred ancestry of each pre-defined population (LOCPRIOR) in each of the three clusters was high: 0.905 for *Q. berberidifolia*, 0.849 for *Q. dumosa*, and 0.938 for *Q. pacifica*. Using the criterion of $q < 0.80$ for admixture, there was limited introgression. Five *Q. berberidifolia*, four *Q. dumosa*, and four *Q. pacifica*, all identified as these species in the field, met this threshold (Table XI).
Table XI: *Q. berberidifolia*, *Q. dumosa* and *Q. pacifica* introgression.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Species</th>
<th>Inferred cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Q. pacifica</td>
</tr>
<tr>
<td>QUBE120</td>
<td><em>Q. berberidifolia</em></td>
<td>0.171</td>
</tr>
<tr>
<td>QUBE123</td>
<td><em>Q. berberidifolia</em></td>
<td>0.330</td>
</tr>
<tr>
<td>QUBE126</td>
<td><em>Q. berberidifolia</em></td>
<td>0.361</td>
</tr>
<tr>
<td>QUBE142</td>
<td><em>Q. berberidifolia</em></td>
<td>0.155</td>
</tr>
<tr>
<td>QUBE157</td>
<td><em>Q. berberidifolia</em></td>
<td>0.541</td>
</tr>
<tr>
<td>QUDU113</td>
<td><em>Q. dumosa</em></td>
<td>0.645</td>
</tr>
<tr>
<td>QUDU114</td>
<td><em>Q. dumosa</em></td>
<td>0.317</td>
</tr>
<tr>
<td>QUDU118</td>
<td><em>Q. dumosa</em></td>
<td>0.231</td>
</tr>
<tr>
<td>QUDU124</td>
<td><em>Q. dumosa</em></td>
<td>0.234</td>
</tr>
<tr>
<td>QUPA211</td>
<td><em>Q. pacifica</em></td>
<td>0.758</td>
</tr>
<tr>
<td>QUPA218</td>
<td><em>Q. pacifica</em></td>
<td>0.794</td>
</tr>
<tr>
<td>QUPA229</td>
<td><em>Q. pacifica</em></td>
<td>0.774</td>
</tr>
<tr>
<td>QUPA239</td>
<td><em>Q. pacifica</em></td>
<td>0.716</td>
</tr>
</tbody>
</table>

Three of the five *Q. berberidifolia* have over 30% of inferred ancestry in the *Q. pacifica* cluster. Over 20% of the inferred ancestry in the four *Q. dumosa* also falls in the *Q. pacifica* cluster, with one sample having over 60% in that cluster. Both of these cases suggest island-to-mainland gene flow. The *Q. pacifica* samples that show significant introgression each have over 20% of inferred ancestry that fall into the *Q. dumosa* cluster. All of these *Q. pacifica* individuals are from Santa Catalina, which is the island closest to the locations at which *Q. dumosa* was sampled, suggesting a mainland-to-island gene flow.
4.4 Discussion

*Quercus* maintains species integrity while readily exchanging interspecific genetic material. These two seemingly conflicting findings have challenged those trying to define a universal species concept since the time of Darwin (Coyne & Orr, 2004, Darwin, 1872), and have presented difficulties to those trying to clarify oak taxonomies (Nixon, 2002). This study substantiates the taxonomic reclassification of three species that had been grouped within a complex of California scrub white oaks. The limited introgression among the three differentiated species leads to questions regarding barriers to gene flow that contribute to retention of species boundaries.

California scrub oaks have long been a taxonomic challenge with species ‘blurred’ by introgression and hybridization (Fryer, 2012). The group known as the ‘*Q. dumosa* complex’ included a number of similar species. Reclassification of this group, based on more stringent criteria, identified a number of species that had been lumped together, including *Q. berberidifolia*, *Q. dumosa sensu stricto*, and *Q. pacifica*. The genetic analysis of this study supports the reclassification that was based on morphology, habitat, and associated plant communities.

While confirming the three species’ genetic boundaries, the data also reveal limited introgression and gene flow among them. Results suggest directions in which gene flow has occurred. There was evidence of limited genetic exchange between the two mainland species, *Q. berberidifolia* and *Q. dumosa*. This low level of introgression
between the mainland species may be explained by the distances of 160-220 km and physical barriers, both natural and human-induced, between the collected samples. There were higher levels of introgression between the two mainland species and the island species. *Quercus pacifica* and the mainland species are geographically separated by at least 24 km of open ocean. *Quercus* is wind pollinated, and studies have shown that pollen often comes from well outside the stand of oaks in which the acorns sprout (Dow & Ashley, 1998, Buschbom et al., 2011, Koenig & Ashley, 2003), so gene flow through pollen movement across this distance is not unreasonable.

Researchers continue to examine mechanisms that may play a role in the conservation of species integrity in the face of interspecific gene flow. To this end, they have looked at an array of possible pre-mating and post-mating reproductive barriers in oaks, including differences in phenology (Cavender-Bares & Pahlich, 2009), pollen tube formation, floral abortion, fertilization success, progeny fitness (Abadie et al., 2012), the impact of ecological and climatic niches (Ortego et al., 2014), and the impact of landscape barriers (Herrera-Arroyo et al., 2013). New genome sequencing methods are emerging and beginning to be used in the search to clarify species-related questions (Butlin, 2010, Gailing & Curtu, 2014) as well as to untangle taxonomic puzzles (Hipp et al., 2014). Reproductive barriers in oaks are diverse and are affected by abiotic as well as biotic factors (Abadie et al., 2012). This is a compelling reason to believe that there will not be a simple, universal explanation for the maintenance of species integrity in the genetically porous *Quercus* genus.
4.5 References


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International Oak Society

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“Distributed Relational Database Architecture (DRDA): What’s It All About?”, DB2 Journal, August 1992

POSTER: ‘The story of a tough little West Texas oak, the threatened Quercus hinckleyi C.H. Mull.’ at ICCB 2013 (International Congress for Conservation Biology), Baltimore, Maryland

PRESENTATIONS: ‘Using microsatellite analysis to assess viability of a rare, isolated Quercus species: Q. hinckleyi C.H. Mull.’, at Botany 2012, Columbus, Ohio

‘Using microsatellite analysis to assess viability and genetic distribution of an endemic Channel Island oak: Quercus pacifica K. Nixon & C.H. Muller’ at 8th California Islands Symposium (2012), Ventura, California