USING GENETIC ANALYSIS TO EVALUATE HYBRIDIZATION AS A CONSERVATION CONCERN FOR THE THREATENED SPECIES *QUERCUS HINCKLEYI* C.H. MULLER (FAGACEAE)

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Premise of research.   Hybridization among oaks is well documented and is of special concern in conservation efforts directed toward threatened or endangered *Quercus*, species such as *Quercus hinckleyi*.

Methodology.   This study uses DNA microsatellite analysis to characterize hybridization between the threatened oak *Q. hinckleyi* C.H. Muller and two putative hybridizing species, *Quercus pungens* Liebmann and *Quercus vaseyana* Buckley. The two potential hybridizers were sampled at Guadalupe Mountains National Park (GUMO), approximately 320 km from the current range of *Q. hinckleyi*. *Quercus pungens* and two possible hybrids located in near proximity to the relict populations of *Q. hinckleyi* were also sampled.

Pivotal results.   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 identified as *Q. pungens* found near *Q. hinckleyi* is genetically distinct from the GUMO samples and has five of eight genets with greater than 90% *Q. hinckleyi* introgression. Two of the 14 identified *Q. hinckleyi* in close proximity to this population had *Q. pungens* introgression. Bayesian clustering analysis showed that 5% of the samples identified as *Q. hinckleyi* in the field were hybrids, and one putative hybrid was confirmed genetically.

Conclusions.   While there is some hybridization in the *Q. hinckleyi* population, we found 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.

Keywords: *Quercus hinckleyi*, *Quercus pungens*, *Quercus vaseyana*, threatened species, hybridization, conservation.

Online enhancements: appendix tables.

Introduction

Hybridization in Oaks

Clearly distinguishable lineages of oaks often hybridize, and the problems this creates for delineating species in oaks dates back to Darwin, who described a proliferation of doubtful oak species (Darwin 1872 [1963]). The application of the biological species concept—which focuses on reproductive isolation as the defining attribute of a species—is obviously confounded by hybridization among oak species (Burger 1975; Mayr 1996; Coyne and Orr 2004). Oaks were acknowledged as the stimulus for the idea of the ecological species concept, which defines a species as a group of individuals that exchange genes

and occupy the same adaptive zone (Van Valen 1976). Although shared ancestral polymorphism has been argued as the reason that closely related oak species have many alleles in common (Muir and Schlötterer 2005, 2006), there is ample genetic evidence of hybridization between sympatric oak species (Craft et al. 2002; Dodd and Afzal Rafii 2004; Lexer et al. 2006; Moran et al. 2012; Tamaki and Okada 2014). Although significant interspecific gene flow occurs, selection at functional loci may maintain species' distinctiveness and adaptive differences (Dodd and Afzal Rafii 2004; Lexer et al. 2006; Zeng et al. 2011; Moran et al. 2012). Frequent introgression among oaks creates challenges for developing a taxonomic hierarchy of *Quercus* species (Nixon 2002). New genomic techniques, however, appear poised to clarify phylogenetic relationships among oaks. For example, restriction site–associated DNA (RAD-seq) has produced a well-supported and strongly resolved framework phylogeny for a subset of North American oaks (Hipp et al. 2014).

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Hybridization as a Conservation Issue for *Quercus hinckleyi*

In this study, we assess potential hybridization between *Quercus hinckleyi* (*Quercus* subg. *Quercus* sect. *Quercus*: white oaks), listed as a threatened species (US Fish and Wildlife Service 1988), and other oaks. The range of *Q. hinckleyi* is limited to sites near Shafter, Texas, and the Solitario formation in Big Bend Ranch State Park (BBRSP), Texas (Nixon et al. 1997; Powell 1998), on predominately limestone substrates in Chihuahuan Desert habitat at elevations of approximately 1000–1400 m. Because this region extends into Mexico, *Q. hinckleyi* may be found there as well (Nixon et al. 1997), although its presence and status are not known. A recent study examined the genetic diversity, population structure, and clonal growth of *Q. hinckleyi* (Backs et al. 2015). Although the species is currently limited to a handful of populations and there is a high level of cloning at two of the smaller sites at which it is located, genetic diversity is high and there is no indication of inbreeding. Structure analysis showed two distinct subpopulations, one of which occupies sites separated by 60 km of desert terrain, an example of biogeographical vicariance caused by a drying climate (Backs et al. 2015).

Hybridization can be seen either as a potential risk to the species integrity of *Q. hinckleyi* or, alternatively, as a way of introducing adaptive genetic material to it. Seen as a risk, threatened species may be genetically swamped by more common sympatric species (Haig and Allendorf 2006; Lopez-Pujol et al. 2012). For *Q. hinckleyi*, with few remaining individuals, this could lead to the loss of its identity as a genetically unique species. On the other hand, the genetic porosity of oaks may enable positive adaptations to spread through a species (Van Valen 1976), allowing it to persist in times of environmental change. Becker et al. (2013) have modeled the adaptive potential of hybridization and argue for conservation of closely related hybridizing species as a means of promoting future phylogenetic and biological diversity. A study of hybridization between a California endemic, Engelmann oak (*Quercus engelmannii* Greene), and sympatric oak species found that while hybridization in some fringe areas of the Englemann oak's range appear to be leading to its extirpation there, overall there was no genetic swamping by the other oaks. They postulate that the introgression may in fact be a means of enhancing adaptive potential (Ortego et al. 2014). In this view, hybridization is a means of conserving species that are not viable in the long term because of changing environmental conditions (Anderson and Stebbins 1954; Briggs 1997; Willis and McElwain 2002), 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 should play a part in conservation strategies.

Other oaks in the white oak section could potentially hybridize with *Q. hinckleyi*. A number have ranges sympatric with *Q. hinckleyi* (e.g., *Quercus laceyi* and *Quercus mohriana* found in the vicinity of *Q. hinckleyi* in BBRSP, Texas; Powell 1998). We focus, however, on two that have been identified as possible hybridizers: *Quercus pungens* and *Quercus vaseyana*. Seedlings germinated from acorns collected at one of the *Q. hinckleyi* sites did not exhibit features of *Q. hinckleyi*, and the Hinckley oak recovery plan suggested that they might

have been the result of hybridization with nearby *Q. pungens* (Kennedy and Poole 1992). The 5-yr recovery review refers to a hybrid that shows both *Q. hinckleyi* and *Q. vaseyana* characteristics (US Fish and Wildlife Service 2009), and a tree has been identified morphometrically as a hybrid of *Q. hinckleyi* and *Q. vaseyana* (Terry and Scoppa 2010). Macrofossils show that *Q. hinckleyi* and *Q. pungens* were both part of a once more extensive pinyon-juniper-oak woodland complex. Because the climate became more xeric over the past 10,000 yr, however, the plant assemblage gradually changed, ranges shifted, and *Q. hinckleyi* became the rare species it is today (Van Devender 1990). *Quercus pungens* and *Q. vaseyana* have wider ranges that overlap with *Q. hinckleyi* (Powell 1998; fig. 1). While ranges overlap, *Q. hinckleyi* differs markedly in leaf morphology and growth habit from the two putative hybridizer species, *Q. pungens* and *Q. vaseyana*, so that it is easily identifiable as a different species (fig. 2).
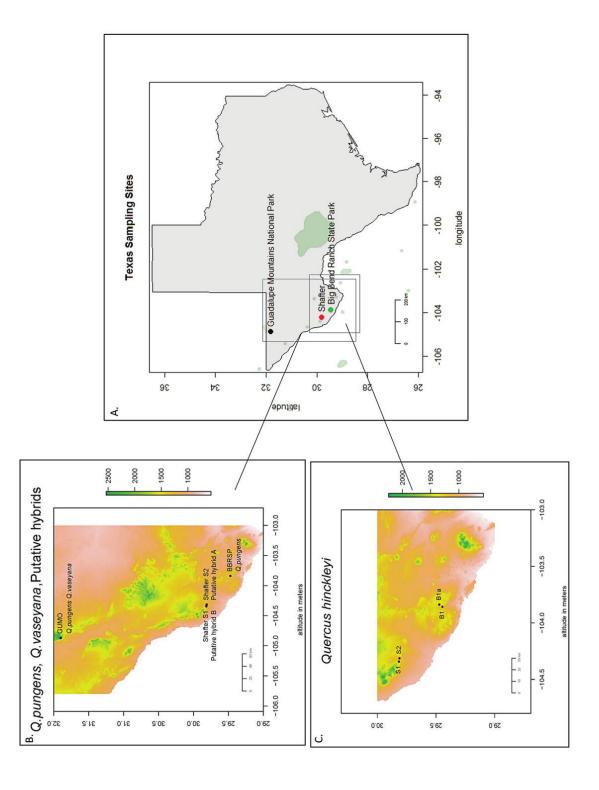
Goals of This Study

The initial Hinckley oak recovery plan listed hybridization as a potential threat (Kennedy and Poole 1992), and the follow-up review continues to list assessment of hybridization as an important recovery action (US Fish and Wildlife Service 2009). This is the first application of genetic analysis focusing on hybridization of *Q. hinckleyi*. Microsatellite genotyping has proven to be a valuable method of genetic analysis in a variety of studies looking at oak hybridization and levels of introgression. It has been used to examine hybridization along multispecies gradients (Valencia-Cuevas et al. 2015), verify levels of introgression in pure individuals (Lee and Choi 2014), investigate levels of hybridization between sympatric species (Abraham et al. 2011), identify distinct taxa even when they readily hybridize (Muir et al. 2000), determine species boundaries between sympatric oaks (Craft et al. 2002), study F_1 hybrids and backcrossing (Burgarella et al. 2009), and understand oak gene flow (Valbuena-Carabana et al. 2005).

Our 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 two putative hybrids between *Q. hinckleyi* and *Q. pungens* or *Q. vaseyana* that were sampled at its relict sites. The first plant clearly shows morphology of both *Q. hinckleyi* and *Q. vaseyana* (Terry and Scoppa 2010; fig. 2C–2E); the second did not exhibit *Q. hinckleyi* growth form but was in close proximity to one of the *Q. hinckleyi* stands and had leaf morphology resembling *Q. hinckleyi* (fig. 2F–2G).
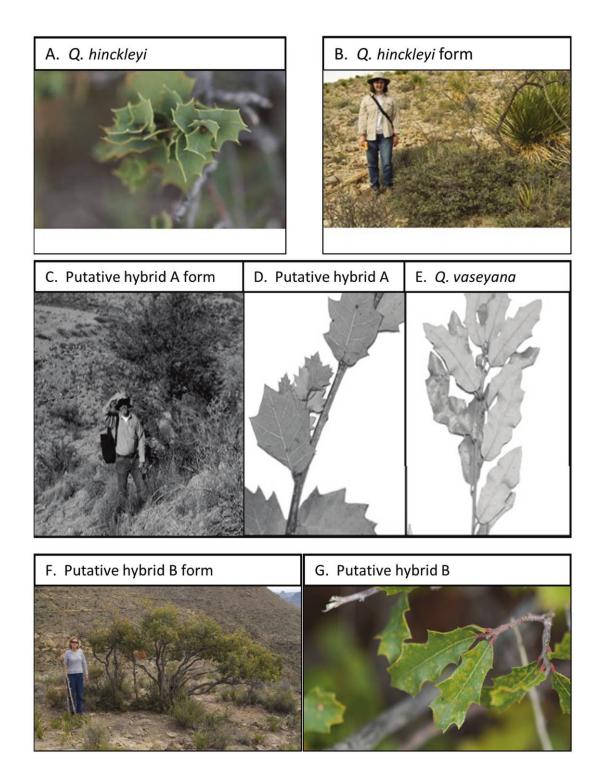
Material and Methods

Study Species

The focal species in this study, *Quercus hinckleyi* C.H. Muller (common name: Hinckley oak), is listed under the US Endangered Species Act and by the state of Texas as a threatened species and is categorized as critically threatened on the

Fig. 1   *A*, Overview of collection sites in the state of Texas. Green patches indicate the range of *Quercus pungens* and *Quercus vaseyana* (adapted from http://gec.cr.usgs.gov/data/little/). *Quercus vaseyana* was considered a variety of *Q. pungens*, Nixon (1997) defined the range of *Q. pungens* as Arizona, New Mexico, Texas, and Mexico (Chihuahua, Coahuila, and Nuevo León). At the time of the taxonomic revision, *Q. pungens* as Texas and Mexico (Chihuahua, Coahuila, and Nuevo León). *B*, *Quercus pungens* and *Q. vaseyana* collection sites at Guadalupe Mountains National Park, *Q. pungens* collection sites at Big Bend Ranch State Park, and sites of two putative hybrids collected near Shafter. *C*, *Quercus hinckleyi* collection sites at Shafter (S1, S2) and Big Bend Ranch State Park (B1, B1a).

Fig. 2 *A*, *Quercus hinckleyi* leaves showing their small holly-like shape. *B*, *Quercus hinckleyi* growth form with reference person to show the size of a typical clump. *C*, Putative hybrid A with reference person. *D*, Leaves of putative hybrid A. *E*, Leaves of *Quercus vaseyana*, A. M. Powell & S. Powell 3733 (SRSC). *F*, Putative hybrid B with reference person. *G*, Leaves of putative hybrid B. Photos in *A*, *B*, *F*, and *G* by B. Backs. Images in *C–E* from Terry and Scopa (2010).

International Union for Conservation of Nature Red List. Small, rotund (1–1.5 cm diameter), gray-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 (Muller 1951; Kennedy and Poole 1992; Poole et al. 2007). While some acorn production 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 US Fish and Wildlife Service identifies a number of threats to the relict US populations, including low population numbers and few individuals, wildlife and insect predation, possible hybridization with *Quercus pungens* Liebmann, and poor regeneration from seed (Kennedy and Poole 1992).

Quercus pungens and *Quercus vaseyana* have been through a taxonomic revision. We treat them as two species, following Nixon (1997), although before this, *vaseyana* was considered a variety of *pungens* (Muller 1951) and is in fact still referenced as such in some publications. *Quercus pungens* Liebmann (common name: sandpaper oak, scrub oak) and *Q. vaseyana* Buckley (common name: Vasey shin oak; previously variety of *Q. pungens*) are both common species across the Trans-Pecos region, although *Q. pungens* is more widespread. At the time of the taxonomic revision, Nixon (1997) defined the range of *Q. pungens* as Arizona, New Mexico, Texas, and Mexico (Chihuahua and Coahuila) and of *Q. vaseyana* as Texas and Mexico (Chihuahua, Coahuila, and Nuevo León). Both are found in the same region as *Q. hinckleyi* as well as in the Guadalupe Mountains (fig. 1). They grow on limestone substrate on desert slopes and form low shrubs and sometimes small trees (*Q. pungens*: 2–3 m; *Q. vaseyana*: 7 m). Leaves of *Q. pungens* are stiff and elliptic to oblong (1–9 cm × 1–4 cm), 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 (2–9 cm × 1–2 cm), either entire or with three to five toothed or lobed margins; upper surfaces are lustrous and lower surfaces pubescent but also somewhat lustrous green (Nixon et al. 1997; Powell 1998; Miller and Lamb 2006). While ranges overlap, *Q. hinckleyi* differs markedly in leaf morphology and growth habit from the two putative hybridizer species, *Q. pungens* and *Q. vaseyana*, so that it is easily identifiable as a different species (fig. 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 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. 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*.
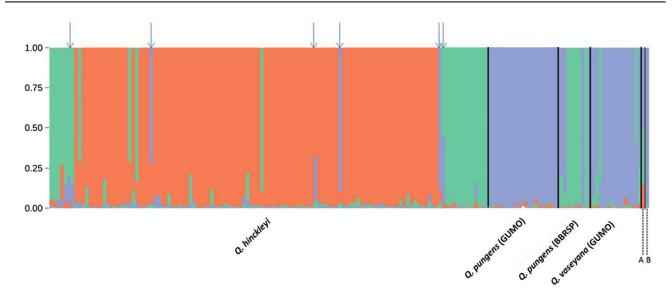
Microsatellite Genotyping

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 (ver. 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.

Genetic Data Analysis

Allele frequency, observed and expected heterozygosity, and fixation index were determined using GenAlEx 6.501 (Peakall and Smouse 2006, 2012). We used three methods to examine levels of hybridization and species differentiation: STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz 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 on the basis of multilocus 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.

For purposes of hybrid analysis, we set STRUCTURE parameters 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 burn-in was 50,000 iterations followed by a Markov chain Monte Carlo (MCMC) of 250,000 iterations. ANCESTDIST options were activated to capture 95% posterior probability intervals (Blair and Hufbauer 2009). Individuals were identified as hybrid if their q value (the posterior probability of an individual belonging to a single genetic cluster) was <0.85 (Abraham et al. 2011). The Q-file output from STRUCTURE HARVESTER was used as input to STRUCTURE PLOT (Ramasamy et al. 2014) to produce a visual representation of the clusters (fig. 3).

To analyze genetic differentiation between the *Q. pungens* and *Q. vaseyana* individuals, we ran the Admixture Model of STRUCTURE with LOCPRIOR (using sampling locations as prior) for $K = 1–7$, with 50,000 burn-in and 100,000 MCMC for 10 reps each. Best K was determined by calculating ln(K) and delta K (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl and vonHoldt 2012). The STRUCTURE procedure was then rerun using best K with burn-in of 50,000 and MCMC of 250,000 to obtain the proportions

Fig. 3 Results of Bayesian assignment of individuals to genetic clusters using STRUCTURE (ver. 2.3.4; Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009) with the options admixture and nolocprior. Each vertical bar corresponds to one individual's inferred ancestry into one of three genetic clusters. *Quercus hinckleyi* forms two clusters shown in orange and green (as described in Backs et al. 2015). *Quercus pungens* and *Quercus vaseyana* from Guadalupe Mountains National Park (GUMO) comprise one genetic cluster shown in blue. Arrows indicate individuals identified as *Q. hinckleyi* that have >0.15 introgression and are therefore considered hybrids. Several individuals identified in the field as *Q. pungens* (Big Bend Ranch State Park [BBRSP]) cluster genetically with *Q. hinckleyi*. A and B are the putative hybrids: A, the individual showing morphology intermediate between *Q. hinckleyi* and *Q. vaseyana*, is confirmed genetically to be a hybrid; B, which shows no evidence of admixture, is assigned to the *Q. pungens/Q. vaseyana* cluster.

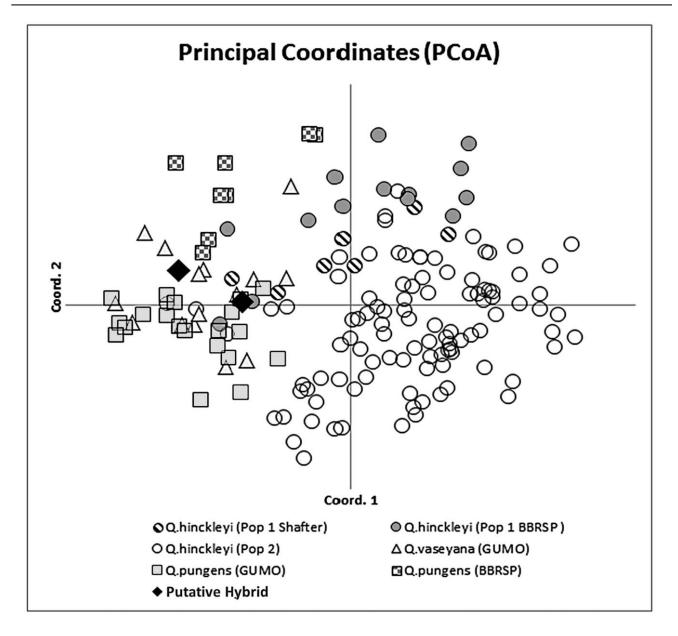of membership of the sampled individuals in the inferred clusters.

Results

Out of 204 ramets, *Quercus hinckleyi* had 123 unique genotypes. All *Quercus pungens* and *Quercus vaseyana* individuals sampled in GUMO were genetically unique. Of the nine *Q. pungens* collected in BBRSP, eight were unique genets. All loci were polymorphic and highly variable for each of the three species (table A1; tables A1–A3 available online). Mean number of alleles, mean observed heterozygosity, and mean expected heterozygosity, respectively, were 17.875, 0.807, and 0.853 for *Q. hinckleyi*; 12.625, 0.734, and 0.851 for *Q. pungens*; and 13.250, 0.789, and 0.869 for *Q. vaseyana*. The mean F_{IS} over all populations was 0.098, indicating no significant deviation from Hardy-Weinberg equilibrium. Each of the species had alleles not observed in the other species; the mean percentage of private alleles across loci was 39.9% for *Q. hinckleyi*, 11.9% for *Q. pungens*, and 14.2% for *Q. vaseyana*.

STRUCTURE results (fig. 3) indicate that *Q. 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 A2). *Quercus vaseyana* shows some admixture at the GUMO site, but because of the distance from the nearest known *Q. hinckleyi*, it is unlikely introgression from *Q. hinckleyi*. Previous research found that *Q. hinckleyi* is differentiated

into two well-defined clusters; one includes individuals from the Shafter sites (S1, S2) and the stand in BBRSP closest to the *Q. pungens* collected there (B1a), and the other is composed of the remaining individuals sampled in BBRSP (B1; Backs et al. 2015). Results of the STRUCTURE hybrid analysis showed that 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 A3).

PCoA results (fig. 4) confirm that *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. *Quercus hinckleyi* and *Q. pungens/Q. vaseyana* separate along axis 1. *Quercus pungens* and *Q. vaseyana* from GUMO cluster together, while individuals identified as *Q. pungens* from BBRSP cluster separately in the upper left of the plot. The two putative hybrids cluster close to the *Q. pungens/Q. vaseyana* GUMO individuals, with the confirmed hybrid located nearer the *Q. hinckleyi* population.

The limited differentiation between *Q. pungens* and *Q. vaseyana* from GUMO was confirmed with a G_{ST} value of 0.007 (Bonferroni-corrected $P = 0.003$) and D_{JOST} of 0.104 (Bonferroni-corrected $P = 0.039$). Genetic distances between *Q. hinckleyi* and the putative hybridizers are greater. For *Q. hinckleyi* and *Q. pungens*, G_{ST} is 0.039 (Bonferroni-corrected

Fig. 4 Principal coordinates analysis of *Quercus hinckleyi*, *Quercus pungens*, and *Quercus vaseyana*. Circles = *Q. hinckleyi*; squares = *Q. pungens*; triangles = *Q. vaseyana*; diamonds = potential hybrids. Principal coordinates 1 and 2 account for 6.79% and 5.44% of the variation, respectively (GenAlEx 6.501). BBRSP, Big Bend Ranch State Park; GUMO, Guadalupe Mountains National Park.

$P = 0.003$) 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 thought to be hybrids, the one that clearly exhibits features of both *Q. hinckleyi* and *Q. vaseyana* 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 physical proximity to *Q. hinckleyi*, was genetically 99% in the *Q. pungens*/*Q. vaseyana* genetic cluster.

Discussion

This research examines three questions related to *Quercus hinckleyi* and the putative hybridizing species *Quercus pungens* and *Quercus vaseyana*. First, is there evidence for introgression from these other oak species within the relict *Q. hinckleyi* populations? We found low levels of hybridization between *Q. hinckleyi* and the *Q. pungens*/*Q. vaseyana* GUMO cluster but no evidence of genetic swamping. Approximately 95% of the samples identified as *Q. hinckleyi* in the field have predominantly *Q. hinckleyi* inferred ancestry. These findings

are in agreement with those found in a study of another threatened endemic oak, *Quercus engelmanii* in California, with a geographical distribution overlapping various oak species. *Quercus engelmanii* also shows no indication of genetic swamping, although it shares alleles with neighboring species. The implication, as with *Q. hinckleyi*, is that the acquisition of these alleles may have adaptive possibilities for the threatened species (Ortego et al. 2014).

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 maintaining the unique genetic identity of *Q. hinckleyi*, the fact remains that they are in a vulnerable position because of small numbers in their native US 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 et al. 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.

Last, is there genetic confirmation for classification of proposed hybrids between *Q. hinckleyi* and *Q. pungens* or *Q. vaseyana* at the relict sites? We resolved the two putative hybrids that were examined, which were both collected near *Q. hinckleyi* at the Shafter sites. The first, which exhibits physical characteristics of both *Q. hinckleyi* and *Q. vaseyana*, was verified to be a hybrid. It is genetically intermediate between *Q. hinckleyi* and *Q. pungens*/*Q. vaseyana* and therefore may be an F_1 hybrid, although more loci would be needed to verify this. Although beyond the scope of this study, research into fertility of F_1 hybrids and their ability to backcross is needed to understand their possible role as conduits of adaptations between hybridizing oaks, as envisioned by Van Valen (1976). F_1 hybrids and backcrossing are also relevant to questions of maintaining species identities and the potential of hybrids to act as repositories of adaptive genetic material (Rieseberg et al. 2003; Olrik and Kjaer 2007; Burgarella et al. 2009). Interestingly, the other putative hybrid fell into the *Q. pungens*/*Q. vaseyana* genetic cluster and did not show *Q. hinckleyi* introgression.

Our study confirmed that hybrid plants do not necessarily exhibit external features of the introgressed species. We found that 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. Although these plants do not exhibit *Q. hinckleyi* morphological characteristics, more than 90% of the inferred ancestry in five of the eight that were genotyped is with *Q. hinckleyi*. This finding concurs with other *Quercus* hybridization studies that found individuals identified as morphologically pure but with high levels of genetic introgression by other species (Ortego and Bonal 2010; Lee and 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; Eaton and Ree 2013); and hybrids, even morphologically unidentifiable hybrids, may be repositories of genetic material of threatened species.

One of the conservation tasks in the Hinckley oak (*Q. hinckleyi*) recovery plan is to examine hybridization as a potential threat. We found 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. Some feel the answer is no if they may potentially overwhelm an endangered species (Vila et al. 2000; Haig and Allendorf 2006; Lopez-Pujol et al. 2012; Zaya 2015). Others recognize that in peripheral zones, the hybrid swarm may represent an area of increased biodiversity (Anderson and Stebbins 1954; Briggs 1997; Willis and McElwain 2002) that should be preserved (Thompson et al. 2010). Some judge hybridization by asking whether it is the result of a natural process, a part of evolution, or the result of human disturbance (Allendorf et al. 2001). Hybrid conservation policy is inconsistent and continues to focus on genetically pure species (Jackiw et al. 2015; Piett et al. 2015). 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 terminology change in 1996 shying away from the actual word "hybrid" and referring to an intercross policy that would provide flexibility in the way that hybrids are treated (US Fish and Wildlife Service 1996). As of this writing, the policy was never adopted nor formally withdrawn (Haig and Allendorf 2006; Jackiw et al. 2015). As shown in this study, 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) that 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.

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