Conserv Genet (2012) 13:117–130
DOI 10.1007/s10592-011-0269-y

RESEARCH ARTICLE

Population genetic diversity and structure of two rare vernal pool grasses in central California

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Received: 14 March 2011 / Accepted: 1 September 2011 / Published online: 5 October 2011
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Abstract Vernal pool ecosystems are declining throughout California, with only 10% of historic habitat remaining. This has endangered many specialist endemic plant species, leaving extant populations fragmented, isolated, and threatened or endangered. Recovery plans for the increasing number of endangered vernal pool species require information on their genetic and ecological status to guide conservation and restoration efforts. Federally threatened *Neostapfia colusana* (Colusa grass) and federally endangered *Tuctoria greenei* (Greene's tuctoria) are two endemic vernal pool grasses of high conservation concern in central California. Remaining populations are highly fragmented due to range-wide habitat destruction. Using five polymorphic microsatellite markers for each species, we performed genetic surveys of 240 individuals from eight vernal pools for *N. colusana*, and 317 individuals from 13 vernal pools for *T. greenei*. We detected high within-population genetic diversity for both species, with average allelic diversities of 24 alleles/locus (mean Hobs = 0.68, mean Hexp = 0.71) for *N. colusana*, and 19 alleles/locus (mean Hobs = 0.77, and mean Hexp = 0.79) for *T. greenei*. Bayesian clustering and AMOVA indicated two genetically distinct population groups for *N. colusana* (Fst = 0.268, $P < 0.0001$), and three for *T. greenei* (Fst = 0.11, $P < 0.0001$). We found very slight temporal genetic structure at one *N. colusana* (Fst = 0.013, $P < 0.05$) and two *T. greenei* (Fst = 0.015, Fst = 0.018, $P < 0.05$) pools. These estimates of population genetic diversity and structure are critical measures for both species that will help inform recovery management actions.

Keywords Genetic structure · Genetic diversity · Endangered species · Vernal pool grasses · Habitat fragmentation · Microsatellite · *Tuctoria* sp. · *Neostapfia colusana*

Electronic supplementary material The online version of this article (doi:10.1007/s10592-011-0269-y) contains supplementary material, which is available to authorized users.

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Introduction

Habitat loss, fragmentation and degradation are major drivers of the decline of species and the current global biodiversity crisis (Young and Clarke 2000). Many geographically or edaphically specialized plant species are in danger of extirpation or extinction (Stone 1990). Vernal pool ecosystems—ephemeral wetlands in shallow impermeable depressions that fill during the winter rainy season and dry slowly to become completely desiccated in the summers—have been declining in recent decades throughout California due to agricultural and urban development (Nikiforoff 1941; Weitkamp et al. 1996; Hobson and Dahlgren 1998). With only 10% of historic habitat remaining, this loss has resulted in an extremely fragmented vernal pool landscape leaving many species vulnerable to extinction (Holland 1978; Griggs and Jain 1983; Baskin 1994; Keeler-Wolf et al. 1998). This trend particularly endangered many specialist endemic plant species that evolved to withstand the extreme seasonal dry and wet conditions of vernal pools. Extant populations now face associated population declines which are likely to lead

to genetic erosion and loss of adaptive capacity (Karron 1987; Elam 1998; Reed and Frankham 2003). To persist, these specialized species will likely require active restoration efforts (Elam 1998; Foin et al. 1998).

Destruction of vernal pool habitat is often mitigated by the creation of new pools or restoration of degraded ones (Black and Zedler 1998; Ferren et al. 1998). The potential genetic ramifications of mixing and distributing seed material of conservation target species are not uniformly considered during mitigation efforts. To do this, managers need information about genetic diversity and structure of vernal pool ecosystems. However, data on genetic patterns within vernal pool species are limited to a small number of taxa (Solomeshch et al. 2007; Ramp et al. 2006; Ramp Neal et al. 2008; Sloop and Ayres in press; Sloop et al. 2011). A meta-analysis of datasets examining a correlation between fitness and heterozygosity by Reed and Frankham (2003) confirmed that loss of heterozygosity has a deleterious effect on population fitness, a conclusion that supports the IUCN (International Union for the Conservation of Nature) designation of genetic diversity as worthy of conservation. As population sizes shrink and isolation increases, inbreeding and genetic drift will increase, resulting in the fixation of alleles and lower genetic diversity (Ellstrand and Elam 1993; Elam 1998). To avoid an extinction vortex (Gilpin and Soulé 1986), human aided gene flow may be warranted in cases where population size and genetic diversity have reached extremely low levels (Tallmon et al. 2004; Frankham 2010; Segelbacher et al. 2010; Sloop et al. 2011). Yet, to ensure that these translocation efforts are effective, a detailed understanding of the underlying genetic structure and diversity of imperiled taxa across their geographic range is imperative (Frankham et al. 2002). In the absence of crossing studies, this genetic information provides a measure of similarity between populations, and may therefore help decrease the risk of mixing seed propagules from very dissimilar populations. Such mixing may cause negative effects from out-breeding depression—the reduction in reproductive fitness due to crossing of individuals from two genetically differentiated populations (Ellstrand and Elam 1993; Elam 1998). In addition to spatial considerations, temporal genetic structure, or the genetic differentiation of populations among years, is also important when devising restoration strategies, particularly for annuals that are dependent on a persistent seed bank for responding to varying environmental conditions over time.

Genetic tools can identify range-wide or regional patterns in the distribution of genetic diversity, distinguishing management units for conservation and appropriate sources for human-aided gene flow (Ellstrand and Elam 1993). Highly variable microsatellite markers, which allow accurate estimation of within- and between-population differentiation, are genetic tools that can be used to detect populations with rare alleles which may be candidates for special protection and populations with low diversity potentially in need of management assistance (Estoup et al. 1995; Cornuet et al. 1999; Pritchard et al. 2000).

In this study, we developed and employed microsatellite markers to identify genetic population structure and diversity in two rare endemic vernal pool grasses, *Neostapfia colusana* (Burtt Davy) Burtt Davy and *Tuctoria greenei* (Vasey) Reeder. We sampled at extant sites of each species across their California Central Valley endemic range in 2007 and 2008. Due to decreasing population sizes and increased potential for inbreeding because of loss of gene flow across their historic ranges, we hypothesize there to be low genetic diversity and high genetic structure. This study provides new genetic tools and an insight into the spatial genetic diversity, temporal genetic diversity and range-wide spatial structure in *N. colusana* and *T. greenei*. This information is useful for recovery plans, and we make general recommendations for conservation and restoration actions.

Methods

Study species

Federally threatened and state endangered *N. colusana,* and federally and state endangered *T. greenei*, together with five other members of the grass tribe Orcuttieae share a number of life history characteristics. They are all wind-pollinated annuals (Griggs 1981) restricted to vernal pool habitats, developing after pools have dried out, generally in late spring or early summer depending on rainfall and flowering typically in June and July (Griggs 1981; Keeley 1998). *Neostapfia colusana* is self-compatible, yet is generally outcrossing where pollen is not limiting (Davis et al. 2009). The breeding system in *T. greenei* is not confirmed, however, a previous study comparing allozyme diversity values within and among two populations suggests high levels of outcrossing (Griggs and Jain 1983). Short distance seed dispersal is facilitated by water. While there is no information in the literature indicating the precise mechanism, rare, long distance dispersal may have historically been carried out by waterfowl, tule elk or pronghorn antelope (Griggs 1981).

As with other vernal pool annuals, populations of *T. greenei* and *N. colusana* can fluctuate widely from year to year by up to three orders of magnitude, and in very dry years when the pools do not fill, there may be no germination at all (California Natural Diversity Database 2006; Alexander and Schlising 1998). Seed dormancy can last many years, germinating after appropriate periods of inundation (Reeder 1982).This seed banking mechanism

allows annual plants to cope with the unpredictability of suitable conditions for germination and growth (Solomeshch et al. 2007).

California Natural Diversity Database records (CNDDB), show that historically, *N. colusana* was known from 60 occurrences throughout the California Central Valley from Colusa County in the north to Merced County in the south (CNDDB 2006). Today, no more than 42 of these are presumed extant, with the majority found in Stanislaus and Merced Counties (Fig. 1). While *N. colusana* has been extirpated from almost everywhere north of Stanislaus County, two extant northern populations are protected, occurring at Yolo County Grasslands and Olcott Lake in Jepson Prairie Preserve in Solano County (Fig. 1). The majority of the remaining documented *N. colusana* populations are located on private land.

Historically, *T. greenei* was known from 41 occurrences over a wide range of the central valley from Shasta County in the north to Fresno County in the south (CNDDB 2006). Of these, 22 are presumed extant today, although our surveys suggest the number is much lower. Extirpated from five counties, *T. greenei* is currently found in four counties, extending from Shasta County in the North, to Merced in the South (CNDDB 2006). The majority of confirmed extant populations are in the Vina Plains region of Tehama County (Fig. 1). All southern populations are in Merced County and are on private land.

Previous allozyme and sequence studies focused primarily on the systematics of the Orcuttieae group, therefore very little information is known about within species or within population genetic diversity (Griggs and Jain 1983; Boykin et al. 2010). An allozyme study examining genetic variation in five Orcuttieae species, including *Orcuttia* and *Tuctoria* genera, found relatively high levels of intrapopulation variation and unique alleles when comparing two populations (i.e., pools, see definition below) of *T. greenei* (Griggs and Jain 1983).

Study design and sampling procedure

Distribution maps for *N. colusana* and *T. greenei* (Fig. 1) show the locations of populations we sampled and presumably extant CNDDB occurrences. Populations, generally synonymous with CNDDB occurrences, are defined

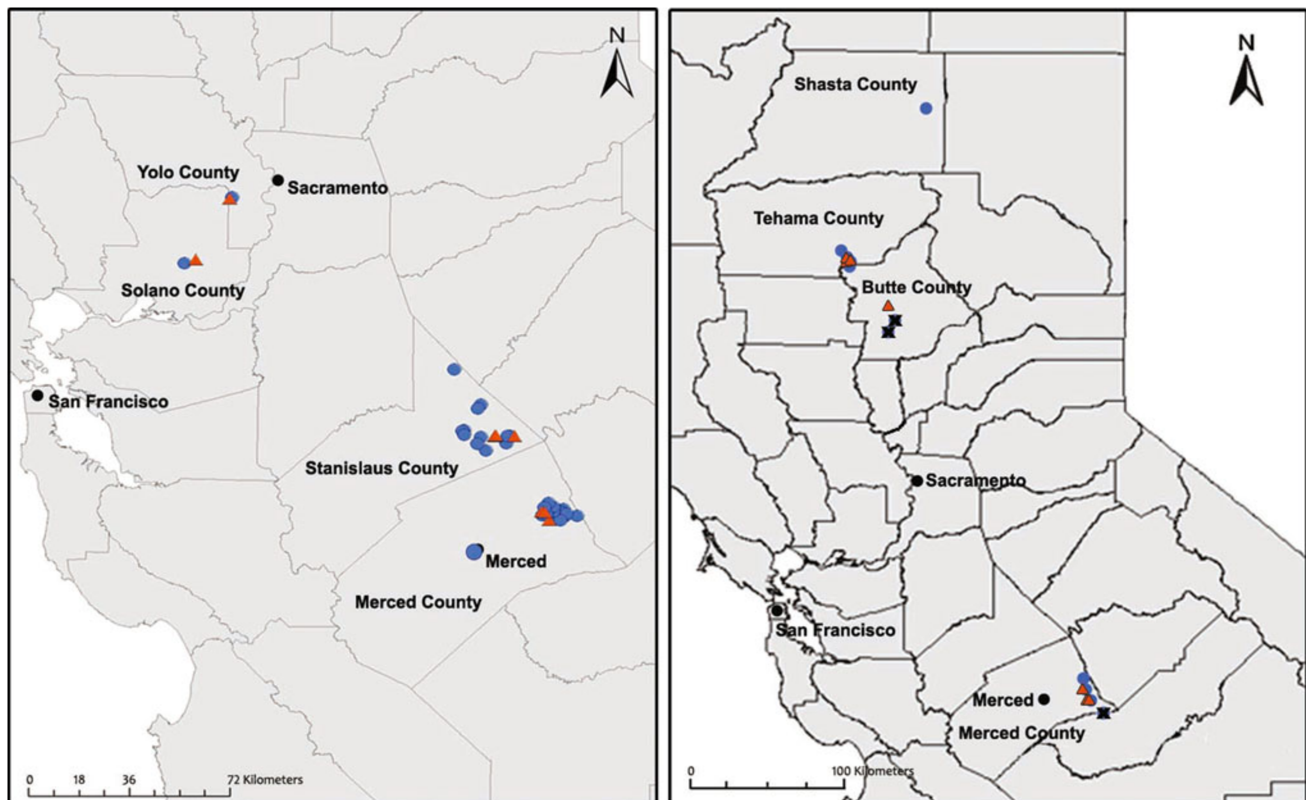

Fig. 1 Distribution maps for *N. colusana* (*left panel*) and *T. greenei* (*right panel*). *Triangles* represent populations collected and included in the genetic analysis. Due to the close proximity of several pools, some *symbol* overlap occurs: one *N. colusana* population was sampled in Yolo County, three *N. colusana* populations were sampled in Merced County, three *T. greenei* populations were sampled in Tehama County, and three *T. greenei* populations were sampled in Merced County. *Circles* represent all remaining CNDDB locations of presumably extant populations. Populations we visited but did not locate plants contain an "x" through the *circle*. *Black circles* represent major cities

here as groups of individuals within a single, discrete vernal pool. To the greatest extent possible, depending on site accessibility, we selected collection sites to span the geographic range of each species, and, in a subset of pools, to examine year-to-year genetic variation within a pool. Haphazardly selecting 30 individuals within each pool, we collected all plant material at the time of flowering to ensure correct identification of species. We froze leaf tissue in individual packets at −20°C until DNA extraction. We roughly estimated population sizes in each pool (Table 1). These data are primarily categorical and should not be used as absolute counts, but as relative comparisons between populations.

For the analysis of spatial genetic structure in *N. colusana,* we included 8 vernal pools sampled in 2008. These collections spanned the maximum geographic range of extant populations, and provided three regional groupings of populations, Solano and Yolo Counties representing the North, and Stanislaus and Merced Counties corresponding to two additional regions in the South (Fig. 1). To assess changes in genetic makeup of vernal pools over time, we collected samples from Olcott Lake at Jepson Prairie Preserve in Solano County over three consecutive years (Table 1).

For the analysis of spatial genetic structure in *T. greenei,* we included individuals collected from 13 vernal pools: 10 from Tehama and Butte County collected in 2007, and 3 from Merced County collected in 2008 (Fig. 1). This multi-year sampling was a result of the low rainfall of the 2006/2007 season. Populations of *T. greenei* south of Sacramento either failed to germinate, or did so in such small numbers, that we could not collect from Merced County in 2007. We visited four additional CNDDB documented pools, but we were unable to locate *T. greenei* plants (Fig. 1). While it is risky to make assumptions about population persistence in annual vernal pool species after a single year's visit, several factors, including adequate rainfall, high numbers of plants found at other sites, and generally degraded habitat in these four pools, suggest that these populations are likely extirpated. To assess changes in genetic makeup of vernal pools over time, and to address the potential effect of the multi-year sampling on spatial genetic structure, we collected samples from three pools at Vina Plains in Tehama County over two consecutive years (Table 1).

Microsatellite analysis

DNA extraction

We extracted DNA using the Sigma Extract-N-Amp Plant PCR kit (XNAP2) following manufacturer recommendations.

Marker development

We contracted with Genetic Identification Services (Chatsworth, California) to develop a combined microsatellite enriched library specifically for *N. colusana* and *T. greenei.* Of the initial 100 primer pairs provided by GIS, we screened 41; 18 of these amplified product in *N. colusana* and 19 primer pairs resulted in product in *T. greenei*, seven of these primer pairs amplified product in both species. We then conducted a secondary PCR screen using a panel of 16–22 individuals per species to assess microsatellite variability (polymorphism) and consistency. This secondary screen eliminated loci that were monomorphic or unscoreable, i.e., primers that resulted in unreliable product amplification or amplification of more than two bands per individual. We were able to identify five reliable and informative loci each for *N. colusana* and *T. greenei*, for a total of 10, to use for genetic analysis (Online Resource 1). It is interesting to note that none of the primers amplified informative loci across both species, a surprising result given their presumed close ancestry. We also initially aimed to use these libraries to transfer and develop primers for three other species in the Orcuttieae tribe (*Tuctoria mucronata*, *Orcuttia viscida*, and *O. pilosa*), but we found that genetic similarity was not close enough to successfully do so.

Genotyping

We generated PCR product in 10 μl reactions using either (a) the Sigma Extract-N-Amp PCR reaction mix following manufacturer instructions, with 0.6 μM each primer and 1.0 μl of genomic DNA extract or (b) custom reactions using PromegaGoTaq Flexi DNA polymerase, containing PromegaGoTaq buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP's, 0.6 μM of each primer, 0.075 units of PromegaGoTaq Flexi DNA polymerase, and 0.50–0.75 μl of genomic DNA extract. While the Sigma Extraction template is optimized for the Sigma PCR mix, we found the custom reactions produced consistent and high quality PCR product and were used for the majority of reactions. In comparisons, we found no differences in product amplification between the two methods. For cycling, we used either a PTC-100 (MJ Research, Inc.), or BIO-RAD DNA Engine thermocycler. We did not observe amplification differences between the two thermocyclers. Thermocycling parameters for nine of the ten primers were as follows: 94°C for 3 min followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 30 s and a final 10 min extension at 72°C, PCR products held at 15°C until further use. One primer, TgC8, has a custom annealing temperature at 59.3°C, all other parameters were the same.

Table 1 Summary of sample collections for genetic analysis

Species/site code	County	Site description	CNDDB occurrence record	Collection year	Estimated population size	Number of samples genotyped
N. colusana/Nc146	Solano	Olcott Lake, Jepson Prairie	19	2006	No data	26
N. colusana/Nc146	Solano	Olcott Lake, Jepson Prairie	19	2007	24	16
N. colusana/Nc146	Solano	Olcott Lake, Jepson Prairie	19	2008	>100,000	30
N. colusana/Nc133	Yolo	Yolo County Grasslands	49	2008	>10,000	30
N. colusana/Nc140	Stanislaus	Private Ranch	4	2008	10,000	30
N. colusana/Nc141	Stanislaus	Private Ranch	62	2008	500	30
N. colusana/Nc142	Stanislaus	Private Ranch	5	2008	>1,000,000	30
N. colusana/Nc143	Merced	UC Merced	68	2008	161	30
N. colusana/Nc144	Merced	UC Merced	69	2008	10,000	30
N. colusana/Nc145	Merced	Private Ranch	60	2008	100,000	30
T. greenei/Tg115	Tehama	Leninger Rd, Private Ranch	4	2007	>1,000	25
T. greenei/Tg113	Tehama	Vina Plains West	*	2007	10,000	24
T. greenei/Tg112	Tehama	Vina Plains West	31	2007	10,000	25
T. greenei/Tg111	Tehama	Vina Plains West	3	2007	>1,000,000	25
T. greenei/Tg111	Tehama	Vina Plains West	3	2008	>1,000,000	24
T. greenei/Tg130	Tehama	Vina Plains East	34	2007	1,000	25
T. greenei/Tg130	Tehama	Vina Plains East	34	2008	>10,000	24
T. greenei/Tg114	Tehama	Vina Plains East	36	2007	>10,000	23
T. greenei/Tg110	Tehama	Vina Plains East	35	2007	>100,000	23
T. greenei/Tg109	Tehama	Vina Plains East	37	2007	>100,000	25
T. greenei/Tg107	Tehama	Vina Plains East	*	2007	>1,000	25
T. greenei/Tg107	Tehama	Vina Plains East	*	2008	10,000	24
T. greenei/Tg116	Butte	Butte County	18	2007	600	25
T. greenei/Tg131	Merced	Fresno Rd, Private Ranch	42	2008	>1,000	24
T. greenei/Tg134	Merced	Cunningham Rd, Private Ranch	11	2008	>1,000	25
T. greenei/Tg135	Merced	State Hwy 140, Private Ranch	29	2008	>100	23

* Indicates pools not linked to a California Natural Diversity Data Base occurrence (CNDDB) record

We separated PCR products using the Applied Biosystem 3100 Genetic Analyzer with a 16 capillary array and the 500 LIZ (-250) size standard following manufacturer recommendations for microsatellite analysis. We assigned allele calls to PCR fragments using GeneMapper 3.7. To verify accuracy, two co-authors reviewed all genotypes independently.

For the study of spatial genetic structure we genotyped 240 *N. colusana* individuals from eight vernal pools, and 317 *T. greenei* individuals from 13 vernal pools (Table 1). For temporal structure analysis in *N. colusana* at Olcott Lake, we included 30 *N. colusana* individuals collected in 2008, 16 individuals collected in 2007 and 26 individuals collected in 2006 (Table 1). For examination of temporal variation in *T. greenei*, we included 25 individuals each from three pools at Vina Plains in 2007 and collected an additional 23, 24, and 25 individuals of *T. greenei* from the same three pools in 2008 (Table 1).

Data analysis

Hardy–Weinberg and linkage disequilibrium

We tested Hardy–Weinberg equilibrium and linkage disequilibrium within all pools using Arlequin 3.11 (Excoffier et al. 2005), followed by a Bonferroni correction for multiple tests (Holm 1979). For all loci within each county, we calculated observed and expected heterozygosity using Arlequin 3.11.

Genetic diversity

We calculated diversity measures using Genetic Data Analysis (GDA v1.1, Lewis and Zaykin 2001) and GENALEX6 (Peakal and Smouse 2006). Diversity measures for each population include proportion of polymorphic loci, mean number of alleles per locus, observed and expected

heterozygosity, private alleles and identification of multilocus genotypes.

Genetic structure

To evaluate the genetic affinity that each individual has to its pool of origin and to test its genetic assignment to each of the sampled pools, we performed assignment tests (GeneClass2, Piry et al. 2004).

To examine spatial genetic structure without a priori hypotheses about population groups, we performed Bayesian clustering analysis using STRUCTURE software (Pritchard et al. 2000; Falush et al. 2003, 2007). This analysis delineates clusters of individuals as populations (K), based on their multilocus genotypes. We selected STRUCTURE run parameters as advised in the users manual and recommended by Evanno et al. (2005). Specifically, we selected the admixture model and correlated allele frequencies with a burn in period of 10,000 and 100,000 MCMC (Markov chain Monte Carlo). To verify the most probable number of clusters (K), we followed the graphical methods and algorithms outlined by Evanno et al. (2005). We performed twenty runs for each data set and each value of K. The range of possible K's we tested was from 2 to the highest number of vernal pools plus 2. For the *N. colusana* analysis, K values we tested ranged from 2 to 10, for *T. greenei*, 2 to 15. This method identified $K = 2$ as the likely true number of clusters in *N. colusana* (Online Resource 2) and $K = 3$ as the likely true number of clusters in *T. greenei* (Online Resource 2).

To test the effect of geographic distance on genetic distance for pools and identify the presence of isolation by distance, we performed Mantel tests (Mantel 1967) using Alleles in Space (Miller 2005) with 10,000 permutations.

To statistically assess the distribution of genetic variation and to partition genetic variation among geographical groups, both among and within pools, we performed Analysis of Molecular Variance (AMOVA) using Arlequin v3.11 (Excoffier et al. 2005). We selected groups based on pools with geographic proximity to each other, as well as testing the results from Bayesian clustering, assignment tests and Mantel tests.

We examined five different spatial groupings for *N. colusana*. Grouping 1 represents all pools belonging to one group with no geographical substructure. Grouping 2 represents the two groups (K) identified through the Bayesian clustering method: Yolo County and Olcott Lake in one northern group, and Stanislaus and Merced Counties in the South. Grouping 3 represents three geographic subdivisions: northern pools (Olcott Lake and Yolo County combined), Stanislaus County pools and Merced County pools. Grouping 4 is similar to Group 3, but influenced by the assignment test results, divides Yolo County and Olcott Lake into two separate groups. Grouping 5 maintains Yolo County and Olcott Lake as separate groups but combines all pools within Merced and Stanislaus Counties.

We tested five different spatial groupings for *T. greenei*. Our selection of groups was guided by geographic locations and the clusters identified through the Bayesian clustering analysis, beginning with the simplest. Grouping 1 represents all pools belonging to one group with no substructure. Grouping 2 represents two geographic subdivisions: northern pools (Tehama and Butte Counties combined) and southern pools (Merced County). Grouping 3 is similar to Group 2, but divides Tehama and Butte Counties into two groups. Groupings 4 and 5 follow two likely structures identified through Bayesian clustering analysis; Grouping 4 represents three groups, southern pools (Merced County), Vina Plains 113 and 114, and all other pools combined; Grouping 5 also examines three groups, southern pools (Merced County), Leninger Rd., and all other pools combined.

The multi-year data collected from Olcott Lake and Vina Plains was intended for the temporal genetic analysis only, therefore 2006 and 2008 data were not "pooled" with 2007 data for inclusion in the spatial genetic structure analysis. This conservative approach eliminates the potential effect of unequal sample sizes on genetic structure and diversity measures. Assignment tests and AMOVA analyses were performed to examine temporal genetic structure.

Results

Primer statistics: Hardy–Weinberg, linkage disequilibrium and heterozygosity

Following a Bonferroni correction for multiple tests, we found two significant departures from Hardy–Weinberg equilibrium, implying non-random mating and potential inbreeding, observed as heterozygote deficiencies in pool Nc146 for locus NcB110, and pool Tg134 for locus TgA8. Of the 80 comparisons in *N. colusana* and 130 comparisons in *T. greenei*, we detected no significant linkage disequilibrium.

The expected heterozygosity for each locus ranged from 0.09 to 0.93 for *N. colusana* and 0.68–0.92 for *T. greenei*; the observed heterozygosity ranged from 0.09 to 0.94 and 0.62 to 0.92, respectively (Online Resource 1).

Neostapfia colusana

Genetic diversity

The mean number of alleles amplified by the five *N. colusana* loci in 282 individuals was 24, ranging from

Table 2 Genetic diversity measures for *N. colusana* and *T. greenei*: proportion of polymorphic loci (P), mean number of alleles per locus (A), mean number of alleles per polymorphic locus (Ap), *He*: expected heterozygosity (He), observed heterozygosity (Ho), and private alleles (Pa)

Population (year)	Region	P	A	Ap	He	Ho	Pa
N. colusana pools used in geographic population structure							
Nc133 (2008)	Yolo	0.8	6.80	8.25	0.56	0.50	7
Nc146 (2008)	Solano, Olcott Lake	1.0	7.60	7.60	0.72	0.69	2
Nc140 (2008)	Stanislaus	1.0	8.40	8.40	0.72	0.68	2
Nc141 (2008)	Stanislaus	1.0	12.40	12.40	0.82	0.86	2
Nc142 (2008)	Stanislaus	1.0	12.40	12.40	0.82	0.84	6
Nc143 (2008)	Merced	0.8	10.00	12.25	0.67	0.58	2
Nc144 (2008)	Merced	1.0	8.40	8.40	0.66	0.61	1
Nc145 (2008)	Merced	1.0	10.40	10.40	0.68	0.68	1
	Mean	0.95	9.55	10.01	0.71	0.68	2.88
Olcott lake *N. colusana* collected in 2006, 2007, 2008 for temporal structure							
Nc146 (2006)	Solano, Olcott Lake	1.0	6.20	6.20	0.70	0.62	0
Nc146 (2007)	Solano, Olcott Lake	1.0	6.60	6.60	0.65	0.58	2
Nc146 (2008)	Solano, Olcott Lake	1.0	7.60	7.60	0.72	0.69	0
	Mean	1.0	6.80	6.80	0.69	0.63	0.67
T. greenei pools used in geographic population structure							
Tg115 (2007)	Tehama, Leninger Rd	1.0	7.40	7.40	0.75	0.75	0
Tg113 (2007)	Tehama, Vina Plains West	1.0	7.20	7.20	0.67	0.68	0
Tg112 (2007)	Tehama, Vina Plains West	1.0	11.00	11.00	0.84	0.86	0
Tg111 (2007)	Tehama, Vina Plains West	1.0	11.20	11.20	0.84	0.82	3
Tg130 (2007)	Tehama, Vina Plains East	1.0	6.40	6.40	0.71	0.66	0
Tg114 (2007)	Tehama, Vina Plains East	1.0	7.20	7.20	0.78	0.71	0
Tg110 (2007)	Tehama, Vina Plains East	1.0	9.60	9.60	0.80	0.81	0
Tg109 (2007)	Tehama, Vina Plains East	1.0	7.80	7.80	0.75	0.77	0
Tg107 (2007)	Tehama, Vina Plains East	1.0	8.60	8.60	0.81	0.86	0
Tg116 (2007)	Butte	1.0	9.20	9.20	0.78	0.80	0
Tg131 (2008)	Merced	1.0	11.20	11.20	0.84	0.73	8
Tg134 (2008)	Merced	1.0	12.20	12.20	0.86	0.74	6
Tg135 (2008)	Merced	1.0	10.40	10.40	0.81	0.80	6
	Mean	1.0	9.18	9.18	0.79	0.77	1.77
T. greenei collected in 2007 and 2008 for temporal structure							
Tg107 (2007)	Vina Plains	1.0	8.60	8.60	0.81	0.86	0
Tg107 (2008)	Vina Plains	1.0	8.40	8.40	0.80	0.84	0
Tg111 (2007)	Vina Plains	1.0	11.20	11.20	0.84	0.82	2
Tg111 (2008)	Vina Plains	1.0	10.60	10.60	0.84	0.86	1
Tg130 (2007)	Vina Plains	1.0	6.40	6.40	0.71	0.66	0
Tg130 2008	Vina Plains	1.0	7.00	7.00	0.72	0.67	0
	Mean	1.0	8.70	8.70	0.79	0.79	0.5

six to 35 alleles per locus (Online Resource 1). The mean number of alleles per locus per pool was 9.55, ranging from 6.8 in the Yolo County pool to 12.4 in Stanislaus pools Nc141 and Nc142 (Table 2). This distribution of diversity is similarly reflected in the observed heterozygosity, which ranges from 0.50 (Yolo County) to 0.86 (Stanislaus pools Nc141 and Nc142), with a mean of 0.68. The expected heterozygosity ranged from 0.56 to 0.82 with a mean of 0.71. Overall, Yolo County showed the least amount of genetic diversity and Stanislaus County pools Nc141 and Nc142 the highest. All the *N. colusana* pools sampled in 2008 possessed private alleles (Table 2), ranging from one (Merced County pools Nc144 and Nc145) to seven (Yolo County).

Mean allele frequencies across all pools ranged from 0.002 to 0.525. Allele frequencies within individual

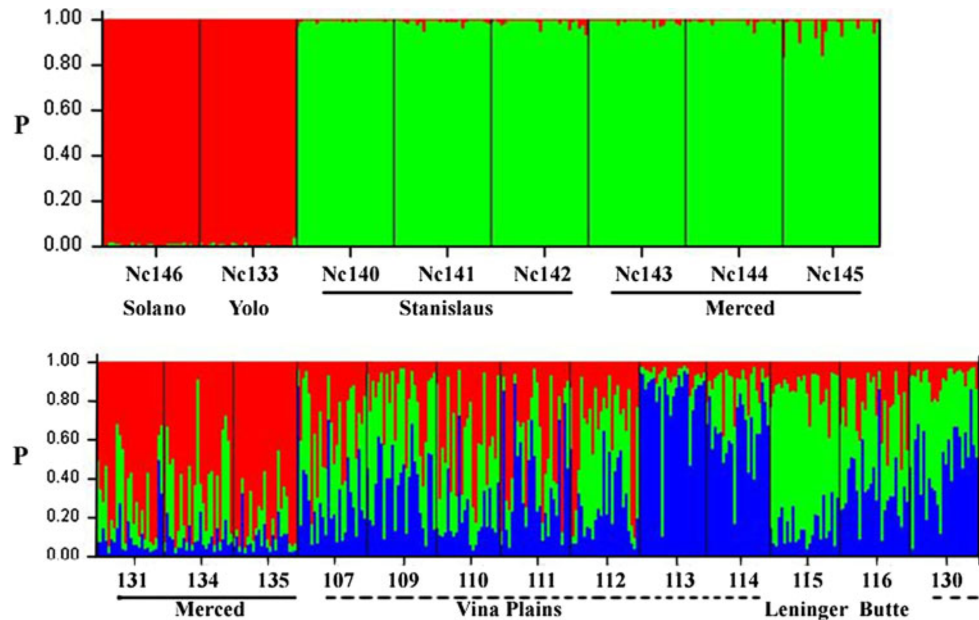

Fig. 2 Membership of *N. colusana* (*top panel*) and *T. greenei* (*lower panel*) individuals to a number of presumed "populations". Population clusters (*N. colusana*, $K = 2$; *T. greenei*, $K = 3$) determined by the a priori Bayesian cluster method in STRUCTURE. Each *vertical line* represents an individual's probability of belonging to one of K clusters (represented by *different colors*) or a combination thereof if ancestry is mixed. Population codes (Table 1) are included and subtended by the geographic location where samples were collected

N. colusana pools ranged from 0.017 (a single expression of a rare allele) to 1.00 (Online Resource 3). One locus, C7, was fixed in two pools, Yolo County and Merced pool Nc143, the remaining loci and pools did not contain fixed loci. All individuals had unique multilocus genotypes.

Spatial genetic structure

The Bayesian clustering analysis distinguished two genetically distinct population clusters for *N. colusana*. These clusters correspond geographically with the two northern pools (Olcott Lake and Yolo County) in the first cluster and all southern pools (Stanislaus and Merced Counties) in the second (Fig. 2a). Very little admixture was detected between these two clusters, indicating very low to no gene flow between the northern and southern regions of occurrence.

AMOVA confirmed overall spatial genetic structure at multiple levels, with strength of regional structure ranging between Fst = 0.18 to Fst = 0.28 ($P < 0.000001$, Table 3, Groupings 1 and 5, respectively). The highest Fst = 0.28 indicates substantial and significant structure among three geographically distinct groups; Olcott Lake (Solano County), Yolo County and the six southern pools from Merced and Stanislaus Counties (Table 3, Grouping 5). Examination of the genetic structure between the North and South regions only, supported by the Bayesian analysis, showed Fst = 0.27 ($P < 0.000001$, Table 3, Grouping 2).

Assignment tests investigating the probability that each individual could be assigned to any of the sampled locations due to their genetic profile varied from pool to pool. All individuals from Yolo County and Olcott Lake were correctly assigned to their pool of origin (Ap = 100%; where Ap is the probability of assignment to correct pool of origin) and the probability of assignment to the correct pool of origin for the Stanislaus and Merced County pools varied from 86.7 to 96.7%, indicating substantial, but less structure among these pools (Online Resource 4). However, an examination of where individuals were incorrectly assigned indicates that pools within geographic regions (i.e., Stanislaus and Merced Counties) have high affinity for their county of origin, and all individuals, with only one exception, were assigned to pools located in their county of origin. These results suggest genetic distinction between four groups: Yolo County, Olcott Lake, Stanislaus County and Merced County.

Our results show a strong correlation between geographic and genetic distances, indicting isolation by distance across the entire range of *N. colusana* (Mantel test, $r = 0.71$, $P < 0.0001$). To examine isolation by distance at a smaller, regional scale, we removed the two northern, most geographically isolated pools from the analysis. We found evidence for isolation by distance across the six pools from Stanislaus and Merced Counties (Mantel test, $r = 0.34$, $P < 0.0001$). These results indicate strong isolation by distance at two spatial levels, across the range (150 km) and within regions (1–40 km).

Olcott Lake temporal genetic structure

Diversity values for the 3 years of sampling at Olcott Lake are listed in Table 2.

The mean number of alleles per locus per population varied annually, from 6.2 in 2006 to 7.6 in 2008. The observed heterozygosity was lowest in 2007 (0.58) and

Table 3 Support for groupings of *N. colusana* and *T. greenei* pools using analysis of molecular variance (AMOVA)

Grouping		Number of groups	Fst	*P*	Fct	*P*	Percentage of variation		
							Among groups	Among pools within groups	Within pools
	N. colusana spatial group structure								
1	[All *N. colusana* pools]	1	0.183	<0.000001	–	–	–	18.28	81.72
2	[Solano & Yolo], [Stanislaus & Merced]	2	0.268	<0.000001	0.183	0.034	18.28	8.54	73.18
3	[Solano & Yolo], [Stanislaus], [Merced]	3	0.213	<0.000001	0.146	0.003	14.61	6.66	78.73
4	[Solano], [Yolo], [Stanislaus], [Merced]	4	0.216	<0.000001	0.189	0.003	18.86	2.73	78.41
5	[Solano], [Yolo], [Stanislaus & Merced]	3	0.278	<0.000001	0.218	0.036	21.83	6.01	72.16
	N. colusana temporal structure								
	[Nc146_2006, Nc146_2007, Nc146_2008]	1	0.013	0.027	–	–	–	1.35	98.65
	T. greenei spatial group structure								
1	[All *T. greenei* pools]	1	0.0846	<0.000001				8.46	91.54
2	[Tehama, Butte], [Merced]	2	0.100	<0.000001	0.02774	0.00228	2.77	7.26	89.96
3	[Tehama], [Butte], [Merced]	3	0.0936	<0.000001	0.01965	0.0413	1.96	7.4	90.64
4	[Tg 113 & 114], [All others], [Merced]	3	0.094	<0.000001	0.024	0.008	2.42	6.97	90.61
5	[Leninger Rd.], [All others], [Merced]	3	0.098	<0.000001	0.029	0.0002	2.87	6.91	90.22
	T. greenei temporal structure								
	[Tg107_2007, Tg107_2008]	1	0.015	0.011	–	–	–	1.54	98.46
	[Tg111_2007, Tg111_2008]	1	−0.007	0.918	–	–	–	−0.71	100.71
	[Tg130_2007, Tg130_2008]	1	0.018	0.045	–	–	–	1.84	98.16

Refer to Table 1 for regions, location descriptions and site codes

highest in 2008 (0.69). Private alleles varied across years, with zero in 2006 and 2008 and two in 2007.

The Analysis of Molecular Variance among the three sampling years at Olcott Lake reveals extremely low, yet statistically significant genetic structure among years (Table 3).

We performed assignment tests for Olcott Lake to assess the affinity of individuals to their respective collection year. Forty individuals (55.6%) were correctly assigned to their sampling year; the remaining 32 individuals (44.4%) were incorrectly assigned, further supporting the assessment of low structure among tested years.

Tuctoria greenei

Genetic diversity

The five *T. greenei* loci amplified 12–28 alleles per locus in 389 individuals, with a mean of 19.0 (Online Resource 1). The mean number of alleles per locus per population was 9.18, ranging from 6.4 in pool Tg130 at Vina Plains to 12.20 at Tg134 in Merced County (Table 2). The observed heterozygosity ranged from 0.66 (Vina Plains Tg130) to 0.86 (Vina Plains Tg107), with a mean of 0.77. Mean expected heterozygosity was 0.79, ranging from 0.67 to 0.86. One pool from Vina Plains and all three southern pools from Merced County possessed private alleles, specifically, four alleles in Tg111 (Vina Plains), eight alleles in Tg131, four in Tg134 and six in Tg135.

Mean allele frequencies across all pools ranged from 0.001 to 0.428. Allele frequencies within *T. greenei* pools ranged from 0.02 (a single expression of a rare allele) to 0.76 (Online Resource 5). No pools were fixed for any allele. All individuals had unique multilocus genotypes.

Spatial genetic structure

The Bayesian clustering analysis determined three genetically distinct clusters (*K*) with fairly homogeneous genetic characters; cluster one is comprised of all three pools in Merced County, cluster two contains two pools in Vina Plains (Tg113 and Tg114) and cluster three contains the Leninger Road pool (Tg115) and the remaining pools in Vina Plains and the Butte County pool (Fig. 2b). However, all northern pools showed a high degree of admixture.

Population spatial genetic structure varied slightly according to geographic grouping of sampled populations (Fst = 0.09–0.10, $P < 0.000001$, Table 3), supporting significant structure between northern and southern pools (Table 3, Grouping 2), and confirming Bayesian clustering

results, including high admixture in northern pools (Fig. 2b; Table 3, Groupings 4 and 5).

Genetic affinity of individuals to their pool of origin varied from pool to pool (Online Resource 6). The highest probability of correct assignment (100%) was in two pools, Vina Plains pool Tg130 and Merced County pool Tg135; the lowest probability of correct assignment (76%) was in Merced County pool Tg134. However, examining where individuals were incorrectly assigned indicates that pools within geographic regions (i.e., North vs. South) have a higher affinity for their region of origin. The probability of correct assignment to region for northern pools increased to 96–100%, while in the South, the probability increased from a low of 76% to 84%. These results are compatible with the AMOVA and STRUCTURE results, indicating low, but significant genetic differences between pools and regions.

Our results demonstrate evidence for isolation by distance across the range of *T. greenei*, showing a strong correlation between geographic and genetic distances across broad geographical scales (330 km) when testing all 13 sampled pools ($r = 0.21$, $P < 0.0001$). However, significant isolation by distance was not detected at the finer, regional scale (primarily within 4 km, $r = 0.038$, $P = 0.096$).

Tuctoria greenei temporal genetic structure

Diversity values for the 2 years of sampling at three pools from Vina Plains in Tehama County are listed in Table 2. The mean number of alleles per pool did not vary much annually, the highest mean difference between years (0.60) is found in Tg111 and Tg130. The observed heterozygosity changed very slightly over the 2 years period; the largest increase was in Tg111 from $H_{obs} = 0.82$ to 0.86. Private alleles across years were only observed in Tg111 (2 in 2007; 1 in 2008).

Temporal genetic structure within pool Tg111 does not show significant structure across 2 years (Fst = 0), however, Tg107 and Tg130 show very small differences, with marginally significant Fst values of 0.015 ($P = 0.01$) and 0.018 ($P = 0.04$), respectively.

Assignment tests assigned 78 individuals (53.1%) to their sampling year; the remaining 69 individuals (46.9%) were incorrectly assigned, again indicating that there is very low to no structure among years.

Discussion

Our study confirmed genetic isolation, yet found high genetic diversity in extant populations of two rare annual vernal pool grasses *N. colusana* and *T. greenei*. As hypothesized, Fst levels and the Bayesian clustering results indicate that range-wide genetic structure exists, and gene flow among most isolated extant populations of both species is low, and is correlated with geographic distance. However, contrary to our hypothesis, most populations of both species exhibited high within population genetic diversity in relation to other vernal pool plants, grasses and insect pollinated plants reported in the literature (Gitzendanner and Soltis 2000; Sun and Salomon 2003; Riley et al. 2010; Martin et al. 2010; Fan et al. 2011; Sloop et al. 2011; Sloop and Ayres in press; Sloop et al. in review), suggesting a low potential for suffering the detrimental effects of genetic drift and severe inbreeding. Predominant outcrossing and seed germination from a persistent soil seed bank probably facilitates the localized high genetic diversity, while the slight temporal structure we identified may indicate diverse cohorts residing in the seed bank (Sloop and Ayres in press), sampling bias or genetic drift.

Genetic diversity

We found *N. colusana* and *T. greenei* genetic results to be consistent with predominantly out-crossing breeding systems and large effective population sizes. This conclusion is supported by high allelic richness and heterozygosity, and very few fixed loci in either species when compared to other microsatellite studies in vernal pools species (Sloop et al. 2011; Sloop and Aryes in press), and other assessments of genetic variation in plants (Gitzendanner and Soltis 2000; Sun and Salomon 2003; Riley et al. 2010; Martin et al. 2010; Fan et al. 2011). It is also consistent with the high allozyme diversity observed within two *T. greenei* populations (Griggs and Jain 1983). Further, in a companion study, *N. colusana* was found to predominately outcross when population density is sufficient (Davis et al. 2009), and a healthy long-lived seed bank can store seeds from many cohorts, effectively maintaining a large dormant population.

We observed a wide variation of diversity among populations, as measured by differing levels of heterozygosity, number of alleles and private alleles, illustrating the need for unique recovery or conservation considerations for individual pools. For example, pools with high diversity are good candidates for conservation of evolutionary potential and fitness, such as one of the pools in Merced County (Nc142). However, *N. colusana* populations at Olcott Lake and Yolo County, with lower than expected heterozygosities and lower than average allelic richness, may require more active management, including potential genetic rescue via supplementation of novel alleles into the population, in particular if population counts show an extreme downward trend. However, other factors, such as erosion of genetic variation due to local adaptation at range

limits, need to be ruled out before such steps can be approved.

Spatial structure

We found evidence of very low to no gene flow between the North and South populations of *N. colusana*. The two major population groups had little admixture between Olcott Lake in Solano County and Yolo County Grasslands in the North, and Stanislaus County and Merced County in the South (Fig. 2a). This is not too surprising given the geographic distance and the absence of intermediately spaced *N. colusana* populations to facilitate stepwise gene flow. When possible, we advise against facilitated out-crossing across these larger distances to avoid the possibility of negative effects of out-breeding depression (Edmands 2007). Genetic rescue should only occur from sources within the range of effective wind pollen dispersal, or from those most similar in genetic makeup (i.e., no strong genetic structure). Additionally, we found strong support for genetic distinction between Yolo County and Olcott Lake in the North and the subdivision of the southern pools between Stanislaus and Merced Counties (Table 3). We suggest that each of the two biogeographic regions and the four subregions should be considered as evolutionary significant units and be managed separately for conservation, monitoring population size closely and implementing recovery actions when appropriate.

In *T. greenei* two major regional divisions exist between North (Butte and Tehama Counties) and South (Merced County), as well as finer spatial structure among several of the pools in Tehama County. With the exception of the Leninger Road pool, and Vina Plains pools Tg 113 and Tg114, overall, there appears to be low genetic structure among the northern *T. greenei* pools. The genetic distinction of the Leninger Road sample is best explained by its isolation from other pools and the Vina Plains pool complex by multiple roads and potentially by different land uses such as grazing. The third genetic cluster identified by the Bayesian analysis is difficult to explain biologically: both pools (Tg 113 and Tg 114) are within the vernal pool matrix of Vina Plains but are themselves separated by the highway that divides Vina Plains into its east and west sections at a distance of less than a kilometer. All the pools at Vina Plains seemingly have the potential for gene flow via seed movement by cattle, which could explain the high degree of admixture. This highlights a possible management need for facilitated out-crossing in pools (1) isolated by human-caused habitat fragmentation and (2) with chronically low population numbers, to counter the effects of inbreeding and lack of gene flow. We believe that the North and South regions should be considered as separate units for conservation. We emphasize the urgency for pool preservation in the South, where all occurrences are on private land and some are substantially degraded. If it is not feasible to protect pool habitat in the South, we highly recommend conserving the unique genetic diversity within these pools by implementing ex situ seed banking across several years to retain maximum diversity in the event these populations are extirpated. We recommend utilizing banked seeds in subsequent restoration efforts as identified by stringent seed re-introduction trials.

Spatial structure in *T. greenei* was examined with individuals collected over 2 years, therefore raising the concern that the genetic structure observed between North and South *T. greenei* could be due to the multi-year sampling. However, the small magnitude of temporal genetic structure (Fst = 0, Fst = 0.015, Fst = 0.018, Table 3) makes its proportional contribution to the overall spatial structure (Fst = 0.10) nominal.

Temporal genetic structure

We found small but significant temporal differentiation for both species. It is impossible to entirely rule out a potential effect of sampling bias or genetic drift due to small population sizes (Table 1). Additionally, the biological significance of extremely small differentiation across years may be low. However, temporal differentiation has also been found in other vernal pool endemics (Ramp et al. 2006; Sloop and Ayres in press). In any particular year, a subset of viable seeds in the seed bank may respond to the specific current environmental conditions, with germinating individuals representing a genetic make-up specific to that year (Ferguson and Ellstrand 1999). In years with very poor conditions germination may not occur at all. Therefore, to obtain a representative measure of population/seed bank diversity, we recommend collecting seed over multiple years for use in restoration activities. Additionally, as the seed bank is an important reservoir of diversity and evolutionary potential that could be utilized in the event of pool destruction, we highly recommend storing seed ex situ at long-term storage facilities by sampling seed production from multiple years and the seed bank directly. Annual surveys of extant population sizes over the next decade and a one-time assessment of seed bank size will provide critical information to guide recovery of the species, inform long-term management and embark on population rescue while there is still time.

The potential deleterious effects of out-breeding depression are serious (Edmands 2007), and mixing of seeds from genetically distinct sources should not be undertaken lightly. However, Frankham (2010) suggests that concern for out-breeding depression may be impeding "rational" genetic management of fragmented populations when populations are small or suffer from severe seed bank

decline and increased inbreeding. Although genetic diversity thus far remains relatively high, the long-term viability and maintenance of evolutionary potential in severely declining populations may ultimately depend on carefully planned human mediated genetic transfer among populations. Updating genetic information on source and target populations, and genetic evaluation of initial low level seed introduction trials are crucial activities to ensure success (Sloop in press).

Recovery planning implications

Recovery plans for the increasing number of endangered vernal pool species require information on their genetic and ecological status to effectively guide conservation and restoration efforts (US Fish and Wildlife Service 2005). To appropriately incorporate genetic factors into the recovery of listed taxa it is critical to (1) estimate the levels of population genetic diversity, using heterozygosity as a correlate of fitness (Reed and Frankham 2003; Frankham 2005, 2010; Gilligan et al. 2005), and (2) assess the levels of population isolation by distance or other gene flow barriers, through their range-wide population genetic structure (Elam 1998; Beardsmore 1983).

Therefore, with the decline of endemic vernal pool plant populations it is imperative to develop effective conservation and management strategies that support the recovery of these species even in the face of ever increasing human-induced threats. Recovery plans for *N. colusana* and *T. greenei* are included in the Draft Recovery Plan for Vernal Pool Ecosystems in California and Southern Oregon (USFWS 2005). Specific recovery plan recommendations are to: (1) Conduct genetic analysis on species slated for reintroduction (*N. colusana*) and introductions (*N. colusana* and *T. greenei*); (2) Conduct biology (genetic) and ecology research needed to inform management and habitat protection decisions; (3) Research genetic relatedness, breeding systems and patterns of gene flow to improve or develop management plans; (4) Implement ex situ seed collection, storage and propagation necessary to preserve rare and unique genotypes or occurrences in danger of extirpation; (5) Develop a prioritized list of sites that need to be enhanced, restored or created to further recovery and long term conservation of vernal pools species; (6) Prioritize list of private landowners interested in pursuing recovery and conservation efforts on their land to retain full range of genetic diversity. This study specifically addresses and informs all recommendations above as it provides site-specific and range-specific genetic information for the target species.

The information on the species presented here will inform their recovery and management needs by giving the current molecular genetic status of extant populations as a baseline for future comparison. In combination with population size, heterozygosity levels can provide an indication of relative fitness status, since the loss of heterozygosity has a deleterious effect on population fitness as shown by a meta-analysis by Reed and Frankham (2003). This will be important in informing potential management actions, spanning from maintaining or increasing population size, prioritizing conservation targets, taking into consideration and protecting the mechanisms of gene flow, removing or bridging potential gene flow barriers, or implementing activities such as assisted migration for genetic rescue.

While the information provided here is unique to the species investigated, general management recommendations, such as the need to conserve diversity unique to individual pools, may be applicable to the recovery of similarly endangered congeners. Streamlined strategies for the recovery and effective management of the growing number of declining specialist species are needed, and information and recommendations presented in this study bring us one step closer to this goal.

Acknowledgments We thank the U.S. Fish & Wildlife Service (Agreement No.: 802706G122) for funding support. We thank Dr. Derek Girman for use of the Sonoma State Core DNA Facility and Dr. Richard Whitkus for providing laboratory and office space. We further acknowledge the many local experts, land managers, and landowners who helped locate and obtain access to a majority of the extant sites, including Joe Silveira (USFWS), Carol Witham (CNPS), John Vollmer and Rich Reiner (The Nature Conservancy).

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