Human impact to vernal pool complexes in Southern California

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This is a final report for the contract "Human impact to vernal pool complexes in Southern California", Tasks 10-12, to Andrew J. Bohonak from SANDAG. (SDSURF Fund 57266A, July 3, 2012 to July 31, 2013). This contract was suggested as matching funds for a USFWS/CDFW Section 6 grant that was originally submitted in 2011. On July 9, 2013, the contract was extended from July 31, 2013 to December 31, 2014. This extension was necessary because 1) there was almost no vernal pool ponding during the 2012-2013 and 2013-2014 winter due to the regional drought, 2) USFWS had not yet renewed Marie Simovich's permit for new field work, and 2) Matching USFWS funds were not yet made available.

The USWFS/CDFW grant was finally awarded on December 27, 2013. Tasks 11 and 12 were jointly funded by SANDAG and USFWS/CDFW.

Background

Southern California's vernal pools support high levels of species diversity and endemism[3, 5, 23, 29] despite ongoing threats from habitat conversion, global change, and the interactions between these two factors[5, 8, 22]. Although these ecosystems once dominated many areas of southern California, it is generally agreed that > 95% of the vernal pools originally present in this region have been lost[5]. Many of those which remain are isolated within small parcels that require ongoing monitoring, and perhaps management, restoration or mitigation. The flagship species for San Diego County's vernal pools is the federally endangered San Diego fairy shrimp (*Branchinecta sandiegonensis*: SDFS). For this species, the most critical management issues are likely to involve population connectivity. Degradation of the landscape, direct damage (often by vehicles), creation of new basins (most often "road ruts"), and increasing biotic connectivity (beyond historic levels) are also important factors.

The specific goals of this study were focused in three areas:
Task 10. Landscape genetics in the San Diego fairy shrimp *B. sandiegonensis*
Task 11. Hybridization between *B. sandiegonensis* and *B. lindahli*
Task 12. Conservation, management and recovery of *B. sandiegonensis*

Task 10. Landscape genetics in the San Diego fairy shrimp *B. sandiegonensis* Completed in 2013, included in July 2013 report

Previous genetic studies of the San Diego fairy shrimp (SDFS) using allozymes[11] and mtDNA[6] had shown high differentiation among populations. These results are typical for freshwater crustaceans in temporary wetlands, but are not intuitive if one assumes that fairy shrimp cysts are being dispersed across the landscape in relatively high numbers by natural

processes (e.g., wind, birds, terrestrial animals). We previously noted that vernal pool complexes disturbed by human activities (e.g., recreation and development) tend to have higher within-pond genetic variation, and less among-pond divergence[6]. This suggests that anthropogenic disturbance artificially homogenizes vernal pool biota among complexes. If true, then SDFS should be well adapted to local hydrology, water chemistry, and other environmental factors, where local is defined at the level of the pool complex. Increased levels of dispersal and gene flow (beyond historic levels) could decrease individual survival and/or reproduction.

10.1. Develop multilocus genetic markers for SDFS (funding not requested).

Because the evolutionary processes of mutation, drift and gene flow are random, only limited inferences are possible from the single genetic marker (mtDNA) used in the previous study[6]. Our goal was to finalize protocol for 10+ microsatellite markers in SDFS, so that our inferences about population genetics in this species will be statistically robust. Microsatellites possess higher polymorphism and quicker mutation rates than other types of genetic markers. In the end, we succeeded in developing robust protocols for seven markers.

Full methods are in Andrews[2], and available from Bohonak upon request. Briefly, genomic DNA from 10 SDFS was sent to Genetic Identification Services (Chatsworth, CA) to develop eight enriched microsatellite libraries. The enriched fragments were ligated into plasmids, electroporated into *E.coli*, and the resulting colonies were screened and sequenced. A total of 98 cloned sequences containing microsatellites were found, 66 of which had suitable flanking regions from primer development. From these, 20 loci were found in preliminary screens by Genetic Identification Services to be polymorphic. All polymorphic loci subsequently underwent a strict quality control screen under a variety of PCR conditions.

Families of known SDFS crosses were genotyped in order to directly test for the presence of Mendelian inheritance and null alleles. Problematic loci with unexpected products, null alleles or non-Mendelian inheritance were either discarded, or the flanking regions were sequenced to redesign primers. At this point, 16 primer pairs representing 12 promising loci were each screened against 2-3 polymorphic families. After validation, seven microsatellite loci were shown to be reliable for genetic analysis in SDFS. This level of protocol validation exceeds the vast majority of published studies that use microsatellites. Final PCR protocols are in Andrews[2], and available from Bohonak upon request.

Using the final optimization, sixteen additional fairy shrimp from two pools (Maddox 2 and AA10 MC5) were genotyped to test for deviations from Hardy-Weinberg expectations and linkage equilibrium at each locus. Within these two pools, eight individuals were from the 2002/2003 wet season and eight from the 2003/2004 wet season. Deviations from Hardy-Weinberg and/or linkage equilibrium were found in some loci when combining data from the two wet seasons. Such deviations are common in freshwater crustacean studies worldwide, and can be due to methodological artifacts (e.g., null alleles) and/or real biological phenomena (e.g., inbreeding, assortative mating or a temporal Wahlund effect). Because we took extreme measures to reduce methodological artifacts, we believe that cohorts from different wet seasons have small but detectable genetic differences.

In summary, we were able to isolate and validate seven informative, reliable and highly variable SDFS microsatellite markers. These markers can be used to quantify population genetic structure and connectivity, and this information is vital for effective conservation and management.

10.2. Quantify genetic variation across the species range at the level of individuals, pools and complexes.

Introduction

Complete methods and results for Task 10.2 are in Andrews[2], and available from Bohonak upon request.

Human disturbance to vernal pool habitat may drastically alter the genetic variation and structure of SDFS populations. Human activities, such as cultivation, grading, urban development and vehicle usage, cause soil disruptions that can alter pool hydrology, drainage patterns, and hydrological connectivity among pools[4]. Humans can also serve as cyst transport vectors[31], and expand the ranges of generalist non-native species[12, 21]. SDFS are endemic to a narrow range of habitat on coastal mesas in San Diego County. The closely related versatile fairy shrimp or Lindahl's fairy shrimp (*B. lindahli*: LFS) is a non-endangered, widespread congener whose presence in San Diego County has historically been limited[14, 28]. LFS can tolerate a wider range of habitat variation, but we believe that historically, co-occurrence in the native range of SDFS was rare. LFS are now being found with SDFS in disturbed conditions with increasing frequency. This is a cause of concern for both the persistence and the genetic integrity of *B. sandiegonensis* populations.

In addition, mitochondrial DNA (mtDNA) sequence data[6] show that pools are often fixed for unique mtDNA haplotypes and that differentiation between regions is high. Pool complexes disturbed by recreation and development also have higher mtDNA variation than those that are relatively pristine. Phylogenetic analysis showed a deep split between two mtDNA clades, named "A" and "B", suggesting historical isolation of these populations with little dispersal between them for up to tens of thousands of years. However, a multilocus assessment using microsatellites is necessary in order to make firm conclusions on this matter.

The current study was designed to:

1. Quantify genetic differentiation among SDFS populations.
2. Interpret the results in terms of human activities that may be modified through management and/or regulations.
3. Provide independent data to support or refute results from the previous mtDNA study.

Methods

Fifty pools in 23 pool complexes across San Diego County were sampled and a total of 501 adult shrimp were successfully genotyped at seven microsatellite loci in order to quantify genetic variation and structure on a range of spatial scales (Figure 1). The majority of pools are from the genetic study that was part of the City of San Diego's Vernal Pool Inventory[6]. The sampling range includes most of the coastal mesa range of San Diego County, from San Onofre in the north to Otay Mesa in the south, and reaching as far inland as Ramona. All shrimp for this study were previously collected under Dr. Marie Simovich's endangered species permit as adults during the wet season, or were hatched from sediment samples. Samples were genotyped at Eton

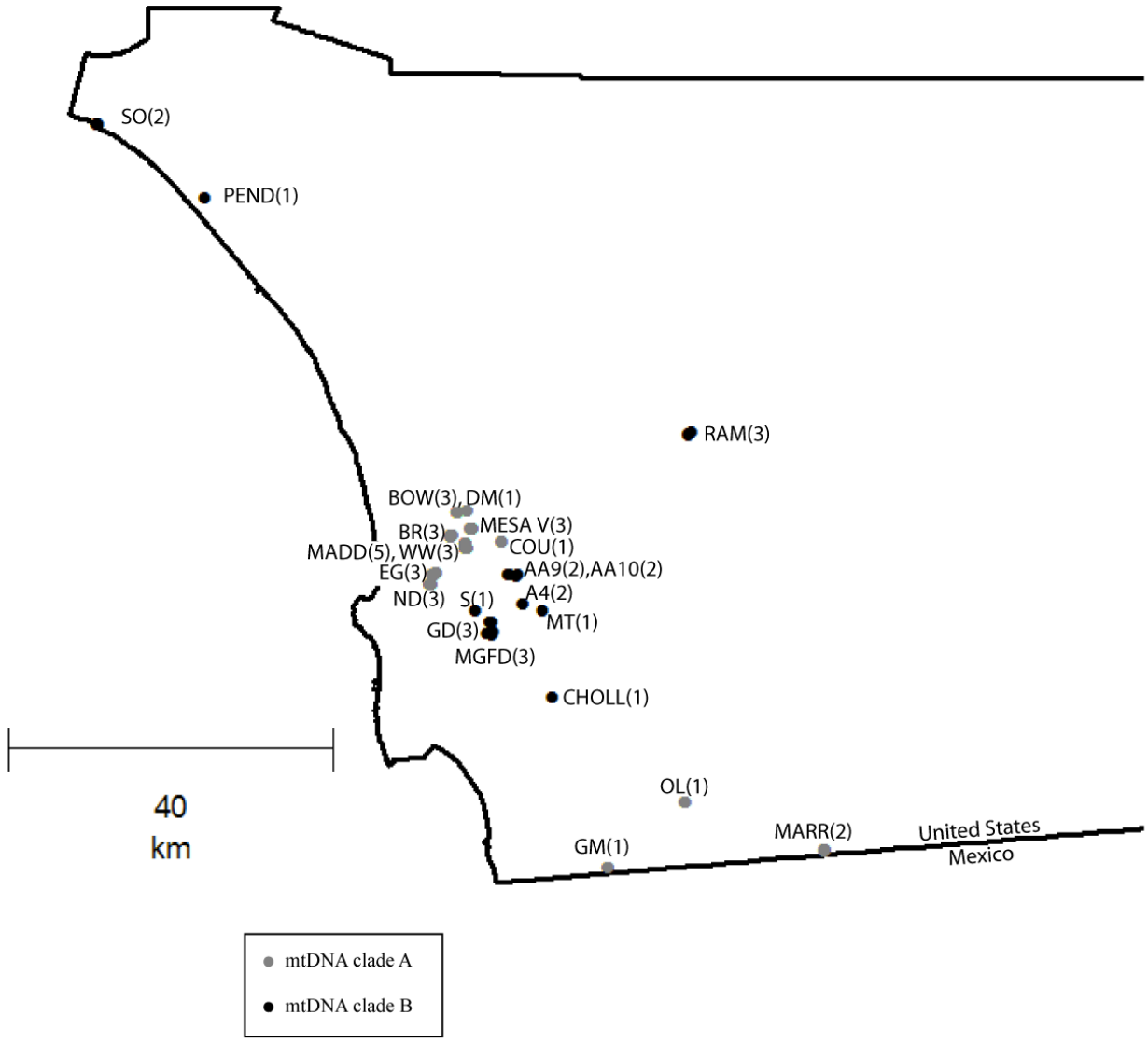

FIGURE 1: Map of sampling locations in San Diego County. A total of 501 individuals were sampled from 50 pools in 23 pool complexes. Labels of each point indicate the name of the pool complex sampled and the number of pools sampled within the complex. Abbreviations are as follows: SO (San Onofre), PEND (Pendleton), RAM (Ramona), BOW (Bowtie), DM (Del Mar Mesa), MESA V (Mesa Verde), BR (Brown), COU (Cousins), MADD (Maddox), WW (Winterwood), EG (Eastgate), ND (Nobel Drive), S (Sander), GD (General Dynamics), MT (Mission Trails), MGFD (Montgomery Field), CHOLL (Chollas), GM (Goat Mesa), OL (Otay Lakes), MARR (Marron Valley).

Biosciences and scored in the Bohonak lab by J. Andrews. Failed samples and those with inconsistent allele sizes were reamplified and genotyped at least once more to determine and confirm the correct allele size. Any scoring errors were checked and re-scored manually.

Results and Discussion

The microsatellite markers used in this study exhibit a variable amount of polymorphism within SDFS. An average of 25.43 alleles was found per locus across all samples genotyped, ranging from four alleles in two of the loci, to a total of 83 alleles in the one with the highest

diversity. Within pools, there was an average of 3.94 alleles per locus. Total expected heterozygosity (H_E) ranged from 0.180 to 0.814 per locus. The average inbreeding coefficient (F_{IS}) per population ranged from 0.031 for locus BsF128, to 0.230 for locus BsH120. Significant deviation from Hardy-Weinberg genotype frequencies occurred in 49 of 350 comparisons ($p<0.05$) due to an excess of homozygotes. After B-Y FDR correction for multiple comparisons, 16 of 350 comparisons were significantly different from Hardy-Weinberg genotype frequencies ($p<0.0078$, Appendix 3), and after Bonferroni correction ($p<0.00014$, Appendix 3), only three of 350 comparisons were significantly different from Hardy-Weinberg expectations. There were no strong indicators of scoring error due to stuttering or allele dropout. Scoring null alleles using the Brookfield (1996) estimator did not significantly affect estimates of population differentiation (see Andrews[2]).

Some populations we analyzed may include SDFS-LFS hybrids. We analyzed the inbreeding coefficient F_{IS} and heterozygosity H_E separately in putative hybrid populations, which were defined based on disturbance levels and the morphological hybrid index we recently published[28]. We found that putative hybrids did not differ from pure SDFS in a statistically detectable manner for the metrics of genetic variation and differentiation that we used. Therefore, we included all individuals for the remainder of the analyses.

Pool disturbance was categorized as low, moderate or high for those in which we had sufficient data and personal experience to make an unambiguous assessment. Genetic variation (as H_E) is significantly lower in low-disturbance pools, confirming our a priori hypothesis that disturbance tends to homogenize populations that normally have low levels of endemic genetic variation.

We used several types of Bayesian clustering analyses at the level of the individual to confirm that pools are the most appropriate minimum biological unit for data analysis. "Cluster" in this sense refers to a gene pool, which is loosely equivalent to an ecological population. Depending on the criteria and software used, the number of statistically significant gene pools ranged from 2-20 in the study area, despite sample sizes that averaged only 10 SDFS per pool. One dominant feature was a clear division between the central part of the study area (Mira Mesa and Del Mar Mesa), and the remainder of the pool complexes. Statistically significant differences are apparent between nearly all complex pairs using these clustering methods.

We then analyzed our data at the level of the pool, because there is no indication that multiple (spatially separate) populations coexist within a single pool. Nearly all pairs of pools show statistically significant genetic divergence, and 1046 of 1225 pairwise F_{ST} estimates were still significant even after correction for multiple tests. Pairwise F_{ST} estimates averaged 0.2 across all comparisons, and the highest values exceeded 0.6. When analyzed with Analysis of Molecular Variance (AMOVA), there was greater differentiation among pool complexes (F_{CT} = 0.18) than among pools within complexes (F_{SC} = 0.03). Pools that are farther away tend to be more different genetically, since log-transformed genetic similarity *M* is negatively correlated with log-transformed straight-line geographic distance ($r = -0.63$, $p < 0.0001$). The A4 pool complex in Miramar showed unusually high divergence from the remainder of the SDFS populations, possibly due to their management and/or mitigation history.

When using pools as the unit of analysis, genetic divergence for microsatellite markers correlates with *overall* genetic divergence in the mtDNA data set ($r = 0.35$, $p < 0.0001$). However, the mtDNA clades A and B described in the previous study do not define biological

units that can be validated with the new data. Pools that are spatially close are indeed very similar for both sets of markers. But after controlling for the geographic distance between populations, microsatellite differences do not correlate with mtDNA *clade membership* ($r = -0.029$, $p = 0.18$).

10.3. Interpret genetic patterns in terms of landscape connectivity, disturbance, recreational activities (e.g., trails, motorized vehicles), and other environmental parameters.

Branchinecta sandiegonensis (SDFS) exhibits genetic patterns that would be typical of ephemeral pond organisms capable of limited passive dispersal. Populations are highly differentiated across the study range, especially among regions and pool complexes. In this broad-scale, county-wide population survey, numerous gene pools were detected and found to be statistically distinct by all methods utilized. Despite our efforts, analysis of the full data set shows that methodological artifacts common in microsatellite studies (especially null alleles) have not been completely eliminated. However, we do not believe that these limitations are pronounced enough to invalidate the qualitative conclusions in this report. We summarize our interpretation as follows:

a. Genetic differentiation among pools is strong and statistically significant. Genetic differentiation increases as geographic distance increases. Pool connectivity is greater within complexes than between complexes, a signature of hierarchical population structure.
b. Populations that are more divergent for microsatellite markers are also more divergent for mtDNA. However, the mtDNA clades A and B do not themselves define spatial units that can be confirmed with microsatellite data.
c. Populations from the Miramar A4 complex are unusually divergent in terms of microsatellite markers, but not mtDNA. We interpret this to mean that the biological isolation of this area is relatively recent (compared to evolutionary time scales).
d. An additional pattern of geographic interest emerged from the microsatellite data set, with Mira Mesa and Del Mar Mesa pools showing higher divergence from the remainder. The southern border of this group is largely coincident with a small portion of the Rose Canyon fault zone, with the exception of Nobel Drive pools just north of the canyon, and Sander pools just south. Fine scale genetic patterns in this area would benefit from additional sampling, and also from expanded sampling within Miramar.
e. Our overall assessment is that significant amounts of biotic connectivity (in terms of SDFS gene flow) are likely to be restricted to pools within complexes, and pools within 5 km of one another. These patterns are assumed to correlate with local adaptation in traits that matter for individual fitness.

Task 11. Hybridization between *B. sandiegonensis* and *B. lindahli* Updated from July 2013 report

Genetic threats to the integrity and persistence of endangered species can be significant and develop rapidly. These threats include the breakdown of locally adapted gene pools, and more extreme phenomena such as the dissolution of species boundaries. Human disturbance in SDFS vernal pools is associated with the increased presence of the widely distributed generalist *B. lindahli* (LFS). Regional sympatry for these closely related species has now become local co-occurrence in anthropogenically created basins and disturbed pools, with possible hybridization.

In the San Diego Fairy Shrimp Five-Year Review[30], the presence of versatile fairy shrimp in disturbed pools is noted, and hybridization between these species is recognized as a threat to the San Diego fairy shrimp. These two species can hybridize in the lab[15, 26]. Consultants have previously speculated about hybrids in field populations (based on unusual morphology), and we have now published a paper demonstrating that hybrids are present in several disturbed pools in coastal San Diego County[27, 28]. This paper also describes a morphological hybrid index to aid in identification.

11.1. Apply the morphological hybrid index to SDFS, LFS and hybrid pools across southern California.

In our recent publication[28], we presented a morphological hybrid index that can be applied to mature *Branchinecta* females. This index ranges from 1.0 for typical LFS adult females to 3.0 for typical SDFS females. ("Typical" means individuals sampled from habitats that are not anthropogenically disturbed, and that are morphologically similar to the published species descriptions.) Index scores in undisturbed habitats are ≤ 1.3 for "pure" LFS and ≥ 2.6 for "pure" SDFS (Figure 2).

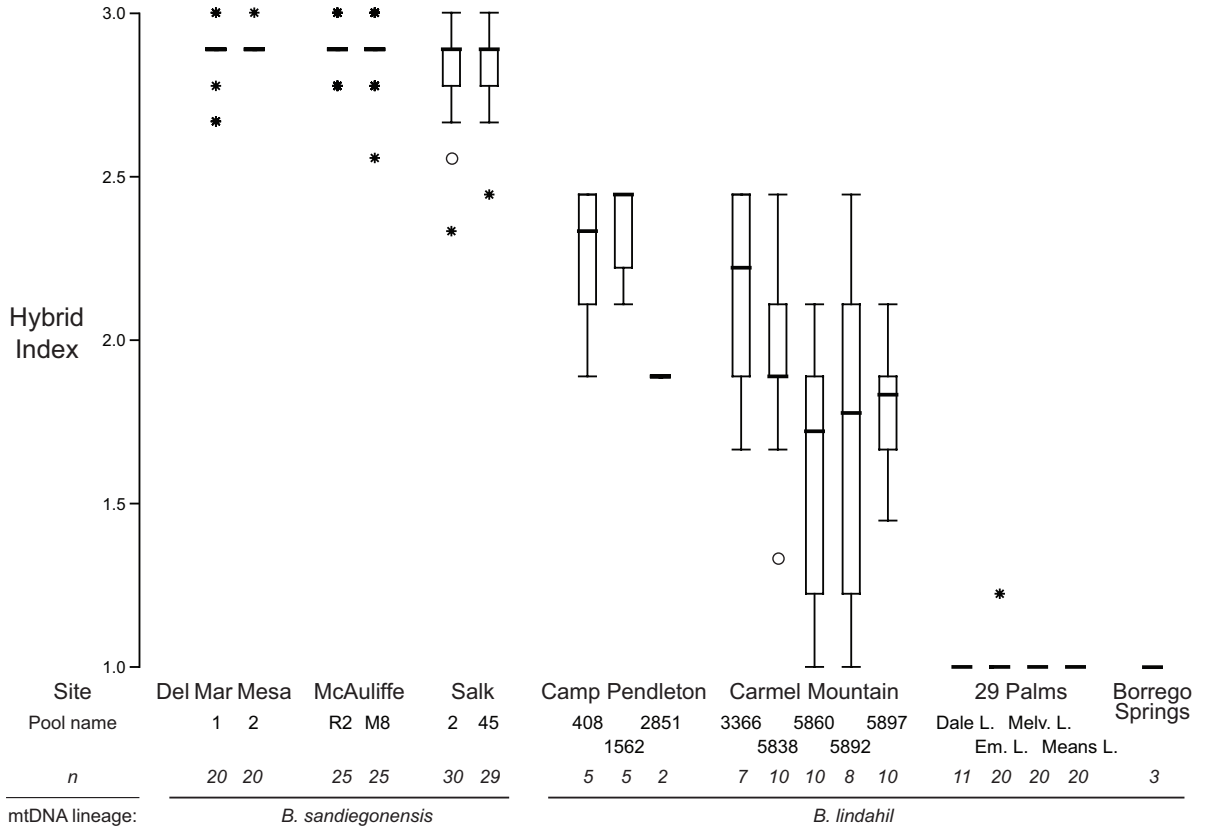

FIGURE 2: Box plot comparing hybrid index scores across the 19 pools from Simovich et al.[28]. Each pool is labeled by site name, pool number (or name) and the mtDNA lineage found in that pool. Only one mtDNA species lineage is found per pool. Scores ≤ 1.3 correspond to "pure" LFS, and scores ≥ 2.6 to "pure" SDFS. Female fairy shrimp from the first six pools correspond morphologically to typical SDFS, and those from the last five correspond to typical LFS. Each box depicts the median for the pool, 25th and 75th percentiles (box hinges), whiskers extending to the minimum and maximum data values occurring within 1.5 x range above and below the box hinges, outliers (circles) and extreme outliers (stars).

We proposed to apply the morphological hybrid index across the entire SDFS species range. We have not completed Task 11.1 because there was insufficient precipitation for most vernal pools to pond during the 2012-2013 and 2013-2014 winters. We were also constrained by a lengthy permitting approval process, and the delay of joint funding from USFWS/CDFW until fall 2013. However, the ongoing regional drought was our biggest limitation.

Assuming adequate rainfall, we will complete Task 11.1 by the end of the USFWS/CDFW contract. We were able to collect some samples after the initial rainfall of the 2014-2015 winter. If necessary, we will supplement whatever new field samples we can obtain with additional specimens from historic collections (see 11.4 below).

11.2. (original) Develop a genetic hybrid index using microsatellites.

Although we have documented the co-occurrence of LFS and SDFS mtDNA (maternal genetic) species lineages in the same pools at MCB Camp Pendleton[7], we cannot yet *genetically* distinguish between hybridization and co-occurrence of "pure" SDFS and LFS. This task requires multiple nuclear genetic markers that work reliably in both species.

The goal of this task was to find a subset of SDFS microsatellite markers that will cross-amplify on BLS individuals and also show no overlap in the alleles found within "pure" types of each species. This set of markers will comprise a genetic hybrid index, which will ultimately be verified on pools known to contain both pure types and hybrids, as inferred from standard taxonomic keys, the morphological hybrid index, and pool disturbance/history.

We first performed a preliminary cross-amplification screen with the final set of microsatellites developed for SDFS. This screen suggested that none of the primer pairs optimized for SDFS could be amplified on LFS samples. Given these results, we stepped back and re-examined other primer pairs that were designed in our lab but discarded during the preliminary optimization stages of SDFS microsatellite development described in Task 10.1. We screened a total of 164 available primer pairs (corresponding to 25 loci) for successful amplification on two DNA cocktails. One contained five SDFS samples from different pools, and the second contained four LFS samples from different pools. This screen was performed under four different sets of PCR conditions (protocols provided on request). The resulting 636 PCR reactions were visualized using standard methods. Those primer pairs showing any potential for identifying hybrids were further tested with fluorescently labeled primers on an automated sequencer. Four SDFS and four LFS individuals were screened, with a minimum of 8 PCR reactions per primer pair. If it was not clear from Stage I which PCR condition was best for both species, we ran replicates under several promising conditions.

After a thorough development and screening phase, we determined that there were an insufficient number of microsatellite primer sets that work reliably on both SDFS and LFS. We modified the task goals to instead develop a genetic hybrid index using modern genomic tools.

11.2. (modified) Develop a genetic hybrid index using SNPs.

After determining that the microsatellite markers would not be reliable (and numerous) enough for cross-species amplification and a genetic hybrid index, we developed genomic SNP markers using the following protocols.

Transcriptome preparation

Species-specific single nucleotide polymorphisms (SNPs) were developed from transcriptomes based on 16 individuals from the following sites.

Species	Site	*n*
B. sandiegonensis	Eastgate, MCAS Miramar	3
B. sandiegonensis	MCR5, McAuliffe Community Park	2
B. sandiegonensis	Day Street, Ramona	2
B. sandiegonensis	Kalbaugh Street, Ramona	2
B. lindahli	Clark Dry Lake, Anza Borrego	5
B. lindahli	DiGeorgio Rd., Borrego Springs	2

The sites were chosen to include broad representation of genetic diversity from across each species' range. The sites were also chosen to minimize the inclusion of hybrids during marker development (based on disturbance at each site and morphological hybrid scores). Samples were preserved in RNA*later*®, and stored (-20°C) for subsequent total RNA extraction. Total RNA was extracted using a phenol chloroform protocol[9, 10] with TRIzol ™ reagent. Extracted RNA was purified using PureLink™ RNA Mini Kit (silica-matrix spin column purification) and quantified using an IMPLEN® NanoPhotometer. Within each species, the extracted RNA was pooled (n = 9 SDFS individuals, and n = 7 LFS individuals). Samples were stored at -20°C prior to being shipped to Hudson Alpha Institute for Biotechnology (HAIB, Huntsville, AL) for RNA library preparation and next-generation sequencing. At HAIB, sample concentration and quality was reassessed using a Qubit® flourometer and Agilent 2100 Bioanalyzer respectively. RNA libraries underwent poly-A tail RNA sequencing, and 100 bp paired end reads were sequenced approximately 25 million times for each species sample.

Transcriptome assembly

High quality reads from both samples were filtered from raw reads using prinseq-lite 0.20.4[25], and sequence adapters remaining on the reads were trimmed using trim_galore_v0 (Babraham Bioinformatics 2014). Filtered reads were then used to assemble transcriptomes *de novo* for each species using Trinity[16]. Probable contaminant sequences were identified using Deconseq 0.4.3[24] and deleted. Following decontamination of each transcriptome, raw reads were mapped to each "clean" transcriptome respectively using Bowtie 1.1.1[17] using default options. Mapped and aligned transcriptomes were indexed and converted to SAM files using Bowtie and then to binary (BAM) files using SAMtools[19]. BCF files were created, then converted to VCF files that were piped to vcfutils.pl with the varFilter -D100 option (filtering out SNPs that had a read depth of 100 or higher). Contiguous sequences containing within-species SNPs were identified, and high probability within-species SNP sites were avoided in panel development (see

below). All bioinformatic analyses were done on the University of California, Riverside, IIGB Linux Cluster with purchased time, storage space and technical support.

Species-specific SNP detection and panel design

After identifying within-species SNP sites, both transcriptomes were reciprocally blasted using the NCBI nucleotide *BLAST*: blastn[1] to identify genetic sequences with high homology. Cross-species matches of 97.0% - 99.9% were chosen, avoiding multiple sequence matches (i.e., paralogs), gaps, deletions, and segments < 200 bp. Multiple sequence alignments with a high likelihood of containing species-specific SNPs were then globally aligned using MUSCLE[13] within the Mesquite package[20], and visualized with AliView[18]. Between-species SNPs were identified, marked according to University of Arizona Genetics Core (UAGC) protocol, and sent to UACG for primer design. Newly designed primer pairs were then blasted against both transcriptomes using nucleotide blast. Candidate sites that had primers with multiple matches were discarded.

Summary

In total, 30 candidate loci are available to screen for putative hybrids between *B. sandiegonensis* and *B. lindahli*. Based on the panel development and putative screen, each contains SNPs that have fixed (diagnostic) differences between the two "pure" species.

11.3. Apply the genetic hybrid index to *Branchinecta* populations across southern California.

We have not completed Task 11.3 because there was insufficient precipitation for most vernal pools to pond during the 2012-2013 and 2013-2014 winters. Together with the range-wide morphological hybrid screen (Task 11.1), the range-wide genetic hybrid screen will be conducted as precipitation and pool filling cycles permit. Unlike Task 11.1, we may be able to rely more on previous collections and archived DNA extractions to complete 11.3. However, no extracted DNA remains for many individuals that were included in Task 10.2, 10.3 and previous genetic work. Whenever possible, we will obtain genetic and morphological data from the same females.

11.4. Review and database vouchered specimens of both species that are held in museum collections. Apply the morphological hybrid index to historic vouchered specimens as possible.

We conducted an inventory of appropriate specimens in Dr. Simovich's previous collections. Our growing database of morphological hybrid index scores now includes 280 individuals from Simovich et al.[28], and 240 new individuals from Simovich's historic collections. The updated results are presented in Figure 3. Based on female morphology, little or no hybridization appears to be occurring in Miramar, Del Mar Mesa, McAuliffe, Nobel Drive and Ramona. We found pools with both pure SDFS and low numbers of putative hybrids at Eastgate, Maddox, Otay Mesa and San Onofre. Higher proportions of hybrids were found throughout each of the pools we analyzed at Pendleton, Carmel Mountain and Pueblo. (The Pendleton samples included only disturbed basins.) Borrego Springs contained both hybrids and pure LFS. The 29 Palms sites contained LFS with little or no hybridization.

We obtained permission from the Los Angeles County Natural History Museum (LANHM) for a limited visit in order to conduct an inventory and analysis of their fairy shrimp samples. LANHM is the preferred repository for voucher specimens from vernal pool surveys throughout southern California. We found that many samples at LANHM were poorly

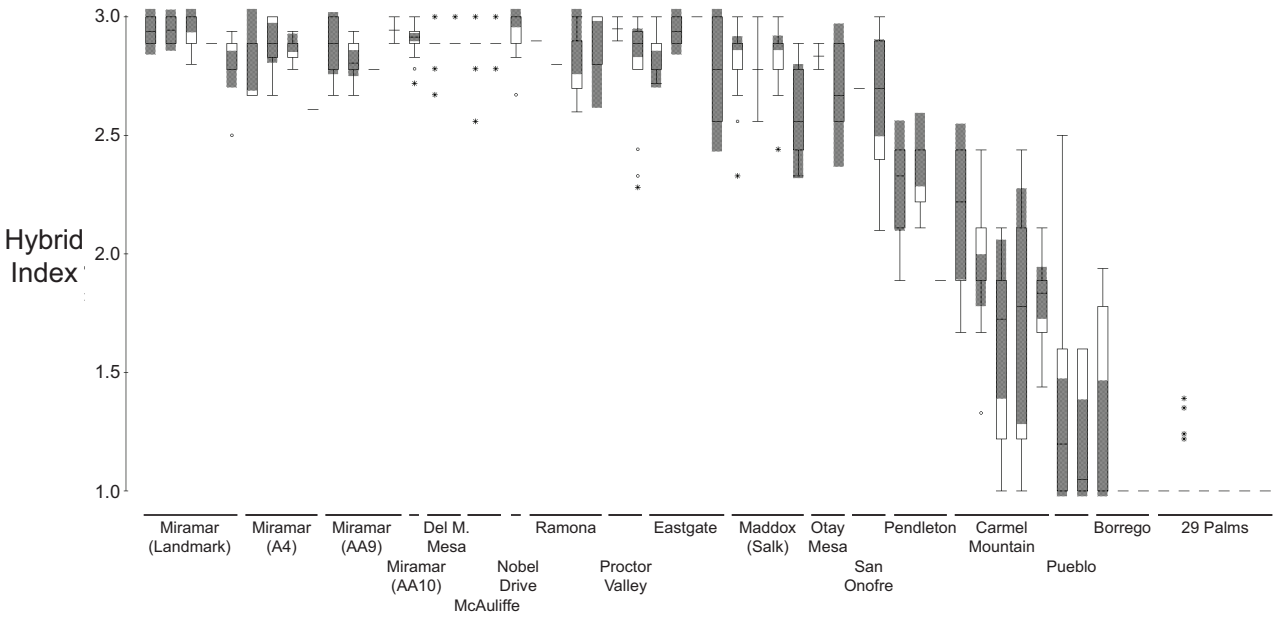

FIGURE 3: Updated box plot including new data collected for Task 11.4. Hybrid index scores are shown for 520 individuals from 56 pools. Scores ≤ 1.3 correspond to "pure" LFS, and scores ≥ 2.6 to "pure" SDFS. Box symbols are as in Figure 2.

preserved, had incomplete sample information and/or were misidentified. The museum has approximately 1400 SDFS males and 320 SDFS females from 325 samples. Less than 100 of the SDFS females are well enough preserved and maintained to score morphologically. LANHM also has approximately 1093 LFS males and 800 LFS females from 335 samples. Approximately 471 of the LFS females appear to be well enough preserved and maintained to score morphologically. Further complicating the analysis, many of the LFS that appear to be hybrids were collected from outside the range of SDFS. It is possible that LFS hybridizes with different *Branchinecta* species in different parts of its range. We have scored approximately 100 specimens from the museum so far, and will coordinate with museum staff for another extended visit to complete analysis of their collections.

The San Diego Natural History Museum has no fairy shrimp samples that we could analyze for this project, as far as we can determine.

Task 12. Conservation, management and recovery of *B. sandiegonensis* Updated from July 2013 report

12.1 Summarize results from Tasks 10 and 11 as recommendations for management, conservation and mitigation, in terms of impacts on the genetic integrity and recovery of SDFS.

Based on the results to date, it is clear that the spatial hierarchical structure of vernal pools (pools nested within distinct and often distant pool complexes) correlates with population genetic differentiation. Female morphology strongly suggests that SDFS hybridizes with LFS in many disturbed sites, and we hope to quantify the extent of hybridization with genetic analyses before completion of the USFWS/CDFW grant. Our specific management recommendations are as follows:

a. Movement of cysts among pools should be minimized, and especially outside of pool complexes. The genetic data suggest that biotic connectivity is very localized. Dispersal and gene flow of SDFS outside of pool complexes is likely to be rare. A common assumption in conservation genetics is that restricted gene flow for markers such as microsatellites correlates with local adaptation for traits that affect survival and reproduction. However, we acknowledge that this assumption must be validated with actual studies of individual fitness.

b. Following from a), newly created pools should be stocked from a single pool complex as close as possible. Stocking single new pools from a single source pool (rather than a multi-pool mixture) is recommended unless logistical or endangered species impacts preclude this.

c. The mtDNA clades A and B from the previous study[6] do not correlate in a statistically measurable way with microsatellite differentiation. Thus, there is not strong support at this time for management units based on these clades.

d. Beyond low levels of connectivity among pool complexes, there may be an even more significant amount of divergence between the pools of {Mira Mesa, Del Mar Mesa} and the remainder of the species range. Additional care should be taken to minimize homogenization of these sites with Miramar and populations south, with Ramona to the east, and with Pendleton to the distant north.

e. Even with the expanded number of individuals and genetic markers available from this study, there is still no single environmental factor such as soil type, latitude or elevation that is an obvious causal factor for overall genetic divergence within SDFS.

f. Intermediate female morphology at highly disturbed sites (including Carmel Mountain, Pueblo and some basins on Pendleton) is consistent with our hypothesis that SDFS and LFS are hybridizing at those sites. The movement of basin sediments (and associated cysts) into and out of those sites on vehicles and boots should be minimized even more than at other sites, if possible. Restoration projects at sites with intermediate female morphology should be carefully reviewed and monitored to make sure that the goals (e.g., establishing pure SDFS populations) have a high likelihood of being achieved.

g. Additional management recommendations will be available after completion of the USFWS/CDFW contract.

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