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Genetic Admixture in Vernal Pool Shrimp: Interspecific Hybridization between *Branchinecta sandiegonensis* and *Branchinecta lindahli*

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Dedication

I always wanted to see how far I could go in academia and how much of an impact I could make toward the conservation of endangered species. I dedicate this dissertation to all who helped me throughout my time here in this program. I thank the undergraduate assistants that have helped me collect data in the field and analyze data in the lab. I thank Tomas Condon, Tim Estep and Lexy Patel for their unbreakable support. I thank Pinkesh Patel, the HBOGO got me through. Finally, I thank Rachana Shah… I know it took a while, but I did it!

ABSTRACT OF THE DISSERTATION

Genetic Admixture in Vernal Pool Shrimp: Interspecific Hybridization Between *Branchinecta sandiegonensis* and *Branchinecta lindahli*

by

Ketan Vasant Patel

Doctor of Philosophy, Graduate Program in Evolutionary Biology
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Dr. Andrew J. Bohonak and Dr. William Walton, Co-Chairpersons

Driven by landscape alteration and the introduction of non-natives through human activities, biotic homogenization is thought to be a significant threat to the survival of endemic taxa. Extensive urbanization in southern California, USA, has converted most of the native coastal vernal pool habitat prompting the conservation of native vernal pool species. Habitat alteration associated with urban expansion in this region has extirpated *B. sandiegonensis* from the majority of its historical habitat. In some artificial basins within the remaining vernal pool habitat, *B. sandiegonensis* hybridizes with *B. lindahli*. Hybrids can be identified through both morphology and newly developed genetic characters (Patel et al. 2017). By using both morphological and genomic hybrid indices, researchers and habitat managers will obtain a relatively holistic perspective on the hybridization process. This not only helps to identify populations where a large-scale introduction of *B. lindahli* has occurred, but also to perhaps predict the future trajectory of species and hybrid distributions.

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Introduction of Study System

Vernal pools in southern California are characterized as hydrologically linked complexes of relatively shallow pools that are seasonally inundated by rainfall. The San Diego fairy shrimp *Branchinecta sandiegonensis* is the most common faunal species found in inundated pools in coastal San Diego County, with a biphasic lifecycle. Encysted embryos (cysts) hatch during a basin's wet phase, live out their adult stage, reproduce, and finally deposit cysts that persist through the dry phase (Erickson & Belk 1999 Ch. 1). Because adult fairy shrimp are bound to their natal pool, the introduction of new alleles can occur through the spatial migration of dormant cysts by other transient organisms/ human activity or by the mixing of individuals from different reproductive events through reproductive bet hedging (Simovich & Hathaway 1996; Simovich et al. 2013).

High habitat heterogeneity and temporal habitat partitioning are thought to prevent hybridization among co-existing species. However, multiple species of shrimp can co-exist in disturbed pools, and morphologically intermediate hybrids have been identified in many localities (Erickson & Belk 1999; Simovich and Bohonak pers. com.). *Branchinecta sandiegonensis* (Fugate 1993) is the most prominent fairy shrimp species found in undisturbed pools in coastal southern California, while disturbed pools contain *B. sandiegonensis* and *Branchinecta lindahli* (Packard 1883) (Fugate 1993; Erickson & Belk 1999; Simovich et al. 2013). Because pool inundation is approximately three weeks, both species develop and reproduce quickly.

The generalist *B. lindahli* is thought to have a competitive advantage because it can mature 1-7 days faster than its competitor, and it produces cysts that are smaller (0.24-0.27µm) compared to *B. sandiegonensis* cysts (0.33µm) (Maynard 1977; Hathaway & Simovich 1996; Erickson & Belk 1999 Ch. 5). Even though *B. lindahli* possesses characteristics that facilitate invasion, lifetime fecundity for *B. sandiegonensis* (up to 479 cysts) can be higher than *B. lindahli* (326 cysts over 77 days) (Simovich & Hathaway 1996; Maynard 1977; Erickson & Belk 1999 Ch. 5). Therefore, invading *B. lindahli* individuals will likely be assimilated into existing *B. sandiegonensis* populations unless the environment maintains a selective advantage of *B. sandiegonensis* alleles over *B. lindahli*.

In coastal San Diego County, vernal pool habitat has undergone drastic fragmentation due to human activity, and currently retains five percent of its historical range (Bauder & McMillen 1998, King 1998). Evidence of *B. sandiegonensis* X *B. lindahli* hybrids in disturbed basins (Simovich et al. 2013) suggests that natural barriers to hybridization have been eroded due to construction and vehicular traffic associated with urban expansion (and these basins in particular). Even though *B. sandiegonensis* and *B. lindahli* are considered to be "good" species, morphological similarity and a relatively porous genome facilitates genetic admixture. Simovich et al. (2013) published a female morphological hybrid index that distinguishes *B. sandiegonensis*, *B. lindahli*, and putative hybrids based on the arrangement of spines displayed on the female's dorsolateral process. This index successfully distinguishes hybrids from parental species as well as parental species from each other. However, adult male hybrids and encysted hybrid

embryos remain morphologically indistinguishable from the "pure" parental species. Conservation of *B. sandiegonensis* and other taxa at risk of extinction through hybridization necessitates the detection of admixed populations, the structure of resultant hybrid swarms, and environmental correlates that facilitate invasion and establishment.

My dissertation is comprised of three chapters that use genetic and morphological markers to investigate hybridization between *B. sandiegonensis* and *B. lindahli* in the context of biotic homogenization. The first chapter details the design and development of a 20-ssSNP (species-specific single nucleotide polymorphism) panel used to distinguish parental pure types from interspecific hybrids and classify hybrids. The first chapter also compares a newly–developed genomic hybrid index to the morphological hybrid index of Simovich et al. (2013). The second chapter of my dissertation focuses on the detection of interspecific hybrids across the native range of *B. sandiegonensis,* and distinguishing admixed localities from non-admixed localities. This chapter investigates genetic admixture across 73 localities using 1,134 individuals. Bayesian and maximum likelihood analyses that assign individuals to genetic clusters (equivalent to gene pools) were utilized to distinguish between admixed and non-admixed localities. To account for the influence of ancestry in hybrid individuals, interspecific heterozygosity was analyzed in further detail (Gompert and Buerkle 2009, 2010). I also analyze patterns of allelic introgression and the frequency of hybridization in the context of cyst bank dynamics over the entire dataset (and with respect to habitat type) using genomic clines for 20 ssSNP loci.

The third chapter of my dissertation analyzes the effectiveness of morphological characters to identify hybrids in natural populations. I analyzed 667 individuals both morphologically and genetically to test how well the 18 characters used in the morphological hybrid index can classify admixed and non-admixed individuals. Simple correlations, a principal components analysis and a local Fisher's discriminant analysis were used to interpret each character in the context of the overall morphological hybrid index. Finally, I document new variation in spinal patterns and provide a detailed workflow on the process of assigning a morphological hybrid score to female shrimp.

Discovery and validation of species–specific diagnostic SNP markers for identifying hybrids between the endangered San Diego Fairy Shrimp (*Branchinecta sandiegonensis*) and the Versatile Fairy Shrimp (*Branchinecta lindahli*)

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Abstract

Because only 3-7% of historically present vernal pool habitat remains in coastal San Diego County, conservation efforts must prioritize both the maintenance of these pools and the genomic integrity of their inhabitants. One inhabitant of the coastal vernal pools found in southern California is the federally endangered San Diego fairy shrimp *Branchinecta sandiegonensis*. To detect hybrids in the wild, we have developed a genomic hybrid index comprised of 20 SNP loci using 16 individuals with no morphological evidence of hybridization, collected from populations unlikely to facilitate introgression.

These loci have alternatively fixed alleles between the endemic San Diego fairy shrimp and the versatile fairy shrimp (*Branchinecta lindahli*). This genomic hybrid index was validated using 421 individuals from 29 localities using morphology and habitat information. Our data suggest that some artificial and disturbed pool basins harbor putative hybrids, and thus have the potential to be stepping-stones for the future spread of hybrids. This genomic hybrid index will be a useful tool for the detection of putative *Branchinecta* hybrids in the wild from both mature and immature life history stages and will be an aid in the monitoring/recovery of non-admixed San Diego fairy shrimp populations.

Introduction

Similar to habitat loss, the alteration of native habitat is often linked to the initial listing of a species as threatened or endangered, and usually decreases the likelihood of a species' recovery. Landscape homogenization, as described in Simovich et al. (2013) in particular, can aid invasive species by breaking down habitat partitions and facilitating novel competition scenarios (Mooney and Cleland 2001; Olden et al., 2004; Olden, 2006; Devictor et al., 2008a, b; Simovich et al. 2013). Along with the decreasing habitat heterogeneity, formerly unique communities will become increasingly similar and novel competition scenarios will favor the range expansion of non-native generalists. Establishment of non-natives into human-altered habitats can also impact biodiversity through a loss of local/endemic genetic and species diversity (Anderson and Stebbins 1954; Rhymer and Simberloff 1996; Ellstrand and Schierenbeck, 2000).

If invasive species readily hybridize with native species, genetic boundaries between both species may erode, resulting in the irrevocable loss of native genetic stock (Rhymer and Simberloff 1996).

Coastal southern California vernal pools are ephemeral wetlands which host an array of plants and animals adapted to the bi-phasic (e.g. wet and dry) nature of the habitat. The most evident faunal elements consist of crustaceans. During periods of pool inundation, formally dormant encysted embryos hatch, develop to their adult stage, reproduce, and deposit cysts for the remainder of pool inundation (Erickson and Belk 1999). The most common of these are several species of fairy shrimp (Anostracans) in the genus *Branchinecta* including the San Diego fairy shrimp, *Branchinecta sandiegonensis* (Fugate 1993; USFWS 1997). This federally listed species is characterized as a narrow-range endemic that is found only in highly functional (Bauder et al. 2009) coastal vernal pools in southern California and Baja California, Mexico (Fugate 1993; Erickson and Belk 1999). Estimates suggest that only 5% of the original coastal vernal pool habitat remains intact (Bauder and McMillen 1998; King 1998) mostly due to urban expansion. Additionally however, the associated construction and vehicular traffic have resulted in the creation of countless artificial basins (e.g. deep impoundments, road ruts, ditches, etc.) that may potentially harbor invasive generalists. For example, *Branchinecta lindahli*, once thought to be restricted to inland playas, now occurs in a variety of artificial pools in and around converted vernal pool habitat (Fugate 1998, Simovich et al. 2013).

In addition to the arrival of *B. lindahli* as a competitor, *B. sandiegonensis* has shown to hybridize with *B. lindahli* in both in situ and ex situ conditions (Fugate 1998; Erickson and Belk 1999; Simovich et al. 2013). Due to the threat posed by interspecific hybridization (Rhymer and Simberloff 1996), hybrid detection and subsequent management must play a crucial part in the conservation and recovery of *B. sandiegonensis*.

To detect hybridization between *B. sandiegonensis* and *B. lindahli*, Simovich et al. (2013) developed a morphological hybrid index based on an adult female's thoracic spine pattern. Spines (dorsolateral processes) on nine thoracic segments of mature females are scored, and the resulting morphological hybrid index distinguishes *B. sandiegonensis* from *B. lindahli* and putative hybrids (Rodgers 2002; Simovich et al. 2013). However, morphological identification of hybrids is limited for several reasons. First, only adult females display the diagnostic characters needed to identify putative hybrids but species identification keys, required by those with federal permits, rely on male characters. Second, the occasional presence of atypical character states in a particular individual could potentially reflect selection on that character, rather than introgression of the entire genome. Third, by relying solely on morphology, misidentification may occur due to transgressive phenotypic variation displayed in highly admixed individuals (Seehausen 2004).

In these instances, hybrid offspring may display extreme phenotype variation compared to the reference phenotypes used to identify either parent species (Seehausen 2004; Arnold 2006; Hedrick 2013). In the worst-case scenario, introgressive hybridization events through multiple generations may render diagnostic morphological markers ineffective.

Genetic markers such as single nucleotide polymorphisms (SNPs) have the potential to distinguish parental species and place hybrid individuals in distinct classes based on multi-locus genotypic patterns (Pritchard et al. 2000; Anderson and Thompson 2002; Li et al. 2015). Characterized by alternatively fixed loci between parent species, species specific SNPs (ssSNPs) can be used as ancestry informative markers that are easily diagnosable, highly reliable, and represent genome-wide patterns of interspecific admixture (VÄHÄ and Primmer 2006; Li et al. 2015). Because hybridization may occur across multiple generations, ssSNP loci may also help to infer the proportion of an individual's genome where one gene copy was inherited from each parental species (i.e. interspecific ancestry). Interspecific ancestry can be then used to distinguish between early, middle, and late-generation hybrids (Gompert and Buerkle 2009; 2010). For example, both F_1 and later-generation hybrids are expected to have a genomic hybrid index value of 0.5 with variance increasing in later-generation hybrids. Specifically, the F_1 generation should be heterozygous for ancestry (e.g. A/T) across the entire genome (i.e. complete or near-complete heterozygosity across the genome).

As subsequent genetic admixture between parental species and hybrid classes occurs, later-generation hybrids will show a reduced interspecific ancestry in that mid-stage hybrids will display fixation of alleles for both parental species at different loci (e.g. F_5, F_7, etc.) and late stage hybrids (e.g. F_{10}, F_{20}, etc.) will display little heterozygosity across the genome, but may show a mosaic pattern where some loci are fixed for one parental SNP and others are fixed for the second parental SNP. In addition, admixed individuals can also be further separated into hybrids and backcross categories. Here, we describe the first assembled *denovo* transcriptomes for *B. sandiegonensis* and *B. lindahli,* and their alignment to discover alternatively fixed species-specific SNP loci. Our objectives for this study are to (i) develop a robust genomic panel capable of detecting both male and female putative hybrids, and (ii) validate the resultant genomic panel with a dataset of morphologically characterized individuals using techniques published in Simovich et al. (2013). This technique will aid in the recovery of *B. sandiegonensis* through detection/ monitoring of hybrids across functional and a variety of disturbed pool types and discerning between admixed and pure *B. sandiegonensis* populations.

Methods

Sample collection, library construction, and RNA-seq with poly A tail enrichment

To obtain representative species-specific genomic diversity for transcriptome assembly, we collected nine reference samples for *B. sandiegonensis* across four coastal

vernal pool sites and seven reference samples of *B. lindahli* across two inland playa sites (denoted by §: Table 1).

These reference sites were chosen because they represent archetypical habitat for each species, have been sampled over multiple seasons, and no individuals displayed hybrid females (based on the index of Simovich et al. 2013). Samples were immersed in separate collection vials containing RNA later® (Ambion, Austin, TX, USA) and were stored at -20°C. Prior to RNA extraction, equal amounts tissue from shrimp of the same species were pooled together to form a species pool (Konczal et al. 2013) and homogenized using a roto-homoginizer at -20°C in the presence of TRIzol™ reagent. Total RNA was extracted using the TRIzol™ extraction protocol (Behera et al. 2002) followed by an RNA purification step using Ambion™ cleanup kit (Ambion, Austin, TX, USA). Total RNA concentrations for both species pools were evaluated by Qubit® flourometer. The resulting two species pools were sent to Hudson Alpha Genomic Services Lab (GSL) (Huntsville, AL USA) for library preparation and subsequent RNA sequencing using poly (A) tail enrichment. Sequencing was carried out using a Illumina HiSeq 2000 with the option of 100 base pair paired-end reads resulting with approximately 25 million reads per species pool.

De novo Transcriptome Assembly and SNP discovery

Data files containing raw sequencing reads in FASTQ format, quality scores, and paired reads information were returned from Hudson Alpha Genomic Services Lab (Huntsville, AL, USA) for the subsequent trimming and transcriptome assembly.

Sequencing adapters were trimmed using Trim Galore! (Kruegar, 2015) and raw reads were filtered for quality control by removing reads with quality scores less that 20 and length below 30 base pairs using prinseq-lite-0.20.4 (Schmieder and Edwards 2011). Reads from each species pool were used to assemble transcriptomes for *B. sandiegonensis* and *B. lindahli* using the Trinity assembler (v.2014-04-13; Grabherr et al. 2011). Trinity employs three methods (named *Inchworm*, *Chrysalis* and *Butterfly*) for transcriptome assembly without a reference genome (i.e. *denovo* assembly). Briefly, *Inchworm* assembles raw sequencing reads by greedy k-mer extension (default is set to k-mer 25) into a single representative (i.e. contig) for a set of variant reads that share k-mers. *Chrysalis* then clusters related contigs, and constructs de Bruijn graphs for each cluster, which represent the complexity of overlaps between variant contigs. In the final step, *Butterfly* analyzes all the paths taken by sequencing reads and read pairings with respect to the corresponding de Bruijn graphs for all clusters and reports all plausible transcript sequences (Grabherr et al. 2011). Following *denovo* transcriptome assembly, raw reads were mapped to each respective transcriptome assembly using Bowtie 1.1.1 set to default options (Langmead et al. 2009).

To isolate SNPs that would serve as diagnosable markers for hybrid identification, we focused our efforts on the discovery of loci that would display fixed-allelic differences between *B. sandiegonensis* and *B. lindahli* (e.g. 'T/T' in *B. sandiegonensis*, 'C/C' in *B. lindahli*, and 'T/C' in F1 hybrids). Therefore, any individuals with at least one

heterozygous genotype or deviation from the genotypes of either pure *B. sandiegonensis* or *B. lindahli* (Table 2) would be considered a hybrid.

Prior to ssSNP discovery, contig sequences containing within species SNPs and/ or having a high probability of containing sequence variants were discovered using SAMtools (Li et al. 2009) and were manually discarded from further panel development. To identify genomic segments of high homology between species, the entire *B. sandiegonensis* transcriptome assembly was blasted against the entire *B. lindahli* transcriptome assembly using NCBI nucleotide *BLAST*: blastn (Altschul 1990). Initially, contigs with matches less than 97.0% (i.e. 97% of base pair matches across the entire length of the contig) were discarded. Contig alignments were then filtered further to discard multiple sequence matches (i.e. hits), gaps, insertions/deletions (i.e. INDELS), and segments less than 200 base pairs in total length. The remaining contig alignments were globally aligned using MUSCLE (Edgar 2004) implemented within the Mesquite program (Maddison and Maddison 2001) and was assessed visually using AliView (Larsson 2014). Any contig sequence alignment that failed to match globally was subsequently discarded. Contig alignments that remained were selected as potential diagnostic markers and carried forward to primer design.

Species-specific panel development and initial testing

Contigs containing putative ssSNP loci were sent to University of Arizona Genetic Core (UAGC) for primer design. Multiplex assays were designed using the MassARRAY Assay Design® software with the goal of multiplexing of 30 SNPs. Only

SNPs with at least a 100 bp flanking region on either side of the polymorphic site were selected for the assay design.

Candidate primer pair sequences were returned and were subsequently blasted against both transcriptome assemblies using NCBI nucleotide *BLAST*: blastn (Altschul 1990). Candidate loci with primers pairs with hits to multiple sites on either transcriptome assembly were discarded. The remaining candidate ssSNP loci were carried forward for marker validation.

To test the genotyping success of the panel, we used 30 candidate ssSNP markers to genotype 46 morphologically identified individuals. Briefly, samples were sent to the University of Arizona Genetic Core (UAGC) facility for genotyping using the Sequenom MassARRAY genotyping platform (Bradić et al. 2011). Noncalls resulting from low probability or bad spectrum were noted and resolved by eye if possible. Individuals with lower than 90% call rates were removed, and failed loci were discarded or redesigned. Primer pairs that successfully amplified target loci were formatted into a final 20-plex ssSNP panel.

Species-specific SNP validation

To validate the quality and performance of the final 20-plex ssSNP panel, we used a dataset of 385 adult female shrimp, morphologically identified as *B. sandiegonensis*, *B. lindahli*, and various interspecific hybrids using the morphological hybrid index developed by Simovich et al. (2013). Arrangement of spines on each thoracic segment were characterized into one of five alphabetic character states (Figure 1). The character

state a is represented by single row of spines projecting laterally associated with *B. lindahli*.

State b is characterized by a single row of spines projecting dorsally, atypical for both species. Character state c is represented by two distinct pairs of spinal processes located on opposing sides of the thorax whereby one process projects laterally, and the other projects dorsally associated with *B. sandiegonensis*. Character state d is marked by one pair of thick bi-lobed processes located on opposing sides of the thorax that project laterally, and appear to represent the fusion of state b associated with *B. sandiegonensis*. Segments absent of spines are placed into character state e. After spine patterns are classified letters are converted in to one of three numbers. Character states congruent with *B. lindahli* are given a score of 1, character states congruent with B. sandiegonensis are given a score of 3, and character states that are atypical for both species are given a score of 2. Numeric scores for each thoracic segment are averaged and the resultant average score is used to categorize individuals as pure *B. lindahli* (1-1.3), hybrids (1.4-2.6), or *B. sandiegonensis* (2.7-3).

To verify that the genomic panel could detect male hybrids, 35 males from a total of five pools were sampled in the range of *B. sandiegonensis*. We selected seven males from Brown Parcel A, six from Proctor Valley Corral side B, nine from Palmdale pool 1, four from Palmdale pool 2, and 10 from Palmdale pool 4. The resultant genomic panel was validated by a total of 421 adult shrimp.

If morphological hybrids were present, pools were characterized as vehicular road ruts, man-made deep impoundments, or artificial pools as a result of habitat remediation efforts. Detailed information regarding sample localities, hybrid presence, and disturbance characteristics are presented in Table 3.

Following the manufacturer's specifications, DNA was extracted and isolated from approximately 10 mg of tissue per sample using the Qiagen DNeasy kit (Qiagen). DNA concentration and purity were estimated using an Implen Nanophotometer™ Pearl. Samples, in batches of 384 were then to the University of Arizona Genetic Core (UAGC) facility for ssSNP panel validation using the Agena Bioscience MassARRAY genotyping platform (Bradić et al. 2011). Diagnostic ssSNP genotypes were subsequently converted into numeric format according to species-specific alleles (e.g. *B. lindahli* = 1, heterozygous loci = 2, and *B. sandiegonensis* = 3) to match assignments used in the morphological hybrid index. A scatterplot was used to compare hybrid indices across a variety of disturbed and undisturbed pools.

Multi-locus genotype data was also formatted for analysis using INTROGRESS (e.g. P1/P1, P1/P2, and P2/P2). Using the function est.h, and genomic.clines implemented in program INTROGRESS, hybrid scores across all loci were used to determine the interspecific ancestry for each individual. Finally, interspecific ancestry as a function of the generated genomic hybrid index using all 20 loci was visualized tri.plot command (Gompert and Buerkle 2009) implemented in R (R Core Team 2016).

Results and Discussion

Transcriptome assembly and SNP discovery

Library sequencing produced 117,154,626 100bp paired-end reads from the *B. sandiegonensis* pool and 73,891,652 100 bp paired-end reads for *B. lindahli* pool. A total of 49,603 and 39,142 contigs (trinity genes) were recovered for *B. sandiegonensis* and *B. lindahli* respectively. Mean contig sizes and N50's were 889.75 bp and 1,700 for *B. sandiegonensis*, and 1,070 and 1,979 for *B. lindahli* (Table 1). Bowtie mapped 54,584,949 and 6,587,601 filtered reads with a total of 30,560,204 and 52,166,678 properly paired reads to the *B. sandiegonensis* and *B. lindahli* respectively. Reciprocal blasting followed by global alignment using MUSCLE (Edgar 2004) yielded a total of 457 unique contig matches that were above 97% similarity, over 200 bp in length, and possessed neither gaps nor INDELs. Data pipeline information regarding transcriptome assembly statistics, and subsequent filtering steps are displayed in (Table 1).

Species-specific SNP Panel development and validation

To determine the accuracy and reliability of this SNP panel as a resource to identify interspecific hybrids, we genotyped a subset of 46 morphologically identified individuals with an initial panel consisting of 30 SNPs optimized in 2 multiplex panels (a 22-plex and an 8-plex; data not shown). From the original 30 initial candidate markers, we selected 20 candidate loci that successfully and reliably amplify for use in the final

multiplex SNP panel. The final panel was validated using a separate dataset of 421 individuals (Table 3).

A genotypic failure rate for each locus was calculated by dividing the number of genotypic failures in the dataset individuals by the total number of individuals (i.e. seven failures across 421 individuals equals a failure rate of 0.016 or 1.6%). Over half (13/20) of the loci tested had a genotyping failure rate of less than five percent, four loci had a failure rate of less than 10 percent, and three loci had genotype failure rates of less than 16 percent. The evident low failure rate suggests that this panel is a robust. Contig ID, species-specific genotypes, and marker failure rate are provided in Table 2.

Both hybrid indices showed similar values for individuals in undisturbed (assumed pristine and pure species) pools. Non-admixed, undisturbed *B. lindahli* localities (Figure 2; bottom-left) show high congruence between the genomic and morphological hybrid index. Non-admixed localities of *B. sandiegonensis* (Figure 2; top-right) also show high correlation between hybrid indices. However, within some non-admixed *B. sandiegonensis* localities, variation in the female morphological hybrid index may suggest some amount of phenotypic plasticity or unreliable character scoring.

Correlation between the genomic and morphological hybrid index seems to weaken in disturbed pools (Figure 2; center). Individuals from disturbed pools with an average genomic score similar to non-admixed *B. lindahli* show a wider range of variance in morphology (Figure 2). Conversely, individuals from a single disturbed pool, Del Mar Mesa 256, displayed morphological scores similar to non-admixed *B. sandiegonensis* yet showed a high variance in genomic makeup. This pool was known to contain three early

stage hybrids and 17 non-admixed *B. sandiegonensis* individuals (Figure 2).

However, very weak congruence between genetics and morphology in many disturbed pools and road ruts is not surprising or unexpected as it suggests that close association between genotype and morphology may dissociate as a result of genetic admixture over many generations. Therefore, repeated introgression may replace distinct species with hybrid swarms that are comprised entirely of admixed individuals (Seehausen 2004).

Focusing solely on the genomic data, the INTROGRES plot shows "pure" *B. sandiegonensis* and *B. lindahli* restricted to opposing corners at the base of the triangle plot, with alternatively fixed alleles at the 20 ssSNP loci (Figure 3). Four early stage hybrids (near the top of the triangle) are the result of ongoing interspecific hybridization in some localities. Admixed individuals at the bottom of the plot (but not in one of the two corners) are the results of past backcrosses among hybrids, or between hybrids and one of the "pure" *Branchinecta* species. Overall, the ssSNP data provide evidence for hybridization and introgression through multiple generations in certain localities. More general conclusions regarding the status of the endangered *B. sandiegonensis* will require additional sampling throughout its range.

The frequency of *B. lindahli* alleles in the coastal vernal pools we surveyed ranged from 0.00 - 0.959. The majority (17/20) contained very low (non-zero but < 0.05 %) *B. lindahli* allele frequencies (Table 3). This suggests very limited past hybridization even in nearly-pure "native" genomic stock. However, a few putative hybrid populations

exist in heavily disturbed ruts as late-stage hybrid swarms, and genomic identity in these habitats is most heavily influenced by the dominant parental species.

Waterkeyn et al (2010) demonstrated that encysted embryos can get trapped in mud and adhere to footwear and vehicle tires and unintentionally be spread from pool to pool. If range expansion of *B. lindahli* has been facilitated through vehicular traffic disturbance in coastal habitats, hybrid establishment and persistence may only be restricted to a limited number of highly disturbed pools (such as road ruts). In our data set, artificial basins that experience high levels of anthropogenic disturbance are most likely to harbor hybrid swarms (e.g. Del Mar Mesa pool 256, Proctor Valley Corral side B, Camp Elliot Village rut, Costa Mesa pool A). Additional studies are needed to confirm whether hybrids in areas not sampled for this study are also restricted to highly disturbed basins (e.g. vehicular road ruts, deep impoundments, artificial pools). It is also unclear whether these types of sites act as "bridgehead" populations to promote the spread of hybridization into native vernal pools (Estoup and Guillemaud 2010).

The accuracy, time- and cost-effectiveness of ssSNPs relative to morphological markers can greatly improve the detection of putative hybrids, and quantify the degree of genetic admixture in natural populations. This study provides a new technique for the identification of both male and female putative hybrids, as well as a quantifiable metric to assess site-specific levels of interspecific ancestry. We encourage the use of this ssSNP panel for use in future studies aimed at mapping the distribution of putative hybrid populations throughout the native coastal range of *B. sandiegonensis*, as well as desert playas and various artificial basins that are more characteristic of *B. lindahli*.

The use of this tool will aid in the detection of male and female hybrids in natural populations and provide a more robust method to characterize admixed localities, thereby aiding in the mitigation of hybrid spread and overall recovery of *B. sandiegonensis* in coastal vernal pools.

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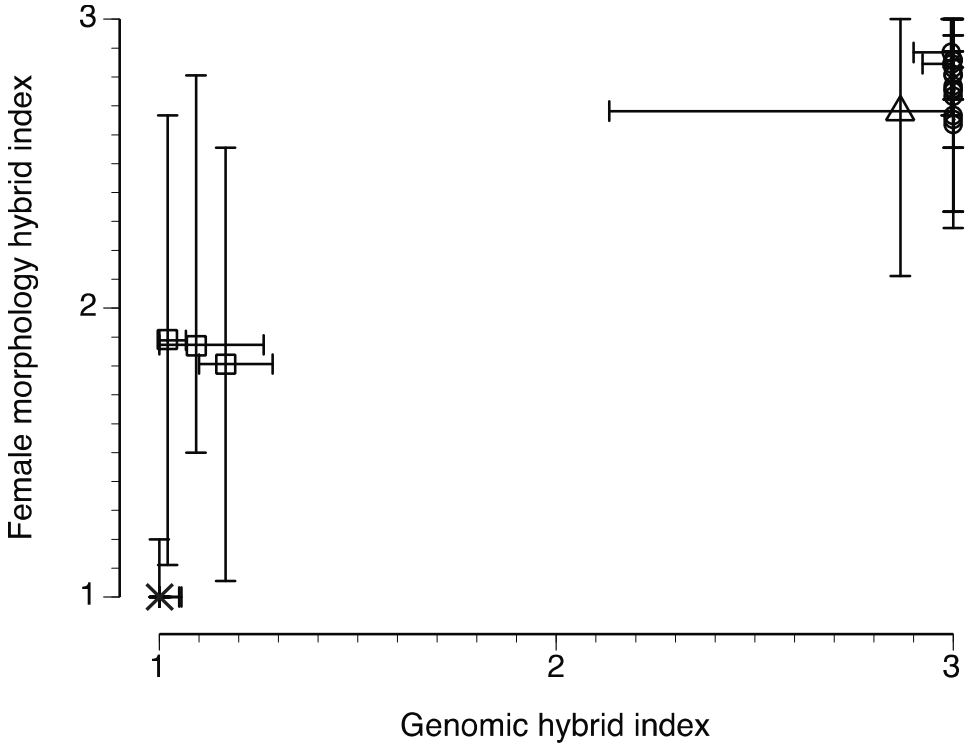
Chapter 1 Table 1. Transcriptome assembly statistics, mapping, and candidate loci filtering summary statistics for *B. sandiegonensis* and *B. lindahli*

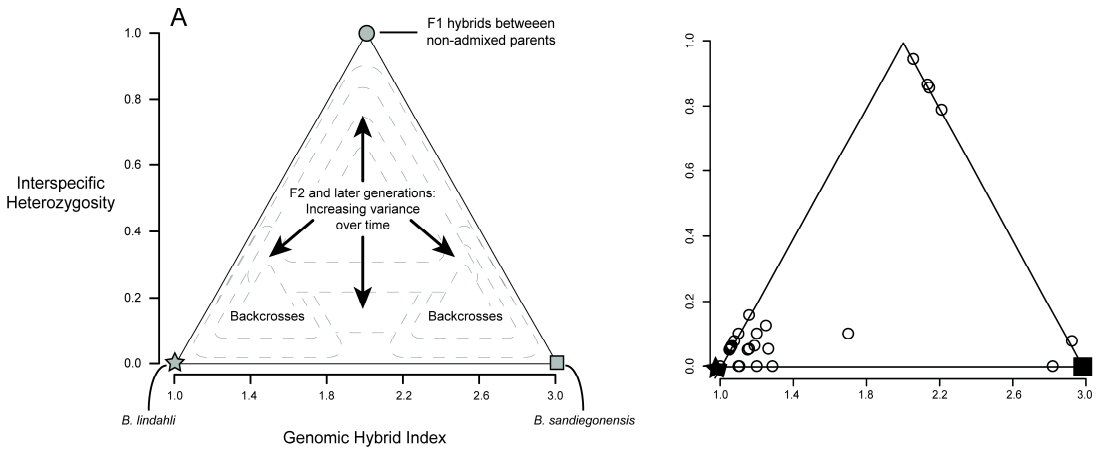
Post RNA sequencing steps	***B. sandiegonensis***	***B. lindahli***
Number of raw 100bp paired reads	117,154,626	73,891,652
Number of contiguous sequences (Contigs)	2,099,012	1,566,647
Total trinity 'genes':	49,603	39,142
Total trinity transcripts:	74,667	39,142
Percent GC:	45.22%	45.18%
Contig N10:	5,117	4,881
Contig N20:	3,572	3,718
Contig N30:	2,775	3,013
Contig N40:	2,178	2,457
Contig N50:	1,700	1,979
Median contig length:	429	566
Average contig:	890	1,071
Total assembled bases:	66,434,959	84,447,374
Number of filter reads mapped	54,584,949	65,876,018
Number of properly paired reads	30,560,204	52,166,678
Alignment using NCBI blastn and MUSCLE		
Post alignment filtering		
Alignments with reciprocal matches 99.9-97% (with duplicates)	3,117	3,397
Alignments with a single match	932	932
Alignments exhibiting zero gaps	742	742
Alignments with a total length greater than 200 bp	457	457

Chapter 1 Table 2. Detailed information of the 20-SNP Agena Bioscience multiplex, including genotypes for *B. sandiegonensis, B. lindahli*, and genotyping failure rate (%)

ssSNP Marker ID	*B. sandiegonensis allele*	*B. lindahli allele*	*Protein Identification via NCBI non-redundant protein database (if applicable)*	*Failure Rate (%)*
RDcomp25015	G	A		0.25
comp1246633	C	T		0.25
comp12974	C	T	LSM domain	0.25
comp28208	C	G		0.49
comp2628	T	C		0.74
comp1209936	A	G		1.23
RDcomp40235	T	C	Ribosomal protein L44	1.47
comp29744	T	C		1.47
RDcomp33135	G	A		1.72
comp19493	C	A		1.72
comp32848	T	A	HMG (high mobility group) box	1.72
comp19136	A	G		2.21
comp37098	G	A	3'5'-cyclic nucleotide phosphodiesterase	3.19
comp678743	G	A		5.15
comp12997	C	A	Ubiquitin-2 like Rad60 SUMO-like	5.64
comp20933	G	A	Neurotransmitter-gated ion-channel ligand binding domain	6.37
comp3767	A	C		6.37
comp31041	A	G	Ion transport protein	12.01
comp977876	G	C	Immunoglobulin I-set domain	13.24



Chapter 1 Figure 1. Comparison of genomic hybrid index and the female morphological hybrid index across 24 localities: 5 (asterisk) inland playas, 3 (square) disturbed coastal pools, 1 (triangle) disturbed pool with three early stage hybrids and 17 *B. sandiegonensis*, and 15 (circle) disturbed and undisturbed coastal pools containing *B. sandiegonensis*. Each symbol represents a population mean, and the bars extend from the minimum to the maximum for each index. Average hybrid scores (comprised of all individuals sampled) of populations range from values consistent with pure *B. lindahli* (1), to admixed (2), to pure *B. sandiegonensis* (3).



Chapter 1 Figure 2. Interspecific ancestry i.e. the proportion of an individual's genome where one gene copy was inherited from each parental species in admixed lineages. (A) Schematic illustration of the total heterozygosity as a function of percent *B. sandiegonensis* alleles in the genomic hybrid score for each individual). Early (F_1), middle (F_2, F_4, F_6) and late stage (F_{10}, F_{20}, F_{30}, etc.) hybrids are enclosed in boxes. Backcross *B. sandiegonensis* and backcross B. *lindahli* are lateral to the hybrid lineage and pure types of both species are clustered at opposite ends of the base. (B) Triangle plot displaying patterns of interspecific ancestry in fairy shrimp hybrid individuals based on genomic hybrid index values (proportion of *B. lindahli* alleles) for individuals used in this study. Non-admixed *B. sandiegonensis* (square; n = 268), non-admixed *B. lindahli* (asterisk; n = 97), early stage hybrids (star; n = 4) and late stage hybrids and introgressed individuals (circle; n= 52).

Chapter 1 Table 3. Sampling locality information, presence of hybrids, description of hybrid pools, number of samples collected females (males), and allele frequency for *B. sandiegonensis* and *B. lindahli* for each sampling locality as determined by SNP genotyping. § denotes that samples were selected for ssSNP marker discovery and * denotes that samples were selected for ssSNP marker validation.

SITE	COMPLEX	POOL	SAMPLES COLLECTED: F (M)	LATITUDE	LONGITUDE	POOL CATEGORY	HYBRID PRESENCE	POOL DESCRIPTION (HYBRIDS PRESENT)	BS ALLELE FREQ	BL ALLELE FREQ
RAMONA *	Town	Main/Hunter St.	20 (0)	33.23277778	-116.9425	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
RAMONA § *	Town	Ramona/Day St.	19 (0)	33.08638889	-117.0797222	Coastal Vernal Pool	No		0.998	0.002
RAMONA § *	Town	Main/ Kalbaugh St.	20 (0)	33.027121	-116.890167	Coastal Vernal Pool	No		1.000	0.000
BROWN *	Parcel	A	5 (6)	32.919815	-117.1725877	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
CARMEL MOUNTAIN *	Carmel Mountain	Football Pool	16 (0)	33.0805556	-117.2905556	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
ORANGE COUNTY*	Costa Mesa	A	15 (0)	33.66	-117.94	Coastal Vernal Pool	Yes	Artificial pool	0.096	0.904
DEL MAR MESA *	Bowtie	A	18 (0)	33.12638889	-117.415	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
DEL MAR MESA*	Del Mar Mesa	256	22 (0)	33.18111111	-117.3563889	Coastal Vernal Pool	Yes	Road rut	0.906	0.094
DEL MAR MESA *	Del Mar Mesa	55	19 (0)	33.12833333	-117.4033333	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
MIRAMAR *	AA10	70.1	17 (0)	32.8763791	-117.0997655	Coastal Vernal Pool	No		1.000	0.000
MIRAMAR *	AA4-7	Cobble Pool 1	18 (0)	32.84005225	-117.1145703	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
MIRAMAR *	AA9	139	19 (0)	32.87629931	-117.1103289	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
MIRAMAR*	Eastgate (I7)	EG-2 (Restored Road Pool)	20 (0)	32.87760071	-117.1911484	Coastal Vernal Pool	No		1.000	0.000
MIRAMAR *	FF1/2 (Flightline)	2	17 (0)	32.87621366	-117.1204777	Coastal Vernal Pool	No		1.000	0.000
MIRAMAR §	Eastgate (I7)	3 (Duck Pond)	5 (0)	32.87747827	-117.1930795	Coastal Vernal Pool	No		1.000	0.000

MIRAMAR *	Camp Elliot	Village Rut	14 (0)	-117.14058	32.88816	Coastal Vernal Pool	Yes	Road rut	0.063	0.937
MISSION TRAILS *	Mission Trails	Shepherds pond	17 (0)	33.06027778	-117.0483333	Coastal Vernal Pool	Yes	Deep impoundment	0.078	0.922
MCAULIFFE §	McAuliffe Community Park	MCR5	2 (0)	32.914283	-117.160195	Coastal Vernal Pool	No		1.000	0.000
NOBEL *	Nobel Dr.	3	19 (0)	33.125	-117.3961111	Coastal Vernal Pool	No		1.000	0.000
OTAY MESA *	Proctor Valley	17	19 (0)	32.80222222	-116.9805556	Coastal Vernal Pool	Yes	Road rut	1.000	0.000
OTAY MESA *	Proctor Valley	Corral Pool (B Side)	12 (6)	32.72361111	-117.0316667	Coastal Vernal Pool	Yes	Road rut	0.040	0.960
LOS ANGELES COUNTY	Palmdale *	Pool 1	10 (9)	-118.170558	34.824174	Inland Desert Playa	Yes		0.056	0.944
LOS ANGELES COUNTY	Palmdale*	Pool 2	13 (4)	-118.170943	34.83131	Inland Desert Playa	Yes		0.057	0.943
LOS ANGELES COUNTY	Palmdale *	Pool 3	20 (0)	-118.170865	34.83315	Inland Desert Playa	Yes		0.051	0.949
LOS ANGELES COUNTY	Palmdale *	Pool 4	8 (10)	-118.170731	34.826899	Inland Desert Playa	Yes		0.050	0.950
SAN BERNADINO COUNTY	Dale Dry Lake *		8 (0)	34.129948	-115.708218	Inland Desert Playa	No		0.000	1.000
SAN BERNADINO COUNTY	Melville Dry Lake *		2 (0)	34.45194444	-115.4258333	Inland Desert Playa	No		0.000	1.000
ANZA BORREGO	Clark Dry Lake §		5 (0)	33.304515	-116.246102	Inland Desert Playa	No		0.000	1.000

What Is Dead May Never Die: A Case Study of De-Extinction of Native Alleles via Biotic Homogenization

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Abstract

Driven by landscape alteration and the introduction of non-natives through human activities, biotic homogenization is thought to be a significant threat to the survival of endemic taxa. Specifically, extensive urbanization in southern California, USA, has developed most of the native coastal vernal pool habitat prompting the conservation of native vernal pool species.

The San Diego fairy shrimp, *B. sandiegonensis*, is the only endangered species of the *Branchinecta* genus found in this habitat and since is recognized as the flagship species for this habitat. Initially listed due to habitat/population loss, the genetic integrity of *B. sandiegonensis* is now threatened by hybridization in altered habitats with *B. lindahli* (Simovich et al. 2013).. Numerous road ruts formed through historic vernal pool habitats are dominated by hybrids resembling *B. lindahli.* In this study, we analyzed 1,134 samples from 73 basins containing *B. sandiegonensis, B. lindahli,* and interspecific hybrids using genetic and morphological hybrid indices. Our goals were to characterize and compare observed levels of genetic admixture and patterns of allelic introgression within distinct habitat types across the native range of *B. sandiegonensis.* Contemporary genetic introgression between *B. sandiegonensis* and *B. lindahli* was found to be rare in undisturbed habitats, therefore background levels of hybridization may be a natural part of the evolutionary history of this group rather than a threat. However, higher levels of hybridization are present in nearly all disturbed basins surveyed, suggesting that hybridization can be fueled by habitat disturbance, but habitat alteration does not always dictate a large-scale influx of *B. lindahli.* In extensively disturbed road ruts, a reduction in the number of *B. sandiegonensis* cysts, large-scale introduction of *B. lindahli* cysts, and environmental conditions conducive to *B. lindahli* result in interspecific hybrids that are genetically more similar to *B. lindahli*. This observation also suggests that in these particular basins, *B. lindahli* dominate the cyst bank and the gene pool.

Because only a fraction of both *B. sandiegonensis* and *B. lindahli* dormant cysts hatch with each inundation cycle, the relative proportions of cysts, differences in hatching cues, and the local environmental conditions can all influence subsequent patterns of recruitment. If the habitat remains abiotically similar to undisturbed vernal pools, annual recruitment of *B. sandiegonensis* is predicted to dominate over relatively few alleles intogressing from *B. lindahli*. Conversely, if the habitat has been extensively converted and no longer retains vernal pool functionality, selection for *B. lindahli* (i.e., higher reproductive success) can increase its relatively frequency so that *B. lindahli* alleles comprise the majority of the cyst bank. Anthropogenic activities may increase both dispersal and hybridization to the extent that they threaten the integrity and recovery of endangered species. Using both published genomic and morphological hybrid indices will allow researchers and habitat managers to better categorize the extent of hybridization across habitat types, quantify the relative contribution of natives and non-natives alleles to the local gene pool, and perhaps predict the short-term trajectory of the hybridization process.

Introduction

Biotic homogenization is defined by the mixing of once disparate biota and the replacement of specific native forms by non-native generalist in space and time (McKinney and Lockwood, 1999).

Although instances of biotic homogenization have occurred throughout the paleontological record (Hewitt 1980; Vermeij 1991, Seehausen et al. 2008), recent human activities have accelerated this process by facilitating biotic exchange among allopatric species. Man-made alterations to the environment and human-mediated introductions of exotic species have sparked notable changes in the global distribution of biota with two predominant consequences: extirpation of regional and endemic native species, and the introduction of cosmopolitan, non-native species (Olden et al. 2004). Both outcomes are most evident with the process of urbanization.

Urbanization is one of the leading causes of species extinction in the United States (McKinney 2006) because habitat loss and alteration from urbanization is both drastic and increasingly widespread. Currently, urbanization is more geographically ubiquitous and endangers more species than any other human activity in the United States mainland (Czech et al. 2000; McKinney 2006). In contemporary urban development, large parcels of land are cleared, paved and dramatically modified in ways that can greatly exceed habitat changes that occur from logging, traditional farming and many other historic land uses (Marzluff and Ewing 2001). Furthermore, because cities typically grow by accretion, long-term landscape alterations often intensify with time so that there is no opportunity for successional recovery. Even though these types of landscape disturbances have been shown to alter selection regimes, many habitats classified as "disturbed" could equally be termed "novel". It is this novelty that renders converted habitats vulnerable to invasion (Simberloff 1998; McKinney 2006).

Because human settlements facilitate the movement of non-native species for several reasons, construction and expansion of towns and cities also promote the replacement of native species by non-native (Mack and Lonsdale 2001). This outcome has been documented in birds (Marzluff 2001), mammals (Mackin-Rogalska et al. 1988), and insects (McIntyre 2000). Furthermore, the introduction of non-natives in association with the urbanization also enhances the potential for the breakdown of reproductive isolating mechanisms and intraspecific hybridization resulting in the genetic assimilation of previously differentiated gene pools (Storfer 1999; McKinney 2006).

Human-mediated dispersal has promoted dramatic hybridization events between formerly allopatric species resulting in either adaptive introgression, speciation, or biotic homogenization (Rhymer and Simberloff 1996; Rieseberg et al. 2003; Olden et al 2004; McKinney 2006; Nolte et al. 2009; Schierenbeck and Ellstrand 2009; Abbott et al. 2013; Gompert et al. 2013, Pacheco-Sierra et al. 2016). Selection against hybrids can be influenced by the degree of reproductive isolation in parent taxa, fitness of the hybrid offspring, linkage relationships among genes, and positive selection in the alternate background or environment (Barton 2001; Wu 2001; Payseur 2010; Gompert et al. 2013; Hedrick 2013). Similarly, migration can be influenced by a variety of factors such as spatial distance between species and the difference between migrant and resident population sizes in populations undergoing hybridization (Burgess et al. 2005; Lepais et al. 2009; Supplementary Figure 1). As the products of selection against hybrids and rate of migration of parental taxa into the hybrid zone, genomic clines can be used to characterize the process of interspecific hybridization.

Genomic clines have been extensively used to study spatial patterns of allelic introgression in in birds (Baldassarre et al 2014), reptiles (Gerrick et al. 2014), mammals (Roca et al 2001), and fish (Lamaze et al. 2012). However, patterns of allelic introgression have been less studied in taxa endemic to temporary fluctuating environments that support organisms with extended variable periods of dormancy where genetic variation can be introduced through the mixing of individuals from different reproductive events through a reproductive bet hedging strategy (Ellner and Hairston 1994).

Ephemeral wetlands, such as vernal pools, desert playas, and mountain meadows, exhibit periodic filling and drying; therefore, aquatic obligates survive non-aqueous periods as dormant cysts and develop quickly during inundation periods (Belk and Cole 1975; Wiggins et al. 1980; Brendonck and Persoone 1993; Simovich and Hathaway 1997, Erickson and Belk 1999). Similar to desert annuals (Pake and Venable 1996), many of these taxa exhibit a reproductive bet-hedging strategy whereby only a fraction of the viable cysts hatch in any single hydration event. This strategy allows large numbers of cysts to accumulate in the soil after several years of reproduction (Simovich and Hathaway 1997) and ensures that the population will not go extinct even if one or more filling events result in complete reproductive failure (Hairston 1996 a. b; Brendonck & Riddoch, 2000). The presence of cyst/seed banks not only allows a prolonged generation time for otherwise short-lived organisms, but also results in the mixing of individuals across many generations (Ellner and Hairston 1994; Hairston 1996 a, b).

Because generations are overlapped, genotypes which may have done poorly (or well) in previous years are reintroduced resulting in a constant reshuffling of genotypes with variable past success (Ellner and Hairston 1994; Hairston 1996 a, b; Simovich and Hathaway 1997; Ripley et al. 2004). This mixing of individuals from various mating events also maintains genetic diversity that would otherwise be expected through spatial gene flow, or introgressive hybridization (Bohoank 2005; Kinnison, & Hairston 2007; Hedrick 2013).

Because premature drying events are common to in ephemeral habitats, many species of vernal pool crustaceans have adopted a bet-hedging strategy where its reproductive effort is partitioned over more than one pool hydration event to avoid local extirpation (Simovich and Hathaway 1997). The San Diego fairy shrimp, *Branchinecta sandiegonensis* (Fugate 1993) inhabits vernal pools in southern California and is characterized as a narrow-range endemic. It is the only species normally found inhabiting functional (or near-functional) coastal vernal pools in southern California and Baja California, Mexico (Fugate 1993). Because nearly 95% of its historic habitat has been urbanized, the San Diego fairy shrimp is federally listed as an endangered species and is recognized as the flagship for conservation of native coastal vernal pool habitat (USFWS 1997; Bauder and McMillen 1998; Bauder et al. 2009; Simovich et al. 2013). In contrast, the Versatile fairy shrimp *B. lindahli* is widespread generalist utilizing a large range of temporary aquatic habitats from seasonally arid regions (alkaline vernal pools, prairie potholes, and slightly saline pools and inland playas; Eng et al. 1990; Erikson and Belk 1999; Maeda-Martinez et al. 2002).

Because of its broad tolerance to water chemistry and temperature, it also inhabits some disturbed "edge" habitats (i.e., roadside ditches, railroad bed drains, road ruts) and has been documented cohabitating with 15 other fairy shrimp species from five genera (Eng et al. 1990; Erikson and Belk 1999; Maeda-Martinez et al. 1997; Aguilar et al. 2017).

Habitat alteration associated with urban expansion in Southern California (e.g., construction and vehicular traffic) has unintentionally extirpated *B. sandiegonensis* from the majority of its historical habitat and created highly connected artificial basins across converted remaining vernal pool habitat. This process has also apparently resulted in the movement of *B. lindahli* cysts into coastal areas (Simovich et al. 2013), *B. sandiegonensis* has been found to readily hybridize with *B. lindahli* in these disturbed habitats and also laboratory conditions (Fugate 1998; Simovich et al. 2013). Hybrids can be identified through both morphology (Simovich et al. 2013) and genetic characters (Patel et al. 2017). Because reproductive barriers are apparently not fully developed across these two species, intermediate hybrids may even have the potential to replace distinct species (Keller et al. 2002). Although disturbed habitats are not always associated with large-scale introduction of *B. lindahli*, road ruts and basins found adjacent to (or in the middle of) dirt roads are associated with extensive construction, military and utility activities are frequently dominated by intermediate hybrids cohabitating with genetically non-admixed *B. lindahli* (Simovich et al. 2013; Patel et al. 2017).

This observation suggests that a large-scale introduction of *B. lindahli* (i.e. greater than the number of extant *B. sandiegonensis* cysts and up to 99% of the total number of viable cysts in the cyst bank) has occurred in association with road construction and continual usage by large tracked construction and military vehicles. In addition, because dirt can originate from inland playa habitat (Lovich and Bainbridge 1999) and from other disturbed habitats in southern California, it is plausible that introduction of dirt adhered to tracked construction equipment can also introduce large numbers of *B. lindahli* or hybrid cysts in converted vernal pool habitat. The introduction of dirt from elsewhere, as a part of road construction, also disproportionately converts vernal pool habitat into turbid, less heterogeneous, artificial basins that are likely more favorable towards a generalist such as *B. lindahli*. Additionally, the frequency of local dispersal of *B. lindahli* and hybrid cysts can also increase as a function of human incursion onto neighboring vernal pool habitats (utility and maintenance vehicle traffic; Waterkeyn et al. 2010; Simovich et al. 2013). The outcome of hybridization is determined by selection on each species and the initial proportion of cysts in the cyst bank. Therefore, if the habitat has been extensively converted so that it does not retain functionality, selection for the invader may increase the reproductive success of the invader to the point that it comprises the majority of the cyst bank.

Characterizing the link between habitat disturbance and *B. lindahli* introductions is necessary to determine how *B. lindahli* alleles can dominate gene pools that were once dominated by *B. sandiegonensis* alleles.

Using species-specific SNP markers (Patel et al. 2017), we investigate patterns of interspecific genetic introgression between *B. sandiegonensis* and *B. lindahli* in response to habitat disturbance. We quantify the extent of hybridization by morphologically and genetically analyzing specimens from undisturbed vernal pools, undisturbed inland playas, disturbed vernal pool habitats, and road ruts. We also present a framework to classify populations based on the extent of genetic and morphological hybridization. This framework can serve as a metric to distinguish low level background hybridization (involving low frequencies of *B. lindahli* alleles) from more extensive introgression that threatens species. Our work emphasizes the roles that habitat connectivity and habitat conversion play in the breakdown of species barriers. Finally, it has conservation implications for a wide variety of endemic taxa that inhabit coastal vernal pools in southern California, where *B. sandiegonensis* serves as the flagship species for conservation.

Methods

Field Sampling

Sample collection was carried out over three winter seasons (2013/14 - 2016/17) when environmental conditions were appropriate for hatching. Because much of the habitat has been converted as a part of urban development, we included samples from populations that are currently extinct.

Because our goal was to assess hybridization with respect to landscape alteration in coastal vernal pool habitat, we concentrated sampling efforts on three habitat categories that differed with respect to degree of habitat disturbance and the dominant species found (intact coastal vernal pools, vernal pools with limited disturbance, and road ruts through vernal pool habitat; Supplementary Figure 2). We additionally sampled eight inland playas (Table 1; Supplementary Figure 2C) where *B. lindahli* are the native species, to determine if hybridization extended into inland playas. In total, we sampled 73 basins (i.e. vernal pools, road ruts, or inland playas) clustered into 30 distinct complexes (Figure 1; Table 1).

Prior to DNA extraction, adult females were classified morphologically as pure species or hybrids using methods published in Simovich et al. (2013). Adult males were identified to species by morphology (Erickson and Belk, 1999). Morphological hybrid index scores for individuals sampled in a locality were averaged to obtain an average population score (MHI score; Table 1). Following morphological measurements, heads of adult females, and bodies of adult males were excised for subsequent genetic analysis. Total genomic DNA was extracted and isolated from approximately 10 mg of tissue per sample using the Qiagen DNeasy 250 Blood and Tissue Kit (Cat. No. 69506; Qiagen, Inc.). DNA concentration and purity were estimated using a Pearl Implen Nanophotometer™. Sample batches of 384 were then sent to the University of Arizona Genetic Core (UAGC) facility for genotyping using the Agena Bioscience MassARRAY genotyping platform (Bradić et al. 2011; Patel et al. 2017).

Genomic scores for all individuals in a population were averaged to obtain an average genomic hybrid score (GHI score) for the population (Table 1). A total of 1,134 adult males and females both were genotyped using this marker set.

Classification of Hybridization in Natural Populations

Using Maximum-likelihood and Bayesian methods identified genetically pure and admixed individuals in the dataset. Maximum-likelihood estimation of hybrid indexes was performed using the program ADMIXTURE v.1.23 with K of 2 (Alexander et al. 2009). Bayesian inference of admixture proportions was performed in STRUCTURE v.2.3.4 (Pritchard et al. 2000) using an admixture model with independent allelic frequencies with K of 2, representing the parental species. Ten replicate MCMC runs (50,000 burn-in followed by 1,000,000 Markov chain Monte Carlo (MCMC) iterations for each run) were conducted with model parameter estimation utilizing the admixture model and independent allele frequencies. Output files from multiple runs were combined and analyzed using STRUCTUREHARVESTER (Earl & von-Holdt 2012) and results from the multiple runs were summarized and subsequently combined using CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007).

Individuals were also classified into one of six classifications using a MCMC algorithm performed with an initial 50,000 cycle burn-in followed by 1,000,000 cycle iterations implemented in NEWHYBRIDS (Anderson & Thompson 2002).

With confidence values inferred from posterior probabilities, individuals were classified as either: *B. sandiegonensis*, non-admixed *B. lindahli*, F_1, F_2, backcrosses to *B. sandiegonensis* and backcrosses to *B. lindahli*. In addition, we also calculated interspecific ancestry for each specimen (i.e. the proportion of an individual's genome where one gene copy was inherited from each parental species) using INTROGRESS implemented in R (Fitzpatrick 2012; Gompert & Buerkle 2010; Pacheco-Sierra et al. 2016; R Core Team, 2016; Patel et al. 2017). First, species-specific SNP genotypes were used to identify admixed and non-admixed individuals based on their genotypes (i.e. P1/P1, P1/P2, P2/P2). Subsequently, the hybrid index score (h) was used to classify individuals based on the genotypes of all 20 loci; a mean h score of 0 corresponded to non-admixed *B. sandiegonensis*, h score of 1 corresponded to non-admixed *B. lindahli* individuals, and h score values between 0-1 corresponded to a variety of admixed individuals. Hybrid indexes and interspecific ancestry coefficients based on these allelic classes were calculated and visualized using the est.h and triangle.plot features implemented in INTROGRESS.

Since the relative composition of groups in the effective population influences the future trajectory for each locality we also classified the degree of hybridization among populations throughout the extant range of *B. sandiegonensis* based on 20 neutral genomic loci (GHI score; Patel et al. 2017) and female morphology (MHI score; Simovich et al. 2013) as a surrogate for the entire genome (Figure 2A). Populations were classified as non-admixed *B. sandiegonensis* (GHI score and MHI score = 3.00-2.60), or limited-hybridization (either GHI score and/or MHI score = 2.59-2.40).

Populations of *B. lindahli* were classified as non-admixed (GHI score and MHI score = 1.00-1.40), or limited-hybridization (either GHI score and/or MHI score = 1.41 -1.8). If both metrics were in the range of extensive hybridization (GHI score and MHI score = 1.81-2.19) we assume that maintenance of species boundaries was unlikely.

Patterns of Asymmetrical Introgression in Natural Populations

To characterize patterns of allelic introgression in our system, we employed the genomic.clines feature in INTROGRESS. This feature estimates genomic clines using multinomial regression (Gompert and Buerkle 2009) and quantifies locus specific introgression relative to the genomic background. We investigated deviation from neutral expectation of introgression of neutral alleles in our marker set by comparing observed clines to an expected distribution (i.e., 95% confidence interval) assuming neutral introgression. Expected genomic clines were generated using 10,000 runs of parametric procedure as a function in the genomic.clines feature. We visualized the proportion of individuals with the homozygous *B. sandiegonensis* genotype (*P*) as a function of mean genomic hybrid index score for all 20 clines using the compare.clines command. To characterize variation in clinal patterns we identified where each locus the midpoint of heterozygosity with respect to the average genomic hybrid index score. This was done for the entire dataset and again for each habitat category.

Results

We amplified 20 species-specific SNP loci for 1,134 individuals from 73 different basins throughout coastal southern California and inland playas. Using the genomic hybrid index criteria of Patel et al. (2017), we identified 842 individual *B. sandiegonensis*, 151 individual *B. lindahli,* and 143 intermediate hybrids (Supplementary Figure 4). In undisturbed vernal pools and inland playas, the vast majority of individuals were non-admixed. However, the occasional presence of hybrids suggests that natural hybridization may occur at a low frequency across the entire range, due to the movement of *B. lindahli* by natural vectors, primarily birds (Supplementary Figure 3A, 3B, 3C). Habitat disturbance was associated with an increased frequency of hybridization, but the morphological and genetic composition of hybrids was starkly different between vernal pools with limited disturbance (Figure 2C) and highly disturbed or newly created road ruts (Figure 2E). Road ruts have a high frequency of hybridization and some are dominated by *B. lindahli* backcrosses. This high frequency of hybridization is likely responsible for the variable dissociation between genetic and morphological index scores recorded across different road rut populations.

Classification of Hybridization in Natural Populations

Both the hybrid index implemented in ADMIXTURE and the Bayesian admixture proportions for K = 2 ($_{qi}$) (from STRUCTURE) suggest that most populations sampled are genetically "pure" (Table 1).

Early stage hybrids (e.gs., F_1, F_2) are rare, and hybridization between non-admixed *B. sandiegonensis* and non-admixed *B. lindahli* is not pervasive throughout the range of *B. sandiegonensis* (Supplementary Figure 3A, 3B). The Bayesian analysis implemented in NEWHYBRIDS suggests that most putatively admixed individuals are later-generation hybrids (i.e. *B. lindahli* backcrosses; Supplementary Figure 3C). When interspecific ancestry was plotted against the overall hybrid index, most intermediate hybrids were also identified as backcrosses into *B. lindahli* (Supplementary Figure 4).

Using population morphology and genomic hybrid index scores (Figure 2A), 67 of the 73 basins we surveyed were classified into four categories: undisturbed inland playas, undisturbed coastal vernal pools, disturbed vernal pools exhibiting limited-hybridization, and road ruts exhibiting extensive hybridization (Figure 2A; Table 1). Genetically non-admixed *B. lindahli* comprised the vast majority of inland playa populations resulting in a high congruence between genetic and morphological characters (Figure 2D; Table 1). There was no observable variation in either set of characters in inland playas. The majority of undisturbed vernal pools were classified as non-admixed *B. sandiegonensis* populations and showed a high level of congruence between genomic and morphological index scores (Figure 2B; Table 1). Twenty-nine pools sampled across Marine Core Air Station at Miramar (MCAS Miramar) were all comprised of non-admixed *B. sandiegonensis,* making this the largest local set of non-introgressed populations of *B. sandiegonensis* we surveyed. However, slight morphological variation in highly-functional (undisturbed) vernal pools could signal low levels of past or current hybridization or measurement error in the morphological characters.

Hybridization that was evident in the only two rock pools we surveyed (Irvine Rock and Culp Valley Teneja) cannot be attributed to anthropogenic disturbance, since both are undisturbed and located in rock crevices.

Vernal pool basins with low or moderate levels of disturbance exhibit low levels of hybridization (Figure 2C; Table 1). The observed correlation between the genomic and morphological hybrid score at the population level was strong because these populations were mostly comprised of *B. sandiegonensis* (Figure 2C). This suggests that habitat alteration alone may not significantly promote hybridization or the breakdown of species integrity but can facilitate the introduction of *B. lindahli* from other areas. In stark contrast, congruence between genetics and morphology dissociated markedly in road ruts that have experienced a large-scale introduction of *B. lindahli* cysts (FP populations, Corral B; Figure 2E). Hybrids were more numerous and more genetically and/or morphologically diverse in road ruts compared to all other habitat types. Eight road rut populations were classified as having extensive hybridization, while five road ruts were either *B. lindahli* with limited hybridization, or non-admixed populations of *B. lindahli* (Table 1).

Although most individuals in undisturbed inland habitats were genetically non-admixed *B. lindahli* (N =110; Figure 3A), two late stage hybrids were found in one inland population (Skunk Hollow). In undisturbed vernal pool habitats, the presence of two early stage hybrids (e.g. DMM 315B) and several backcrossed individuals suggests a very low level of background hybridization (Figure 3B). However, most individuals in undisturbed vernal pool basins (N = 803) were non-admixed *B. sandiegonensis*.

In disturbed vernal pools, intermediate hybrids were less common than natives and were mostly congruent with the expected genetic makeup of *B. sandiegonensis* (Figure 3C). Even though three early stage hybrids were found in DMM 256, a total of 40 individuals from disturbed vernal pool habitats were genetically and morphologically identified as *B. sandiegonensis* (Figure 2C, 3D). Conversely, *B. lindahli* backcrosses were common in road ruts (73/173 individuals) (Figure 3C). Backcrosses into *B. lindahli* were more common and diverse in road ruts (Figure 3C), suggesting a large-scale introduction of *B. lindahli* or hybrid cysts at some point in the past.

Patterns of Asymmetrical Introgression in Natural Populations

We evaluated interlocus variation in hybrid clines using cline midpoints (i.e., the mean GHI score corresponding $P = 0.50$; Figure 4A). Because our sampling efforts focused on coastal habitats where *B. sandiegonensis* alleles were more common, the majority of loci cross the $P = 0.50$ threshold at a mean genomic hybrid index greater than 2.0. We found marked heterogeneity in locus-specific patterns of introgression (Figure 4A, 4B). Five loci had cline midpoints less than 2.00 (Figure 4B), suggesting unusually high penetration of *B. lindahli* alleles into *B. sandiegonensis*, for these loci, and perhaps different evolutionary pressures for these loci. Genomic clines differed among habitat categories (Figure 4C). Minor clinal variation was observed in undisturbed inland playas and undisturbed coastal vernal pools. In native *B. lindahli* habitats, clines for 10 loci crossed the $P = 0.50$ midpoint at a mean genomic hybrid score between 1.4 -1.6, and one crossed between 2.0 - 2.2 signifying some clinal variation in native *B. lindahli* habitat.

In undisturbed vernal pools, 18 loci were homozygous and the remaining two crossed the midpoint between 2.4 and 2.8. Disturbed basins showed greater variation in patterns of allelic introgression. Interlocus cline variation in disturbed vernal pools was low; 15/18 variable loci crossed the midpoint when the mean genomic hybrid index score was between 2.4-2.6. Road ruts exhibited considerably more variability than the other habitat categories. Nine locus clines crossed the midpoint at a mean genomic hybrid index score between 2.4-2.6, but the remaining 11 were spread evenly when the mean genomic hybrid index score was between 1.0- 2.0. Furthermore, *B. lindahli* backcrosses and non-admixed *B. lindahli* were the dominant groups in 15/17 road ruts (Table 1), and only two genetically non-admixed *B. sandiegonensis* individuals were found. In summary, repeat bouts of hybridization in each pooling event drive variability in patterns of genomic introgression in established road rut populations. This even spread in locus variability seems to stem from road ruts being individually unique in terms of the relative number of founders (of each species), and selection regimes in the local environment during hatching and growth. However, along with laboratory cultures (to determine the relative hatching fraction of each species) more work focusing on the vegetation availability, temperature fluctuations, and measurement of water chemistry is needed to determine the relative influence of these factors.

Discussion

As a product of increasing urbanization, instances of biotic homogenization are becoming more common. Therefore, predicting the species-specific (or site-specific) consequences of homogenization requires an understanding of how species boundaries are maintained. It is clear that passive dispersal of freshwater crustacean cysts can occur through biotic (e.gs. birds and salamanders; Bohonak and Whitman 1999) and abiotic (e.gs. water and wind; Graham and Wirth 2008; Haliburton and Graham 2017) vectors, and this could lead to low levels of hybridization between differentially adapted allopatric species (Figure 3A, 3B; Supplementary Figure 3A, 3C). However, anthropogenic activities may increase both dispersal and hybridization to the extent that they threaten the integrity and recovery of endangered species (Hitt et al. 2003; Allendorf et al. 2004; Seehausen 2008).

Classifying Hybridization in Natural Populations

Undisturbed Habitats

When adaptive divergence follows reproductive isolation, selection will constrain each incipient species in a separate niche. Furthermore, drift will eventually lead to fixation of different species-specific alleles for selectively neutral genes. Successive bouts of introgressive hybridization in zones of contact can fuel the recombination of distinct genomes, resulting in countless combinations of genotypes and phenotypes (Fitzpatrick and Shaffer 2004; Seehausen 2004, 2008, 2014).

In undisturbed habitats, *B. sandiegonensis* and *B. lindahli* maintain genomic and morphological divergence despite low and infrequent rates of interspecific hybridization in both coastal vernal pools (Figure 2B; Figure 3B) and inland playas (Figure 2D; Figure 3A). Because vernal pool crustaceans serve as a diet staple for transient bird and amphibian species, the transport of cysts across short (and sometimes long) distances can occur as a function of the distance between pools and habitat similarity (Bohonak and Jenkens 2003; Green and Figuerola 2005). For example, the two rock pools that we sampled were not accessible to vehicular traffic; hybridization in these populations is most likely facilitated by the dispersal of non-natives via transient birds that use rock pools as sources of water. *B. sandiegonensis* is not thought to inhabit inland habitats due to its strict tolerance for water chemistry (Gonzalez et al. 1996) however, in may be able to inhabit the Tenaja rock formation since the Teneja does not have the same water chemistry as other desert pools. (i.e. high solute levels found in dry lakes). Over very long periods of time, spatial transfer of cysts by avian and other natural vectors may help to replenish and potentially introduce alleles that may have gone extinct in isolated populations. However, these low levels of connectivity do not seem to compromise species integrity in undisturbed habitats.

Disturbed Vernal Pools

The frequency of *B. lindahli* alleles was greater in populations that have experienced some amount of habitat disturbance.

The nine disturbed vernal pools we surveyed showed a slight breakdown in the association between genetic and morphology, but population means were similar to undisturbed vernal pool populations (Figure 2B, 2C; Figure 3D). Even if habitat disturbance via vehicular traffic reduces *B. sandiegonensis* cysts or introduces *B. lindahli* cysts into these basins, the abiotic habitat should still favor the remaining *B. sandiegonensis* cyst bank if the habitat still resembles native vernal pool habitat. Furthermore, despite *B. lindahli* having a faster development time (7-13 days compared to 10-20 for *B. sandiegonensis*; Erickson and Belk 1999), and potentially being able to disproportionately contribute to the remaining cyst bank, *B. sandiegonensis* populations can recover in optimal inundation periods because of their relatively high fecundity (164-479 cysts compared to *B. lindahli* maximum of 326; Simovich & Hathaway, 1997, Erickson and Belk 1999). Therefore, hybrids become more genetically and morphologically similar to *B. sandiegonensis* (Figure 3D) but beneficial alleles from *B. lindahli* may integrate into the gene pool by "surfing" and subsequently be maintained or increase in frequency due to positive selection.

Road Ruts

Even though road ruts are temporary wetlands and may contain threatened or endangered crustaceans, they rarely support the biologic, hydrologic, or water storage functionality that is expected from intact vernal pools (Bauder et al. 2009).

Furthermore, they present a more complex pattern of hybridization than disturbed vernal pools, which can vary as a function of *B. lindahli* introduction through tracked construction/military vehicles (large-scale), *B. lindahli* introduction via utility/maintenance vehicle traffic (small-scale) or change in the original *B. sandiegonensis* cyst bank size (if any), and the abiotic environment that is created through habitat disturbance.

It is unlikely that large-scale introductions of *B. lindahli* occur naturally into temporary basins in coastal southern California, but multiple introductions of cysts in dirt adhered to vehicular tires and undercarriages can facilitate an overall large-scale introduction of *B. lindahli* into newly formed road ruts. Some road ruts contain a relatively large frequency of *B. sandiegonensis* in the adult population, and therefore while disturbed, have not experienced a significant introduction of *B. lindahli* migrants even if hybrids may be found (e.g. DMM 256 and Ramona Day St.). However, the relative population sizes for *B. sandiegonensis* and *B. lindahli* are likely to change in road ruts after their initial creation as a result of recruitment in successive reproductive events. This is because each road rut is unique in terms of background and human-induced connectivity, cyst bank composition, and local conditions resulting from habitat disturbance (i.e., vegetation community, local temperature fluctuation, water chemistry). Conversely, road ruts in local landscapes that never had vernal pools may only contain *B. lindahli,* likely introduced by vehicular traffic (e.g. CM Pool 2).

Ripley et al. (2004) showed that the maintenance of an existing cyst bank requires successful reproduction event every two or three filling events, therefore small-scale introductions of *B. lindahli* into novel road ruts are likely to result in extinction if they are shallow and have a short hydroperiod, but may establish if they fill sufficiently and frequently

If roads are constructed adjacent to (or through) existing vernal pools, a more complex pattern emerges. The strong genetic pattern found in extensively hybridized populations signals a significant and abrupt change in the number of *B. lindahli* relative to the number of *B. sandiegonensis* after habitat alteration (FP populations; Table). As a result of this transition, an increase in heterospecific mating events results in the production of a variable "swarm" of genetically and morphologically hybrid individuals (sensu Seehausen et al. 2008; Figure 3C). This high proportion of intermediate hybrids is likely responsible for the observed marked dissociation between genetic and morphological characters (Figure 2E). An example of this would be the village rut pool located near the center of MCAS Miramar. Initially formed between the 2016-2017 sampling season by traffic associated with military activity (C. Black pers. obs.), samples were collected in the winter/spring of 2017 and results showed that five of were classified as hybrids and nine individuals being classified as genetically non-admixed *B. lindahli*. Indeed, known instances of *B. lindahli* and intermediate hybrids in vernal pools are associated with inoculum from disturbed populations being added into or adjacent to artificially created basins as part of restoration projects (e.gs. Costa Mesa A and C, located near Fairview Park, and Shepherd's pond, located in Mission Trails Park).

Furthermore, because *B. lindahli* often co-occurs with other fairy shrimp species in anthropogenically disturbed habitats (Eng et al. 1990; Aguilar et al. 2017), it is reasonable to conclude that human activities associated with urbanization may often facilitate its range expansion.

Patterns of Asymmetrical Introgression

In cases of human-mediated range expansion, the frequency of interspecific hybridization will increase as the relative abundances of distinct species become sharply unbalanced (Wirtz 1999; Burgess et al. 2005; Chan et al. 2006; Lepias et al. 2009; Saarman et al. 2015). Despite slight variability among loci, genomic clines generally show introgression of *B. lindahli* alleles into the genome of *B. sandiegonensis* (Figure 4A). Selection for *B. lindahli* alleles in the same loci (for closely linked genes) may occur because road ruts resemble native *B. lindahli* habitats abiotically, or because generalists have a selective advantage over specialists in disturbed/novel habitats (Anderson and Stebbins 1954; Sakai et al. 2001; Simovich et al. 2013). Variation in allelic heterozygosity suggests that recurrent hybridization can maintain *B. sandiegonensis* alleles either because *B. sandiegonensis* alleles potentially confer a selective advantage or *B. lindahli* backcrosses may develop novel genetic combinations that confer equal or greater fitness to non-admixed *B. lindahli* (Figure 3C; Figure 4A, B; Fitzpatrick 2012).

Because cline variation is not uniform across habitat categories, patterns of allelic heterozygosity can be potentially influenced by the differences in local environmental cues that signal *B. sandiegonensis* and *B. lindahli* cysts to hatch, and their relative population sizes in basins. Theoretical models suggest that bet-hedging strategies should follow three predictions. First, the number of individuals in the initial hatching fraction is proportional to the probability of a long and stable inundation period (Brown and Venable 1986; Venable 2007). Second, dormant seeds/cysts must remain viable until favorable conditions return, such as a rain event (Phillipi and Seger 1989). This suggest that in road ruts where there has not been a large-scale introduction of *B. lindahli,* a portion of dormant *B. sandiegonensis* cysts can hatch and reintroduce genetic variation to the effective population for multiple inundation periods. In road ruts that have experienced a large-scale introduction of *B. lindahli* or hybrid cysts, eventual replacement of *B. sandiegonensis* genomic diversity may be inevitable. Because the gene pool in these populations will be mostly comprised of *B. lindahli* alleles, intermediate hybrids will genetically be more similar to *B. lindahli* (Figure 3C, 4C) and locus-specific clinal variation in road ruts may be the signature of this ongoing conversion (Figure 4C). However, the flat distribution of clinal variation in road ruts is due to their individuality in terms of their original species composition, current levels of connectivity (dispersal and gene flow), and and selection during both the hatching and growth phases. More insight into the invasion and slection proceses will require laboratory culture experiments (to determine the relative hatching fraction of each species), and comparative studies of vegetation availability, temperature fluctuations, and water chemistry.

This will help to determine the relative influence of each factor on the success of each species' alleles in recruitment and dominance of the local gene pool.

Third, variation in hatching likelihood among cysts is also due to phenotypic variability among mothers of the same genotype, or even within single clutches (Philippi and Seger 1989; Hairston 1996 a, b; Ripley et al. 2004). Like many migrant populations, the number of cysts and overall genetic diversity of *B. lindahli* in coastal road pools should be initially lower than what is expected in inland playas where *B. lindahli* have historically existed (Sakai et al 2001; Holway et al. 2002; Olden et al 2004; Aguilar et al. 2017). Bet-hedging is thought to maintain genetic diversity in both extant *B. sandiegonensis* and *B. lindahli* populations and potentially introduce alleles previously thought to be extinct. In road ruts that have experienced a large-scale introduction of non-natives, recurrent hybridization, as a result of reproductive bet-hedging from both species, may be beneficial to *B. lindahli* and hybrids. Similar to the colonization of North America by the Caribbean anole *Anolis sagrei* (Kolbe *et al*. 2004, 2007; Seehausen et al. 2008), recurrent hybridization with *B. sandiegonensis* from different mating events may also introduce genetic diversity that increases the adaptive potential of *B. lindahli* and hybrids to persist in road ruts by exploiting underused or open niches.

Conservation Implications

While habitat loss is most frequently the reason species become vulnerable of endangered. Hybridization can then be a secondary threat to remaining populations. Hybridization presents one of the most challenging problems for protecting and managing species under the Endangered Species Act because it can cause the extinction of distinct genetic, phenotypic, and/or evolutionary units (Taylor et al. 2006; Doremus 2010; Keller 2014; Bohling 2015). Vernal pools represent taxonomical hotspots for many species of endemic crustaceans, all of which may potentially be adversely affected by habitat alteration (King et al. 2008). Since its initial discovery, *B. sandiegonensis* has been designated as a flagship species for vernal pool habitats, and has become an "umbrella" for the protection of a variety of taxonomically rich, co-occurring groups found in coastal vernal pool habitat (Bauder 1996; Bauder & McMillan 1998, King et al. 1998, Erikson & Belk 1999 Ripley and Simovich). Habitat disturbance does not always result in the introduction of non-natives or increased frequency of hybridization, but it may lead to a significant reduction of historic cyst banks and/or local extirpation of *B. sandiegonensis* populations by the destruction of historically intact cyst banks. However, road ruts represent an extreme type of disturbance, and do not resemble native coastal vernal pools in terms of the diversity and ecological roles of native species. Undisturbed vernal pools in coastal southern California have a high biodiversity with respect to crustaceans (with at least 6 indicator species and 10 or more total species).

In pools that are highly disturbed, crustacean diversity declines to 10-20 percent of the historic diversity and *B. sandiegonensis* is replaced by *B. lindahli* (Ripley and Simovich, 2008; Bauder et al., 2009). By using both morphology and genomic hybrid indices, researchers and habitat managers should be able to obtain a relatively holistic perspective for any particular basin. This not only helps to identify populations where a large-scale introduction of *B. lindahli* has occurred, but also to perhaps predict the trajectory of species and hybrid distributions.

With respect to maintaining natural biodiversity, this study provides three considerations for the conservation of *B. sandiegonensis*. First, as urbanization continues, the extent of hybridization is likely to also continue. Hybridizing populations are likely to increase as a function of the continued introduction of *B. lindahli* as a result of construction and vehicular traffic. Short-distance dispersal will also become more frequent as increased vehicular traffic enable recurrent movement of cysts among adjoining road ruts. In this study, most admixed basins were formed as the result of habitat alteration by significant construction activities and/or vehicular traffic. Limiting vehicular incursion into functional vernal pool landscapes should limit the introduction of non-natives and hybrid spread. In addition, using dirt only from genetically and morphologically non-admixed pools for pool creation and restoration efforts will limit the change of hybrid spread and unintentional formation of admixed vernal pools.

Second, future hatching rate and/or reproductive output may not be equal between species because *B. sandiegonensis* and *B. lindahli* are adapted to different selection regimes and life histories.

Because *B. sandiegonensis* is adapted to the premature drying events commonly experienced by coastal vernal pools, only a small portion of cysts will hatch during a given inundation event. Conversely, because *B. lindahli* is a generalist inhabiting a wide variety of habitat types and tolerating a variety of environmental conditions, *B. lindahli* are likely to hatch more readily (compared to *B. sandiegonensis*) during any particular filling event as part of the evolutionary strategy of a generalist species, however it is likely that a portion of encysted embryos will remain dormant. Combined with a faster development rate and the ability to reproductively bet-hedge, *B. lindahli* seems poised to rapidly colonize road ruts, disproportionately transform the cyst bank, and persist even in sub-optimal inundation events. If future patterns of rainfall become sporadic, water temperatures become hotter (i.e. summer rainfall), and the frequency of successive premature drying events increases, climate change may promote the replacement of *B. sandiegonensis* by *B. lindahli* and hybrids in marginal habitats in coastal southern California. An aggressive strategy that limits construction/vehicular traffic adjacent to undisturbed vernal pools and eradicates the most compromised basins may be best to limit the overall spread of hybridization because it is difficult to keep *B. lindahli* from disproportionately contributing to the cyst bank and to convert road ruts into functional vernal pool habitat.

Although we surveyed 73 basins across 30 complexes, additional coastal vernal pool habitat remains to be genetically surveyed throughout the region. We hope that our results drive investigation of hybridization in basins that were not sampled to better understand the overall link between urbanization and invasive hybridization.

With the help of continued efforts to protect habitat, limit the large-scale introduction of non-natives, and short distance dispersal of hybrids, *B. sandiegonensis* may be able to persist indefinitely.

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Chapter 2 Table 1. Data summary table for each locality including: locality organizational structure, latitude and longitude, population morphological score, and genomic score with corresponding numbers of individuals (n) and Population classification.

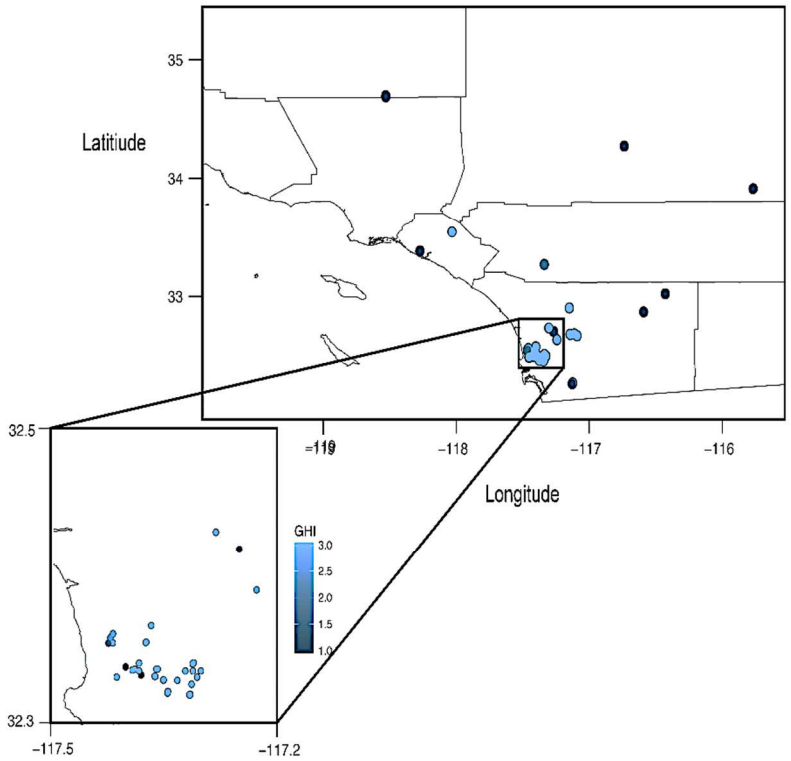
Sites	Complex	Pool	Longitude	Latitude	MHI score (n)	GHI score (n)	Classification Category
Anza Borrego	Clark Dry Lake	CDL	-116.30028	33.34222	1.000 (3)	1.000 (3)	Non-admixed *B. lindahli*
Anza Borrego	Culp Valley	Tenaja	-116.44472	33.20583	1.000 (2)	1.013 (23)	Non-admixed *B. lindahli*
Carmel Mountain	Carmel Mountain	39	-117.27556	33.10889	2.244 (3)	1.022 (3)	Extensive hybridization
Carmel Mountain	Carmel Mountain	Football Pool	-117.27944	33.07806	2.741 (33)	2.974 (33)	Non-admixed *B. sandiegonensis*
Carmel Mountain	Carmel Mountain	Pool 2	-117.23917	33.17750	1.592 (3)	1.104 (3)	*B. lindahli* with limited introgression
Del Mar Mesa	Bowtie	A	-117.31500	33.12639	2.830 (18)	3.000 (18)	Non-admixed *B. sandiegonensis*
Del Mar Mesa	Del Mar Mesa	55	-117.30113	33.06167	2.859 (17)	3.000 (17)	Non-admixed B. sandiegonensis
Del Mar Mesa	Del Mar Mesa	167	-117.39472	33.17139	1.132 (5)	2.566 (4)	Extensive hybridization
Del Mar Mesa	Del Mar Mesa	256	-117.35639	33.18111	2.683 (25)	2.833 (25)	Non-admixed *B. sandiegonensis*
Del Mar Mesa	Del Mar Mesa	315B	-117.29889	33.13139	2.514 (6)	1.567 (6)	Extensive hybridization
Del Mar Mesa	Del Mar Mesa	53	-117.15250	32.93530	2.20 (5)	1.050 (5)	Extensive hybridization
Los Angeles County	Palmdale	Pool 1	-118.17056	34.82417	1.000 (9)	1.002 (19)	Non-admixed *B. lindahli*
Los Angeles County	Palmdale	Pool 2	-118.17094	34.83131	1.000 (13)	1.006 (17)	Non-admixed *B. lindahli*

Los Angeles County	Palmdale	Pool 3	-118.17087	34.83315	1.015 (20)	1.000 (20)	Non-admixed *B. lindahli*
Los Angeles County	Palmdale	Pool 4	-118.17073	34.82690	1.000 (9)	1.000 (18)	Non-admixed *B. lindahli*
Mira Mesa	Brown Parcel	A	-117.17259	32.91982	2.769 (5)	3.000 (12)	Non-admixed *B. sandiegonensis*
Mira Mesa	Brown Parcel	B	-117.27639	32.95917	NA	3.000 (4)	Non-admixed *B. sandiegonensis*
Mira Mesa	Pueblo	Pueblo	-117.12606	32.88377	1.000 (1)	1.069 (3)	Non-admixed *B. lindahli*
Mission Trails	Mission Trails	Pool 1	-117.18222	32.88806	2.805 (6)	3.000 (10)	Non-admixed *B. sandiegonensis*
Mission Trails	Mission Trails	Shepherd's Pond	-117.04833	33.06028	1.806 (17)	1.166 (17)	Extensive hybridization
Nobel	Nobel Dr.	2	-117.21210	32.86719	2.807 (12)	3.000 (18)	Non-admixed *B. sandiegonensis*
Nobel	Nobel Dr.	3	-117.21210	32.86719	2.803 (58)	2.996 (58)	Non-admixed *B. sandiegonensis*
Orange Co	Fairview	A	-117.94189	38.66334	NA	1.241 (15)	NA
Orange Co	Fairview	C	-117.94281	33.66090	NA	1.050 (11)	NA
Orange Co	field pool	field pool	-117.94186	33.66172	NA	1.000 (3)	NA
Orange Co	Irvine Rock	Rock pool	-117.72780	33.80478	NA	2.937 (16)	NA
Otay Mesa	Proctor Valley	12	-116.98028	32.80750	2.858 (22)	2.977 (22)	Non-admixed *B. sandiegonensis*
Otay Mesa	Proctor Valley	17	-116.98056	32.80222	2.666 (19)	3.000 (19)	Non-admixed *B. sandiegonensis*
Otay Mesa	Proctor Valley	Corral Pool (B Side)	-117.03167	32.72361	1.872 (12)	1.092 (18)	Extensive hybridization
Poway	Poway	Poway	-117.02539	32.99840	2.733 (3)	2.888 (9)	Non-admixed *B. sandiegonensis*
Ramona	Grasslands	8	-116.94806	33.07806	2.601 (9)	3.000 (17)	Non-admixed *B. sandiegonensis*

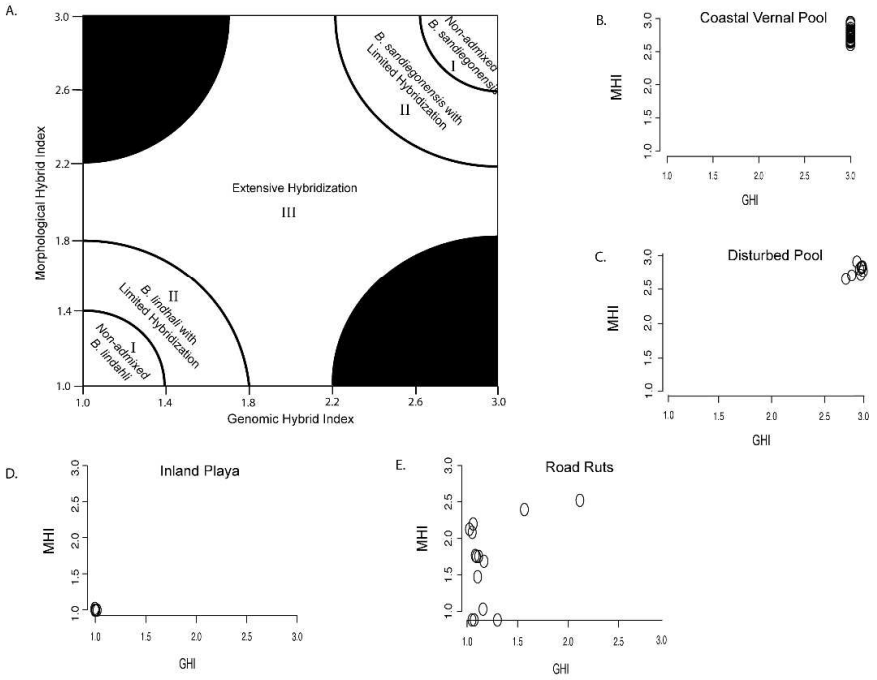
Ramona	Grasslands	17B/H1	-116.91039	33.03163	2.858 (12)	3.000 (17)	Non-admixed *B. sandiegonensis*
Ramona	Grasslands	Corner of Airport	-116.94694	33.07833	2.809 (15)	3.000 (15)	Non-admixed *B. sandiegonensis*
Ramona	Grasslands	Rangeland	-116.97861	33.07944	2.939 (11)	3.000 (11)	Non-admixed *B. sandiegonensis*
Ramona	Ramona	55 (Ramona Day)	-117.07972	33.08639	2.866 (39)	2.989 (39)	Non-admixed *B. sandiegonensis*
Ramona	Ramona	Main St/Kalbaugh St	-116.89017	33.02712	2.805 (19)	3.000 (19)	Non-admixed *B. sandiegonensis*
Ramona	Town	Main St/Hunter St	-116.94250	33.23556	2.856 (20)	3.000 (20)	Non-admixed *B. sandiegonensis*
Riverside County	Skunk hollow	Skunk hollow	-117.11000	33.56000	NA	1.754 (3)	NA
Twenty-Nine Palms	Dale lake	Dale lake	-117.07972	33.08639	1.000 (8)	1.000 (8)	Non-admixed *B. lindahli*
Twenty-Nine Palms	Melville lake	Melville lake	-116.57465	34.45498	1.000 (2)	1.000 (2)	Non-admixed *B. lindahli*
MCAS	AA1	1	-117.11087	32.88722	2.800 (19)	3.000 (19)	Non-admixed *B. sandiegonensis*
MCAS	AA1	2	-117.10928	32.88862	2.799 (19)	3.000 (19)	Non-admixed *B. sandiegonensis*
MCAS	AA10	70	-117.09977	32.87638	2.808 (17)	3.000 (17)	Non-admixed *B. sandiegonensis*
MCAS	AA4-7	Cobble Pool 1	-117.11457	32.84005	2.635 (18)	3.000 (18)	Non-admixed *B. sandiegonensis*
MCAS	AA4-7	Cobble Pool 2	-117.11505	32.84071	2.739 (19)	3.000 (19)	Non-admixed *B. sandiegonensis*
MCAS	AA4-7	Road Rut (New)	-117.11375	32.84111	2.770 (8)	3.000 (8)	Non-admixed *B. sandiegonensis*
MCAS	AA9	139	-117.11033	32.87630	2.760 (35)	3.000 (35)	Non-admixed *B. sandiegonensis*

MCAS	AA9	C3	-117.11033	32.87630	2.784 (8)	3.000 (8)	Non-admixed *B. sandiegonensis*
MCAS	Camp Elliot	Footing 2	-117.14980	32.86281	2.916 (19)	3.000 (19)	Non-admixed *B. sandiegonensis*
MCAS	Camp Elliot	Rifle Range	-117.14938	32.86287	2.648 (18)	3.000 (18)	Non-admixed *B. sandiegonensis*
MCAS	Camp Elliot	Village	-117.14386	32.84535	1.885 (14)	1.08 (15)	Extensive hybridization
MCAS	Eastgate	1	-117.19115	32.87760	2.814 (34)	3.000 (34)	Non-admixed *B. sandiegonensis*
MCAS	Eastgate	2	-117.19308	32.87748	2.750 (20)	3.000 (20)	Non-admixed *B. sandiegonensis*
MCAS	EE1	1	-117.14980	32.86281	2.709 (18)	3.000 (18)	Non-admixed *B. sandiegonensis*
MCAS	EE1	2	-117.14938	32.86287	2.721 (30)	3.000 (30)	Non-admixed *B. sandiegonensis*
MCAS	EE1-2	3	-117.13119	32.86226	2.652 (20)	3.000 (20)	Non-admixed *B. sandiegonensis*
MCAS	FF1/2 (Flightline)	2	-117.12048	32.87621	2.733 (18)	3.000 (18)	Non-admixed *B. sandiegonensis*
MCAS	FP (568)	1	-117.17948	32.87035	NA	1.121 (10)	NA
MCAS	FP (569)	1	-117.17948	32.87035	1.150 (2)	1.157 (8)	Non-admixed *B. lindahli*
MCAS	FP (570)	1	-117.17948	32.87035	1.000 (1)	1.300 (7)	Non-admixed *B. lindahli*
MCAS	FP (702)	1	-117.17948	32.87035	2.659 (8)	3.000 (20)	Non-admixed *B. sandiegonensis*
MCAS	FP (703)	1	-117.17948	32.87035	1.000 (5)	1.048 (13)	Non-admixed *B. lindahli*
MCAS	FP1 (Railroad)	1	-117.17948	32.87035	2.316 (5)	1.060 (5)	Extensive hybridization
MCAS	FP1	3	-117.17948	32.87035	2.640 (13)	2.117 (13)	Extensive hybridization
MCAS	FP1 (Nursery)	2	-117.17948	32.87035	1.871 (21)	1.114 (21)	Extensive hybridization

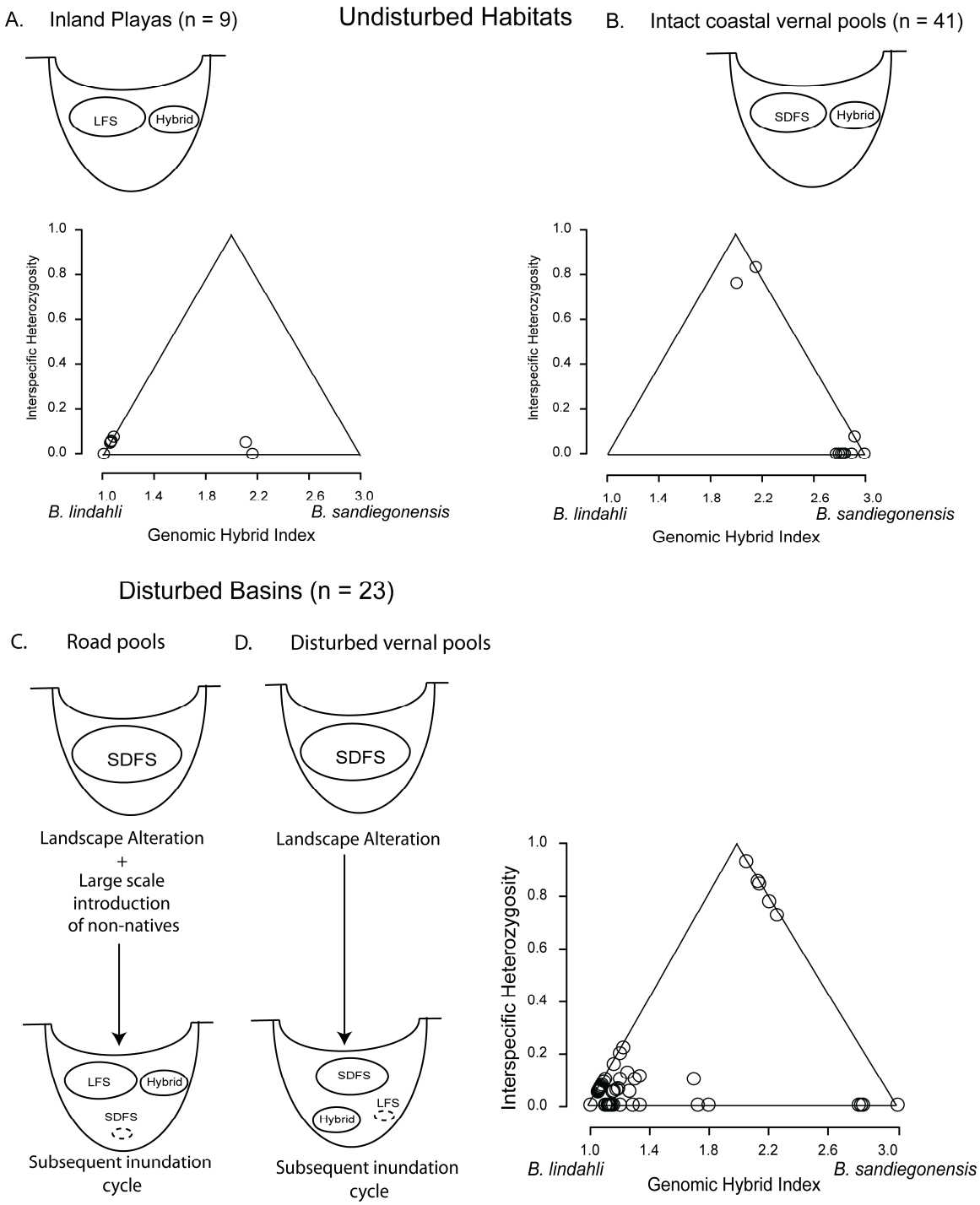
MCAS	HH1	1	-117.16057	32.86829	2.832 (6)	2.962 (6)	Non-admixed *B. sandiegonensis*
MCAS	HH1	2	-117.16141	32.86852	2.916 (4)	3.000 (4)	Non-admixed *B. sandiegonensis*
MCAS	Landmark	2	-117.12048	32.87621	2.805 (3)	3.000 (3)	Non-admixed *B. sandiegonensis*
MCAS	Landmark	3	-117.14354	32.84530	2.679 (17)	3.000 (17)	Non-admixed *B. sandiegonensis*
MCAS	Landmark	4	-117.14386	32.84535	2.745 (12)	3.000 (12)	Non-admixed *B. sandiegonensis*
MCAS	X1-4	3	-117.18227	32.87656	2.850 (35)	2.984 (35)	Non-admixed *B. sandiegonensis*
MCAS	Z1-3	1	-117.15724	32.87913	2.950 (20)	3.000 (20)	Non-admixed *B. sandiegonensis*
MCAS	Z1-3	4	-117.15912	32.87929	2.794 (13)	3.000 (13)	Non-admixed *B. sandiegonensis*



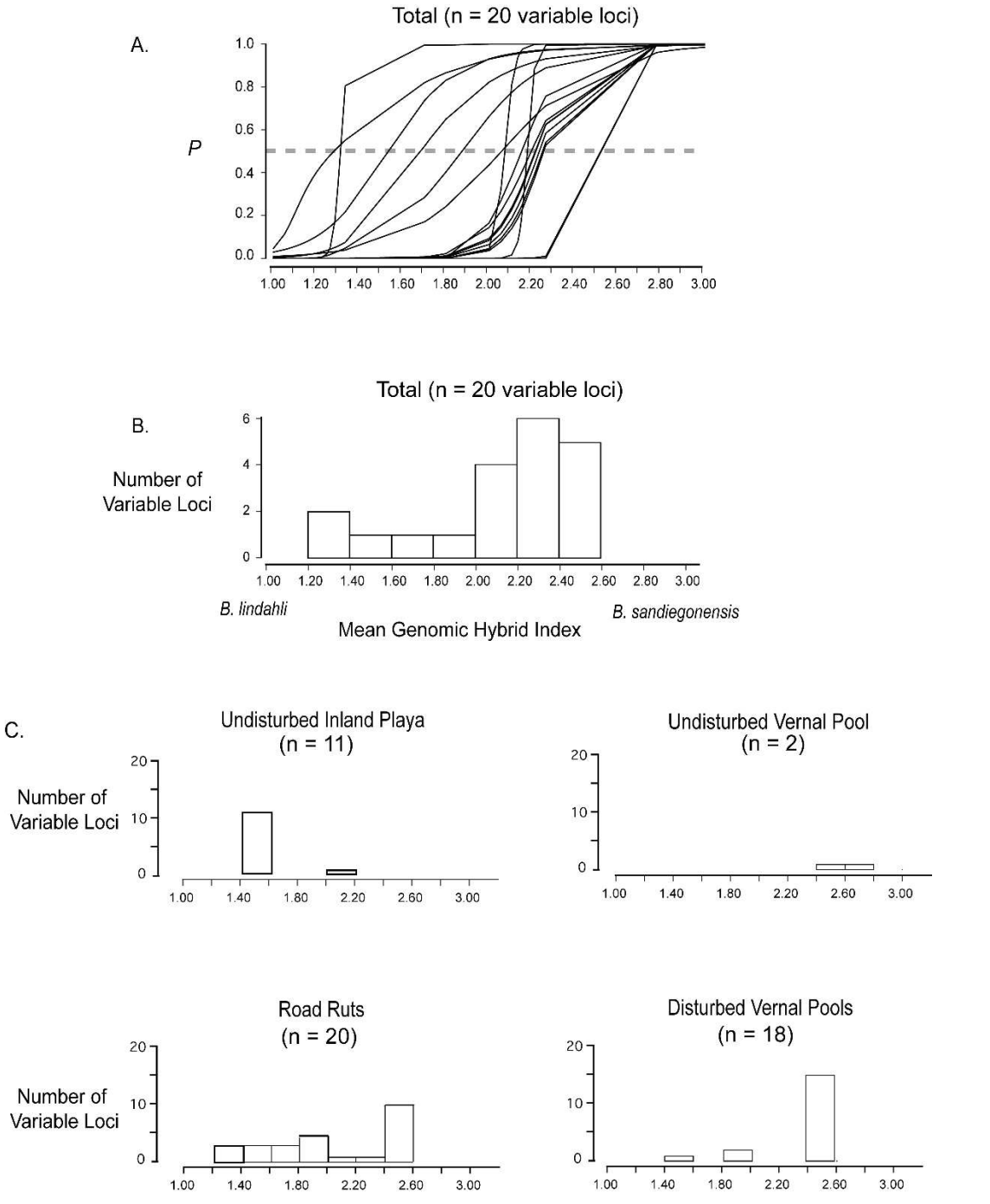
Chapter 2 Figure 1. Sampled basins (n = 73) colored by genomic hybrid index score for each locality. Scores range from 1(non-admixed *B. lindahli*; black) to 3 (non-admixed *B. sandiegonensis*; light blue).



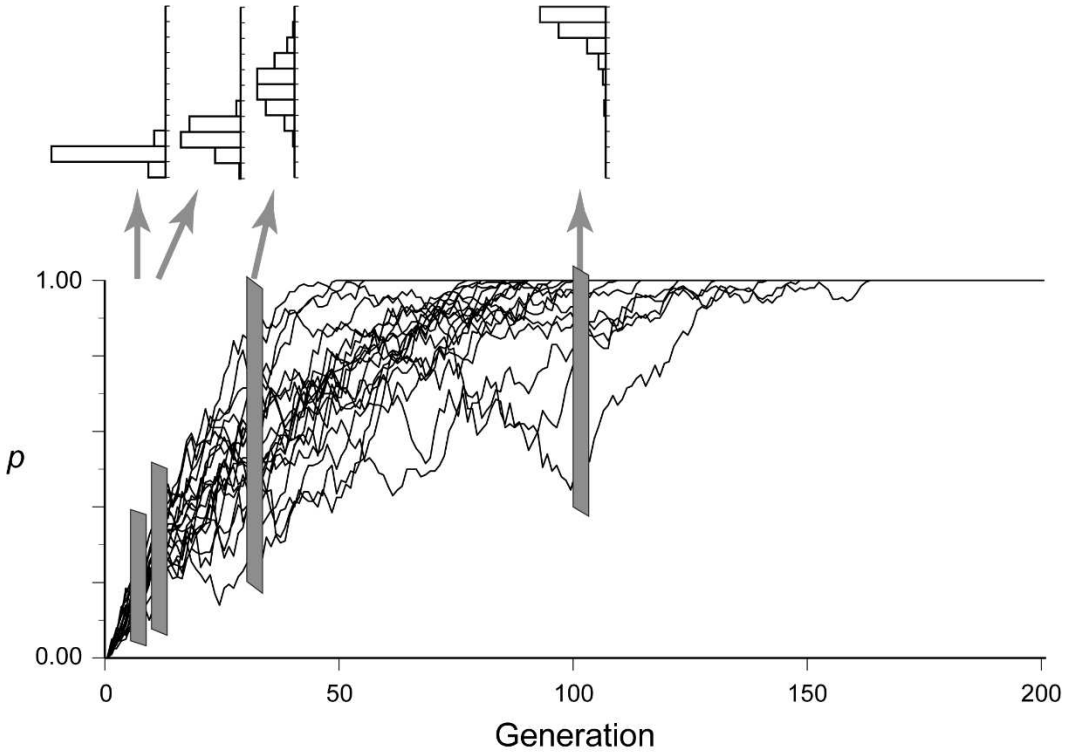
Chapter 2 Figure 2. A. Diagram showing classification of populations based on levels of hybridization inferred by genomic (x-axis) and morphological (y-axis) scores with each point representing the mean scores for a basin (population). Ranges for both indices (Genomic Hybrid Index score: Morphological Hybrid Index score) span from 1:1 (*B. lindahli*) to 3:3 (*B sandiegonensis*). Range of scores for non-admixed species (I) are 1-1.4 (*B. lindahli*) and 2.6-3.0 (*B. sandiegonensis*). Ranges for limited hybridization (II) are 1.41-1.8 (*B. lindahli*) and 2.2-2.59 (*B. sandiegonensis*). Ranges for extensive hybridization (III) are 1.81-2.19 for either species. Regions in black (top-left and bottom-right) were not observed in our study. B. Genomic and morphological hybrid index scores for undisturbed vernal pool habitat, where *B. sandiegonensis* is the native species. C. Genomic and morphological hybrid index scores for disturbed vernal pool habitat. D. Genomic and morphological hybrid index scores for undisturbed inland playa habitat, where *B. lindahli* is the native species. E. Genomic and morphological hybrid index scores for road ruts in coastal vernal pool habitat. Genomic and morphological congruence breaks down significantly in these road ruts, and *B. lindahli* makes up a larger proportion of the population even though *B. sandiegonensis* is the native species.


Undisturbed Habitats
A. Inland Playas (n = 9)
B. Intact coastal vernal pools (n = 41)
LFS
Hybrid
SDFS
Hybrid
Interspecific Heterozygosity
1.0
0.8
0.6
0.4
0.2
0.0
1.0 1.4 1.8 2.2 2.6 3.0
B. lindahli
B. sandiegonensis
Genomic Hybrid Index
Disturbed Basins (n = 23)
C. Road pools
D. Disturbed vernal pools
SDFS
SDFS
Landscape Alteration
+
Large scale
introduction
of non-natives
Landscape Alteration
LFS
Hybrid
SDFS
SDFS
LFS
Hybrid
Subsequent inundation
cycle
Subsequent inundation
cycle
Interspecific Heterozygosity
1.0
0.8
0.6
0.4
0.2
0.0
1.0 1.4 1.8 2.2 2.6 3.0
B. lindahli
B. sandiegonensis
Genomic Hybrid Index

Chapter 2 Figure 3. A. Species makeup of an inland playa and the likely contribution of the predominant species *B. lindahli* to the cyst bank compared to non-natives and hybrids. B. Species makeup of an inland playa and the likely contribution of the predominant species *B. sandiegonensis* to the cyst bank compared to non-natives and hybrids. Both triangle plots depict data collected in this study showing genomic uniformity of most individuals found in intact vernal pool habitat and low levels of interspecific hybridization indicated by points not at either end of the triangle base. Habitat disturbance in coastal vernal pools can occur with (C) or without (D) a large-scale introduction of *B. lindahli* into converted basins. The creation of road ruts not only requires destruction of existing cyst banks but also the introduction of dirt used in road construction. Because fill dirt from inland areas can harbor *B. lindahli* cysts, road construction can facilitate in large-scale introduction of non-natives. C. In road ruts *B. lindahli* makes up the greatest proportion with intermediate hybrids showing varying degrees of genetic assimilation. However, since road construction may not eradicate native cyst completely, B. sandiegonensis and hybrids may re-occur as a part of their reproductive bet hedging strategy. In disturbed vernal pools (D), habitat disturbance is not associated with a large-scale introduction of non-natives therefore *B. sandiegonensis* dominates makes up the greatest proportion. However, the existing cyst bank is reduced therefore non-natives and hybrids can disproportionately contribute to the breeding population and make up a greater proportion of the cyst bank.

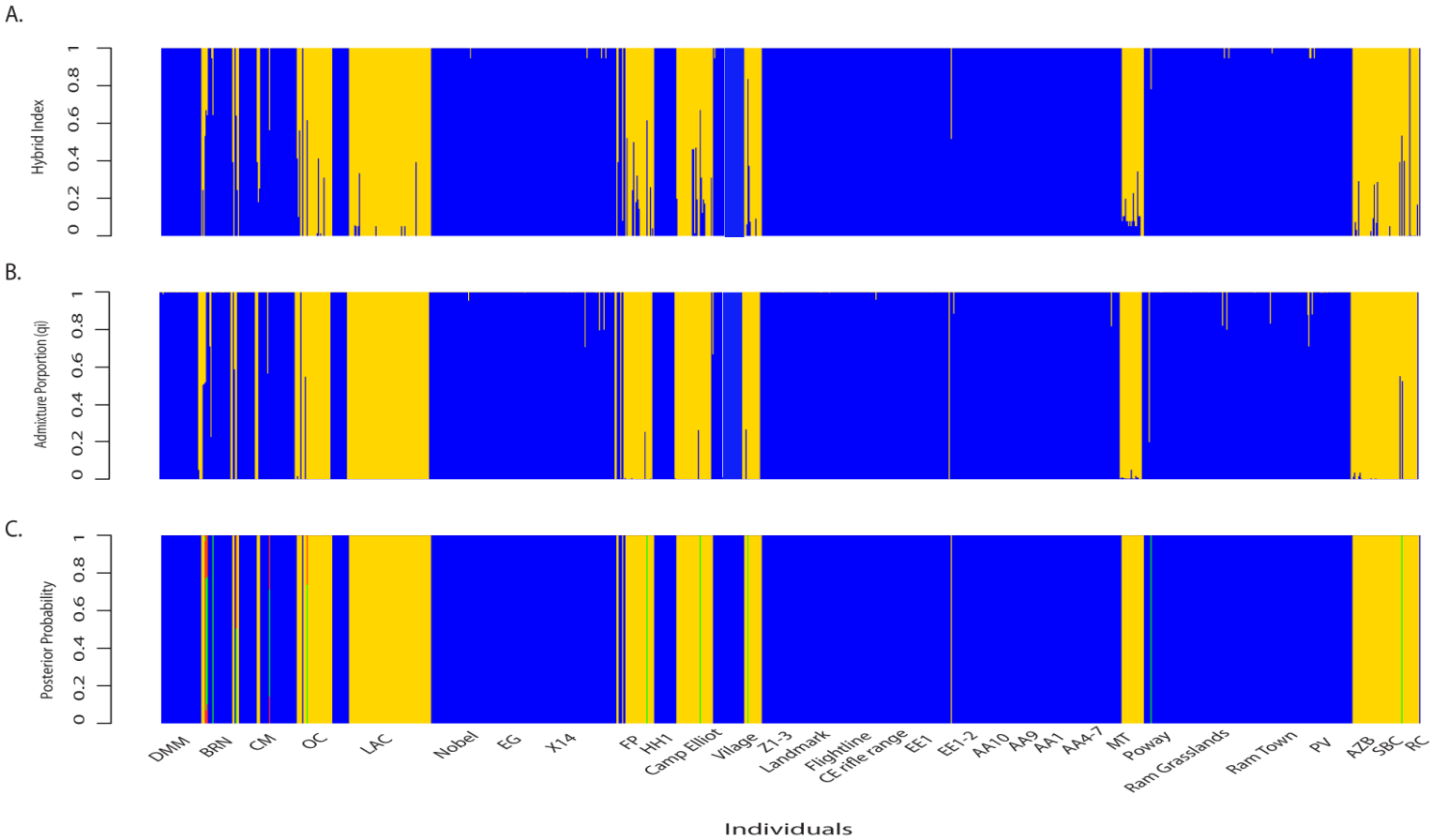


Chapter 2 Figure 4. A. *P* (Proportion of individuals with the homozygous *B. sandiegonensis* genotype) as a function of mean genomic hybrid index score, for all 20 loci. B. Distribution of cline midpoints (where *P* = 0.50), for all 20 loci. C. Distribution of cline midpoints by habitat type, showing only the number of variable loci within each type.

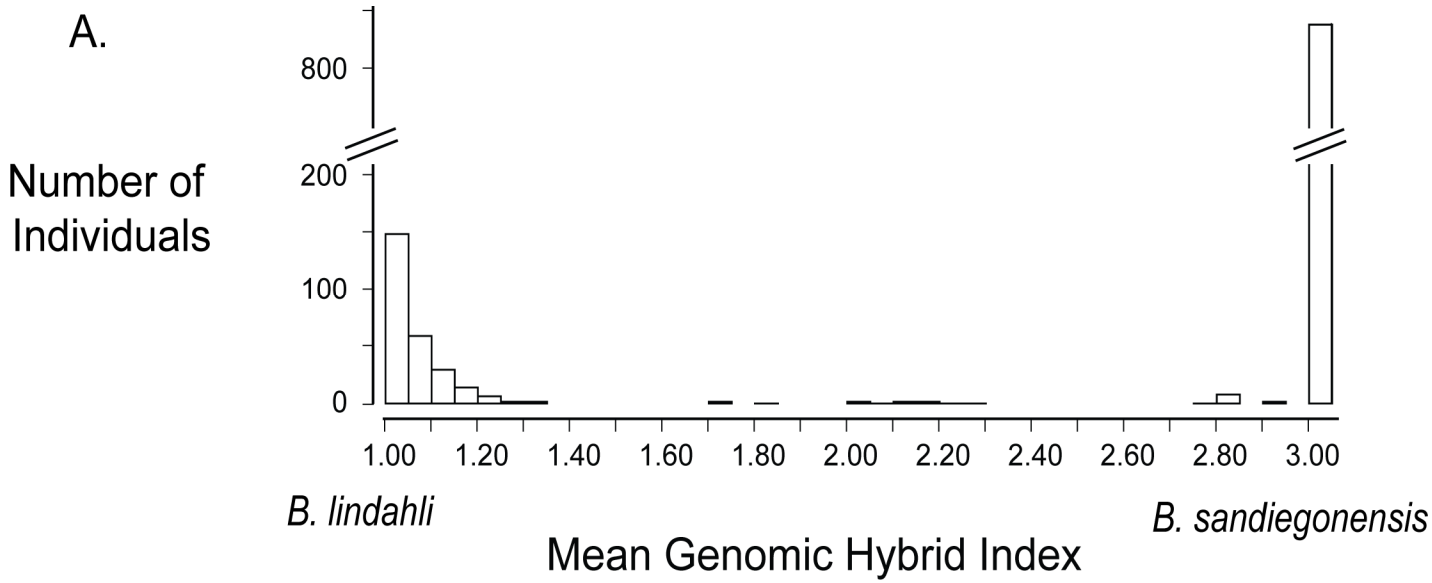


Chapter 2 Supplementary Figure 1. Simulated genetic introgression of species A ($p = 1$) into species B ($p_{t=0} = 0$) under neutrality ($N_A = 100$, $m = 3\%$). Twenty representative loci are shown; histograms represent the distribution of 100 loci. Variance among loci is generally unimodal, except in the earliest and latest stages. Simulations conducted in RedLynx (Cartwright 2009).

Chapter 2 Supplementary Figure 2. Pool types classified in this study: A. Intact coastal vernal pool (as reviewed in Ericson and Belk, 1999), B. Coastal vernal pool with limited disturbance; this pool still maintains habitat heterogeneity and supports native biodiversity, C. artificial basin similar to inland playas (highly-connected road ruts), D. Inland playas (as reviewed in Erickson and Belk, 1999).



Chapter 2 Supplementary Figure 3. Genetic admixture of 1,134 individuals sampled across 30 complexes. (A) Admixture (Maximum Likelihood) hybrid index bar chart of; *B sandiegonensis* (blue), *B. lindahli* (yellow). (B) Bayesian admixture proportion plot bar chart; *B sandiegonensis* (blue), *B. lindahli* (yellow). (C) New Hybrids posterior probability bar chart with *B. sandiegonensis* (blue), *B. lindahli* (yellow) F1 (red) F2 (green) backcross *B. sandiegonensis* (orange) backcross *B. lindahli* (purple).



Chapter 2 Supplementary Figure 4. Number of individuals with respect to mean genomic hybrid score for non-admixed *B. lindahli* (n = 151), non-admixed *B. sandiegonensis* (n = 840) and interspecific hybrids (n = 142); the majority of hybrids are found with backgrounds associated with *B. lindahli*. Individuals were classified as non-admixed *B. lindahli* if all 20 loci were homozygous for *B. lindahli* alleles. Individuals were classified as non-admixed *B. sandiegonensis* if all 20 loci were homozygous for *B. sandiegonensis* alleles. Individuals were classified as hybrids if any locus showed heterozygosity or if not all 20 loci were homozygous for *B. lindahli* or *B. sandiegonensis* allele.

A Review of Morphological Characters for Identifying Hybrids between *B. sandiegonensis* and *B. lindahli*

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Abstract

We validated the morphological hybrid index (MHI) described by Simovich *et al.* (2013) for detecting hybrids between the endangered San Diego fairy shrimp *Branchinecta sandiegonensis* (Fugate, 1993) and the versatile fairy shrimp *B. lindahli* (Packard, 1883) through morphological and genetic analysis of 662 individuals. This index uses species-specific character states for "spines" (dorsolateral projections) on thoracic segments 3–11 in adult females. In non-admixed (non-hybrid) populations, the character states for segment 8 correlate poorly with the remaining segments, and the

overall morphological hybrid index score (MHI score).

Only 22% of non-admixed *B. sandiegonensis* display the expected character state for segment 8 compared to nearly all *B. lindahli*. Eliminating this character in principal component analyses increases the proportion of variation explained by PC1 from 57.3% to 69.5%. The revised MHI, however, still shows slight variation in populations of *B. sandiegonensis* and *B. lindahli* that have no genetic evidence of admixture. Hybrid populations possess considerably more variation in MHI score, and this variation is heavily skewed towards *B. lindahli* phenotypes. We suggest using the revised MHI and genetic characters in future experimental field studies to improve an understanding of the processes that initiate and maintain hybridization in this system.

Introduction

The San Diego fairy shrimp *Branchinecta sandiegonensis* (Fugate, 1993) was listed as a species of concern in 1997 (U.S. Fish and Wildlife Service, 1997) due to habitat destruction. Only approximately 10% of its original estimated habitat, coastal vernal pools, remains intact (Bauder & McMillan, 1998). Despite existing only in a fraction of its historic distribution, the San Diego fairy shrimp is the typical *Branchinecta* species found in coastal vernal pools in southern California, presumably due to its intolerance of more alkaline inland waters (Gonzalez *et al.,* 1996). Fugate (1993), Erickson & Belk (1999), and Simovich *et al.* (2013) have nevertheless documented *B. sandiegonensis* cohabiting with versatile fairy shrimp *Branchinecta lindahli* (Packard, 1883) in some disturbed pools.

The versatile fairy shrimp is a geographically widespread generalist that is known for its ability to tolerate a wide range of pool conditions (Eng *et al.,* 1990; Aguilar *et al.,* 2017). These species can be differentiated by male secondary antennae (Fugate, 1993; Erikson & Belk, 1999) and spine morphology on female thoracic segments (Rodgers, 2002). Genetic analyses using allozymes (Fugate, 1992) and mitochondrial sequence data (Bohonak, 2005; Vandergast *et al.,* 2009) have also shown that *B. sandiegonensis* and *B. lindahli* are genetically distinct species. More recent work has shown that *B. sandiegonensis* can hybridize with *B. lindahli* in laboratory conditions and in nature (Erickson & Belk, 1999; Simovich *et al.,* 2013; C. Shanney, R. Clark & A. J. Bohonak unpublished data). Therefore, the ability to accurately identify hybrids is paramount to study interspecific hybridization between *B. lindahli* and *B. sandiegonensis* and to the conservation and recovery of the endemic and endangered *B. sandiegonensis.*

Simovich *et al.* (2013) published a morphological hybrid index that distinguishes adult female *B. sandiegonensis*, *B. lindahli*, and putative hybrids based on the arrangement of spines displayed on each thoracic segment (Fig. 1). This morphological hybrid index provides a cost-effective method to identify hybrids in natural populations and has also served as a framework to develop a genomic hybrid index (GHI) (Patel *et al.*, 2017). The correlation between both indices dissociated in disturbed localities was characterized by significant genetic admixture when morphological hybrid index scores were compared to genomic hybrid index scores in 24 localities (Patel *et al.,* 2017).

This finding suggests that abiotic disturbances or introgressive hybridization could contribute to variation in spine morphology of genetically non-admixed and admixed individuals, respectively. Conflict between morphological and genomic hybrid indices motivates a re-examination of the morphological hybrid index of Simovich *et al.* (2013) in terms of both intraspecies and interspecies morphological variation. We analyzed the robustness of spine morphology on each thoracic segment as a metric for morphologically detecting hybrids, using individuals of known genomic ancestry.

Methods

A subset of the total ($N = 663$) 561 genetically non-admixed *Branchinecta* females - were used to test the robustness of spine morphology on thoracic segments as metrics to distinguish *B. sandiegonensis* from *B. lindahli* (Supplementary material Table S1). Adult females were scored morphologically as described in Simovich *et al.* (2013). The body of each specimen was rotated so that spine morphology could be observed on multiple sides. Spine morphology on each of the nine segments was characterized based on Simovich *et al.* (2013), and scores for each thoracic segment were averaged to generate a composite morphological hybrid score (MHI score) for each specimen (Fig. 2). Individuals were classified morphologically as *B. lindahli* (MHI score =1–1.39), admixed (MHI score = 1.4–2.59), and non-admixed *B. sandiegonensis* (MHI score = 2.6–3.0) based on morphological score.

We quantified the correlation between thoracic segment scores with the MHI score of individuals using pairwise Pearson Product Moment correlations (Flegel *et al.,* 1999) implemented in R (R Core Team, 2013). We found in preliminary analyses that segment 8 had the lowest correlation with the MHIs as well as with all other segments (Table 1). To further quantify the departure of segment 8 from correlations with all other segments and the MHI score, we performed principal components analyses (PCAs) using 561 individuals from 44 populations with no evidence of genetic admixture using the genomic hybrid index developed by Patel *et al.* (2017). The genomic hybrid index is comprised of 20 single nucleotide polymorphism (SNP) loci that display fixed-allelic differences between *B. sandiegonensis* and *B. lindahli* (e.g. 'T/T' in *B. sandiegonensis*, 'C/C' in *B. lindahli*, and 'T/C' in F_1 hybrids). Non-admixed populations should therefore display genomic hybrid index scores (GHI score) fixed for either *B. lindahli* (e.g. GHI score = 1) or *B. sandiegonensis* (GHI score = 3). We analyzed this dataset of non-admixed populations using data from all nine segments, and again after excluding segment 8. Principal component (PC) scores (Jackson, 1993), implemented in R (R Core Team, 2013), were compared to quantify the influence of data from segment 8 on interspecific morphological variation.

We finally analyzed morphology in a more inclusive dataset of 662 individuals that also included 12 admixed populations (Supplementary material Tables S1, S2, and S3). Segment 8 was not included in the MHI scores for this analysis.

Results and Discussion

Genetically "pure" *B. sandiegonensis* (GHI score = 3) showed the most variation at segment 8, with only 22% of individuals showing expected character states (i.e. two dorsolateral spines). In addition, only half of the individuals displayed typical character states (i.e. two dorsolateral spines) for segment 9 (Table 3). There was little or no variation in any segment in non-admixed populations of *B. lindahli* (Table 3). Indeed, illustrations of *B. sandiegonensis* in its original description showed spines on segment 8 that were much smaller than those on segments 5–7 (Fugate, 1993). This difference in size was nevertheless not included in the schematic diagrams of Erickson & Belk (1999). Consistent with initial characterization of *B. sandiegonensis* by Fugate (1993), we believe that segment 8 represents a transitional point in morphology between dual spines or fused spines in segments 3–7, and no spines in segments 9–11. We found that segment 8 in non-admixed *B. sandiegonensis* can display double spines (22% of individuals), single spines (38%), or a combination of double, single, or no spines on opposing sides (17%) (Fig. 1).

Correlations among individual thoracic segments and the MHIs ranged from 0.66–0.95 when segment 8 was included and 0.65–0.96 when segment 8 data was excluded (Table 1). When segment 8 was removed, the correlation strength increased for all segments except 9, which decreased slightly from 0.66 to 0.65. More importantly, the proportion of variation explained by PC1 increased from 57.3% to 69.5% when segment 8 was removed.

Given our findings, we excluded data from thoracic segment 8 in all subsequent analyses. We found no *B. lindahli* from genetically "pure' populations with MHI scores above 1.3 but 21 of 528 (4.0%) *B. sandiegonensis* from genetically pure populations had MHI scores below 2.6. Even after excluding segment 8, slight morphological variation thus exists in populations of *B. sandiegonensis* with little or no genetic admixture (Table 3; Fig. 3). The range of morphological variation in these 40 populations is limited to 2.375–3, with nearly all individuals scoring between 2.8–3 (Supplementary material Table S1). We nevertheless found that in non-admixed *B. lindahli* populations ($N = 4$) all but two individuals, had MHI scores of 1 (Supplementary material Table S2).

The 12 genetically admixed populations we surveyed exhibited considerable variation in morphology (Supplementary material Table S3). Patel *et al.* (2017) found that interspecific hybrids appear to persist beyond the F_1 generation, with most individuals genetically similar to *B. lindahli* (Patel *et al.,* 2017). Morphological variation shows similar patterns; in admixed populations, MHI scores range from 1 to 3 with 28/44 genetically admixed individuals (64%) scoring as typical *B. lindahli* (Fig. 3, Supplementary material Table S1). This skewed distribution in MHI score can be due to at least four causes. The first cause could be attributed to sampling bias, which is unlikely because admixed localities were geographically disparate. Moreover, specimens were haphazardly sampled in all localities so that discriminating individuals based on spine morphology was minimized. The second cause can be attributed to assortative mating based on spine patterns, could also disproportionately produce offspring that resemble *B.*

lindahli; C. Shanney, R. Clark & A. J. Bohonak (unpublished data) have shown that *B. lindahli* will readily mate with *B. sandiegonensis* and most intermediate hybrids found in extensively admixed localities resemble *B. lindahli*. A third cause could be that, admixed pools may have been founded with a higher frequency of *B. lindahli* than *B. sandiegonensis*. This is quite possible because genetically non-admixed individuals of *B. lindahli* were present in half of admixed localities sampled and are commonly found in disturbed habitats elsewhere (Aguilar *et al.,* 2017). Admixed individuals from surveyed localities likely experienced introgressive hybridization with genetically "pure" *B. lindahli* (Seehausen, 2004). A fourth cause could be that natural selection in hybrid pools could also favor individuals with higher *B. lindahli* genomic content, which is also possible because admixed individuals were sampled from severely disturbed localities (e.g. vehicular road ruts and artificial deep impoundments) resembling abiotic conditions similar to inland playas (Simovich *et al.,* 2013). Furthermore, because *B. lindahli* can tolerate a wide variety of environmental conditions, admixed individuals that are genetically and phenotypically more similar to *B. lindahli* than to *B. sandiegonensis* may be more fit because disturbed habitats resemble inland playas more than coastal vernal pools (Simovich *et al.,* 2013).

Because of their cost efficacy and ability to serve as a metric to assess overall genetic makeup, morphological characters are commonly used to detect admixed individuals and identify hybrid populations in nature. These characters are most useful when their state variation shows consistency within each species and complete divergence among species.

Because spine morphology on thoracic segment 8 correlates poorly with all other segments and genetic determination of the species, we advise that the data from thoracic segment 8 be excluded from the morphological hybrid index of Simovich *et al.* (2013). We also find that a low frequency of morphological variation present in genetically non-admixed populations, due to very low levels of background hybridization or to inherent character variation. Populations with high levels of hybridization tend to resemble *B. lindahli* more than in *B. sandiegonensis*, possibly due to founder effects and greater fitness in disturbed localities.We suggest that an improved understanding of the processes that initiate and maintain hybridization in this system will require the use of this revised female morphological hybrid index to initially detect hybridization and subsequent genetic hybrid index to characterize hybridization in natural populations if present.

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Chapter 3 Table 1. Pairwise correlations matrix comparing correlation strength for each thoracic segment as well as composite morphological hybrid index values including segment 8 (MHI score +8) and excluding segment 8 (MHI score -8).

Pearson Product-Moment Correlation

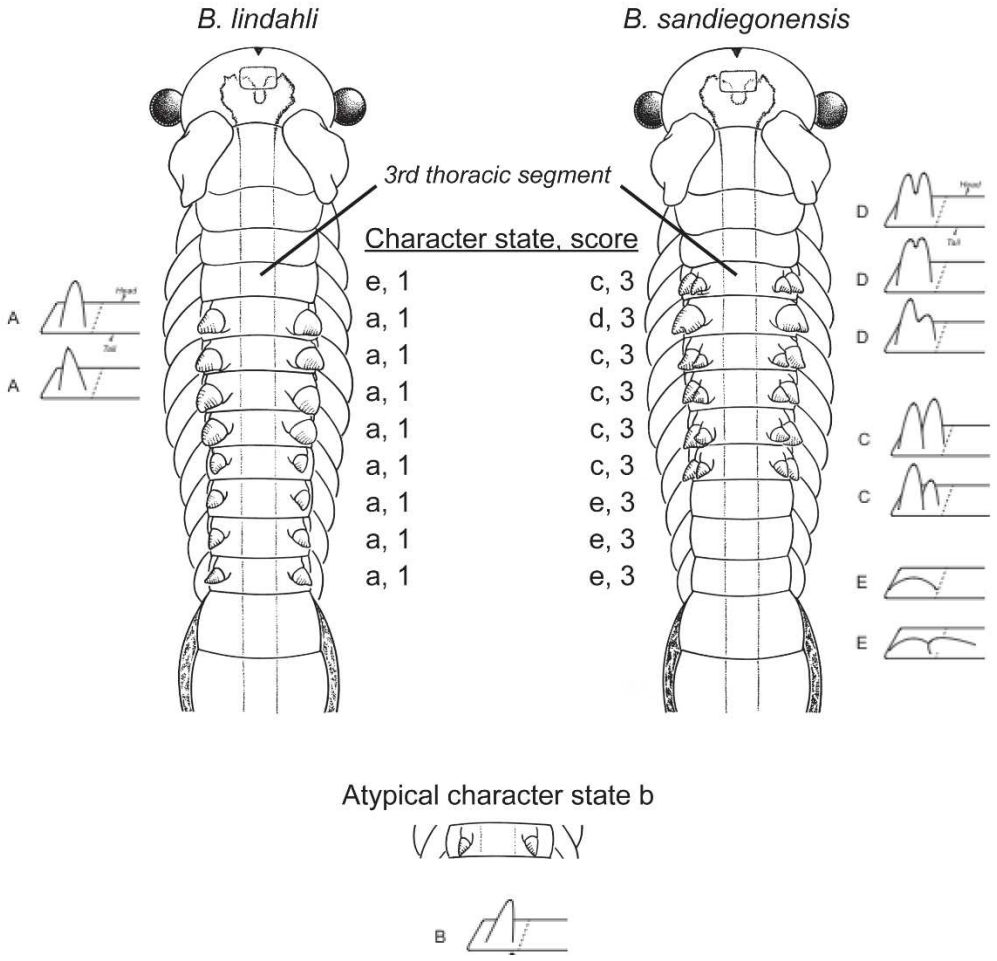
Thoracic segments	T3	T4	T5	T6	T7	T8	T9	T10	T11	MHI score + 8
AT4	0.895	1.000								
AT5	0.884	0.974	1.000							
AT6	0.856	0.941	0.938	1.000						
AT7	0.816	0.890	0.912	0.931	1.000					
AT8	0.276	0.284	0.298	0.322	0.335	1.000				
AT9	0.420	0.495	0.512	0.483	0.473	0.278	1.000			
AT10	0.712	0.786	0.809	0.785	0.760	0.168	0.586	1.000		
AT11	0.765	0.826	0.850	0.828	0.795	0.242	0.510	0.880	1.000	
MHI score +8	0.874	0.937	0.949	0.937	0.916	0.454	0.658	0.864	0.890	1.000
MHI score -8	0.884	0.950	0.961	0.944	0.919		0.654	0.893	0.907	0.988

Chapter 3 Table 2. The first and second principal component scores for non-admixed: "pure" populations of both species with all segment data, and excluding segment 8.

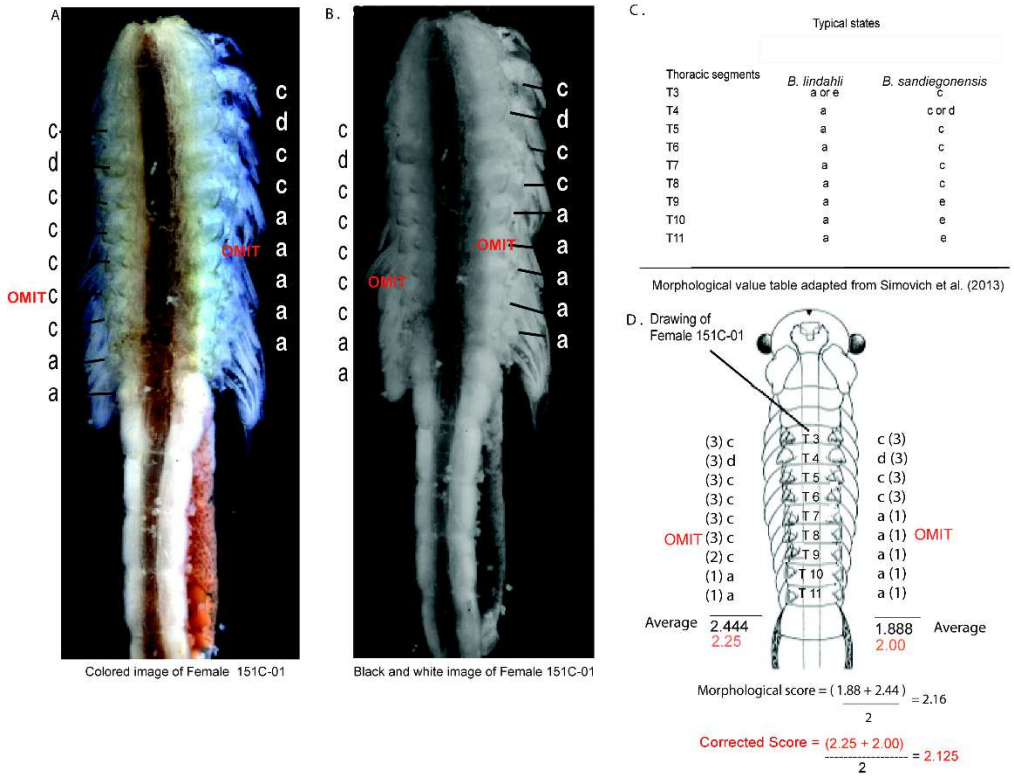
	All segment data		No segment 8 data	
Thoracic Segments	PC1	PC2	PC1	PC2
T3	-0.338	-0.116	-0.347	-0.215
T4	-0.339	-0.105	-0.348	-0.138
T5	-0.339	-0.099	-0.347	-0.132
T6	-0.346	-0.077	-0.353	-0.155
T7	-0.352	-0.062	-0.357	-0.170
T8	-0.213	0.923		
T9	-0.347	0.220	-0.347	0.922
T10	-0.358	-0.192	-0.374	0.020
T11	-0.344	-0.139	-0.355	-0.122
Proportion of variance	0.573	0.192	0.695	0.182

Chapter 3 Table 3. For non-admixed populations, the percentage of *B. sandiegonensis* and *B. lindahli* individuals that displayed expected character states. For admixed populations, the percentage of individuals displaying typical *B. sandiegonensis*, typical *B. lindahli* or atypical scores.

Thoracic Segment	*B. sandiegonensis* (n=528)	*B. lindahli* (n=33)	Admixed (n = 46)		
			B. *sandiegonensis*	*B. lindahli*	Atypical
3	88.5%	100%	28.6%	56.1%	15.3%
4	97.9%	100%	41.8%	50.0%	8.2%
5	98.1%	100%	42.9%	51.0%	6.1%
6	98.1%	100%	38.8%	52.0%	9.2%
7	96.2%	100%	38.8%	54.1%	7.1%
8	22.1%	100%	14.3%	73.5%	12.2%
9	50.2%	100%	20.4%	61.2%	18.4%
10	92.8%	97%	22.4%	68.4%	9.2%
11	96.2%	97%	22.4%	68.4%	9.2%



Chapter 3 Figure 1. This figure depicts the thoracic segment variations found across 667 adult female shrimp. Character D shows the most variation. Characters A, C, and E show the same number of variations. These variations can be the result of a variety of factors including pool effects such as nutrition quality and duration of inundation.



Chapter 3 Figure 2. Complete workflow describing the process of assigning morphological scores to adult females. Figure 2 A shows a high contrast image of an adult female shrimp using a Cannon EOS 5D single-lens reflex camera (SRL) mounted on a Visionary Digital BK + imaging system. A series of images were stitched together into a composite and edited using Adobe Photoshop CS3 to visualize the left and right thoracic segments. Figure 4B shows the same image in black and white to better visualize spines on the left side Numbers in black show the original scoring guidelines including segment 8. Corrected scores omit segment 8 from the process and the corrected score is sown in red. Figure 4 C shows the conversion table found in Simovich et al. 2013. Figure 4 D depicts a sketch of the female morphology, character assignment and corrected (red) and uncorrected (black) composite scores.

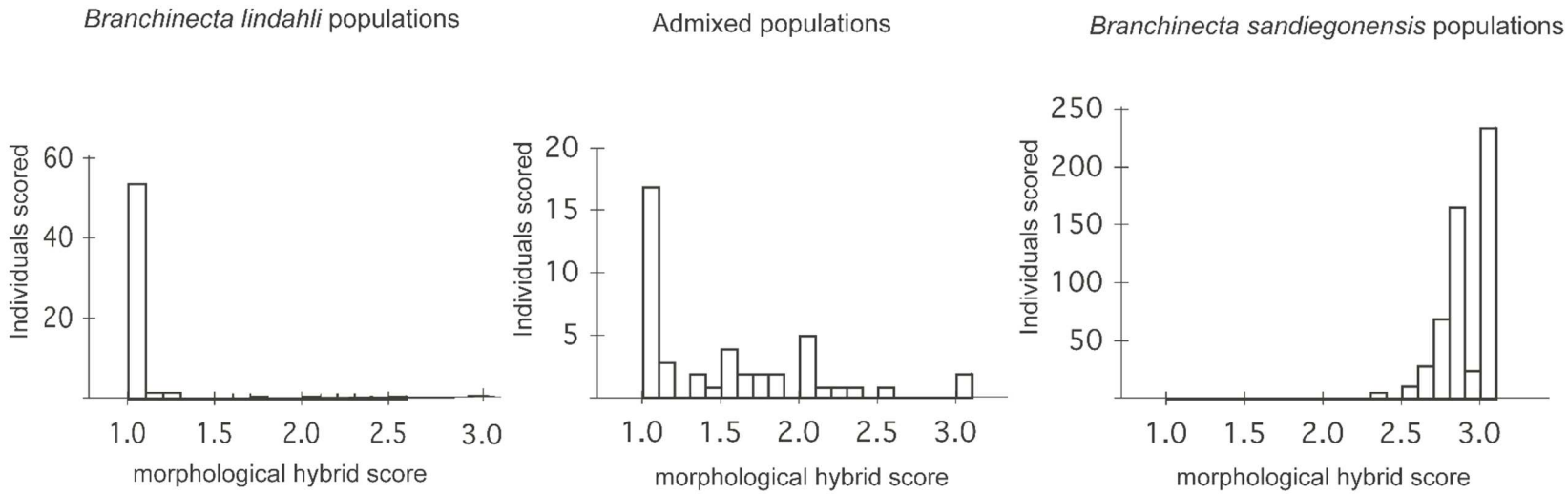


Figure 3. Histograms depicting the range in morphology found in non-admixed populations of *B. lindahli*, admixed, and non-admixed *B. sandiegonensis*, excluding segment 8. Values for non-admixed *B.lindahli* center around 1 with very little skew and the the highest score around 1.25. Morphological variation in admixed populations show that most speciments scores are 1 with a signeificant number of individuals scoring between 1 and 2.5, scores of 3 can also occur but are rare. Morphological varaition in non-admixed *B. sandiegonensis* pools are limited to 2.3- 3 with the majority being 2.8-3. Individulas can score less than 2.6 but never below 2.

Chapter 3 Supplementary Table 1. Non-admixed *B. sandiegonensis* localities. Name, genomic hybrid score (GHI) number of individuals sampled, morphological score (MHIs) with segment 8 data and without segment 8 data, and for each locality sampled in this study.

Localities	Average GHI Score	Individuals Sampled	Average MHI Score Without Segment 8	Average MHI Score With Segment 8
MCAS (7472)	3.000	17	2.893	2.797
MCAS (5248)	3.000	8	2.867	2.771
MCAS (5265)	3.000	19	2.898	2.749
MCAS (5289)	3.000	18	2.865	2.688
MCAS (7269)	3.000	19	2.862	2.754
MCAS (7269)	3.000	8	2.891	2.764
Bowtie A	3.000	18	2.969	2.840
Brown A	3.000	5	2.875	2.800
Brown B	3.000	4	2.984	2.778
MCAS (509)	3.000	18	2.826	2.654
Carmel Mountain Football	3.000	16	2.762	2.660
Del Mar Mesa 255	3.000	16	2.965	2.872
Del Mar Mesa 256	3.000	14	2.848	2.722
MCAS (5188)	3.000	15	2.900	2.722
MCAS (5152)	3.000	20	2.944	2.750
MCAS (1482)	3.000	18	2.906	2.716
MCAS (1421)	3.000	19	2.796	2.658
MCAS (1157)	3.000	20	2.841	2.644
MCAS (3310)	3.000	19	2.829	2.734
MCAS (702)	3.000	8	2.648	2.646
MCAS (2136)	3.000	4	2.938	2.917
MCAS (4654)	3.000	3	2.917	2.852
MCAS (5766)	3.000	17	2.809	2.673
MCAS (5670)	3.000	12	2.906	2.745
Mission Trails Pool 1	3.000	7	2.964	2.817
Nobel 2	3.000	12	2.974	2.806
Nobel 3	3.000	19	2.789	2.693
Poway	3.000	3	2.875	2.741
Proctor Valley Pool 12	3.000	10	2.788	2.778
Proctor Valley Pool 17	3.000	17	2.710	2.693

Ramona Day Street	3.000	19	2.947	2.836
Ramona Grasslands 17b/H1	3.000	13	2.990	2.902
Ramona Grasslands 8	3.000	9	2.764	2.623
Ramona Grasslands Airport	3.000	15	2.992	2.841
Ramona Grasslands Rangeland	3.000	11	2.977	2.944
Ramona Main/Hunter	3.000	20	2.925	2.900
MCAS (3615)	3.000	5	2.950	2.756
MCAS (4693)	3.000	20	2.994	2.972
MCAS (4874)	3.000	16	2.930	2.802

Chapter 3 Supplementary Table 2. Non-admixed *B. lindahli* localities. Name, genomic hybrid score (GHI) number of individuals sampled, morphological score (MHIs) with segment 8 data and without segment 8 data, and for each locality sampled in this study.

Localities	Average GHI Score	Individuals Sampled	AVERAGE MHI Score Without Segment 8	AVERAGE MHI Score With Segment 8
Clark Dry Lake	1.000	2	1.000	1.000
Culp Valley	1.000	2	1.000	1.000
Palmdale Pool 3	1.000	20	1.019	1.017
Palmdale Pool 4	1.000	9	1.000	1.000

Chapter 3 Supplementary Table 3. Admixed localities. Name, genomic hybrid score (GHI) number of individuals sampled, morphological score (MHIs) with segment 8 data and without segment 8 data, and for each locality sampled in this study.

Localities (Genetic Determination)	Average GHI Score	Individuals Sampled	Average MHI score Without Segment 8	Average MHI score With Segment 8
Carmel Mountain Pool 2				
Admixed	1.157	2	1.750	1.667
B. lindahli	1.000	1	1.625	1.556
MCAS (706)				
Admixed	1.133	2	2.063	2.000
B. lindahli	1.000	8	1.650	1.600
MCAS (569)				
Admixed	1.090	2	1.188	1.167
MCAS (570)				
Admixed	1.053	1	1.000	1.000
MCAS (703)	1.044	5	1.025	1.022
Admixed	1.074	3	1.042	1.037
B. lindahli	1.000	2	1.000	1.000
Palmdale Pool 1	1.006	9	1.000	1.000
Admixed	1.050	1	1.000	1.000
B. lindahli	1.000	8	1.000	1.000
Palmdale Pool 2	1.009	13	1.000	1.000
Admixed	1.056	2	1.000	1.000
B. lindahli	1.000	11	1.000	1.000
Proctor Valley Corral B				
Admixed	1.090	6	1.479	1.426
Pueblo				
Admixed	1.056	1	1.000	1.000
Ramona Main/Kalbaugh	2.996	20	2.978	2.869
Admixed	2.923	1	3.000	2.889
B. sandiegonensis	3.000	19	2.977	2.868
Shepherd's Pond				
Admixed	1.167	17	1.489	1.484
Village Pool	1.091	14	1.902	1.881
Admixed	1.213	6	1.875	1.870
B. lindahli	1.000	8	1.922	1.889

Conclusion

Vernal pool ecosystems host tremendous biodiversity in both plant and animal assemblages. In addition to providing resources for a variety of taxa, a significant number of species inhabiting these ecosystems have geographically restricted ranges. Species found in temporary wetlands exhibit unique adaptations and strategies to avoid local extinction due to characteristic variability in patterns of seasonal rainfall, fluctuations in temperature, and duration of pool inundation. Crustaceans including fairy shrimp within the genus *Branchinecta* employ a diversified reproductive bet-hedging strategy, whereby only a portion of dormant encysted embryos hatch during a pool-filling event. This strategy safeguards local extinction and contributes to a growing population of dormant cysts after establishment. Therefore, genetic diversity in vernal pool systems is maintained through the migration of individuals from different generations, as well as spatially separated gene pools. Spatial gene flow in this system can occur with the passive dispersal of encysted embryos through biotic vectors (i.e., birds or amphibians) or abiotic forces (wind or water movement). Even though successful dispersal in space may be limited to a small number of migrants, interspecific hybridization is quite plausible.

Recently, anthropogenic activities such as vehicular traffic, road constriction and military activities have been hypothesized to unintentionally disperse encysted embryos in mud adhered to in vehicle tires, and undercarriages.

The rate of human-mediated cyst dispersal is magnified with the growth of urbanized areas. In southern California, growth of urban areas has resulted in the loss of 95-97 % of historical coastal vernal pool habitat as well as the creation of countless "artificial" temporary basins (i.e., road ruts, ditches, cattle ponds) through frequent vehicular incursion on native vernal pool habitat. In disturbed basins, landscape homogenization results in a pulverized soil layer, a simplified vegetation community, and altered hydrology. Furthermore, habitat disturbance facilitates the cohabitation of disparate biota, which may engender novel competition scenarios and/or promote biotic homogenization at the genetic and taxonomic levels.

Since its first documentation (Simovich et al., 2013), interspecific hybridization between *B. sandiegonensis* and *B. lindahli* has raised many questions about the process of hybridization in *Branchinecta*, and concerns regarding the genetic integrity of the San Diego fairy shrimp. This dissertation investigates interspecific hybridization using revised morphological markers and novel genomic markers. The morphological hybrid index developed by Simovich et al. (2013) was shown to successfully detect adult female hybrids using spine morphology. However, inherent variation in morphological characters contributes to scoring variability and the overall likelihood of incorrect classification. Variation in thoracic segment 8 was the most influential, with only 22% of 561 individuals showed the expected phenotype for this character. The removal of segment 8 reduced a significant portion of statistical variation found within *B. sandiegonensis*. By modifying the workflow to exclude scores from this segment 8, female hybrid characterization has become more accurate and precise.

However, this method still includes considerable variation and is limited to only one sex and one life stage (adult female). Further, these characters do not permit accurate inference regarding the specific degree of hybridization (e.g., F1 generation, F2 generation, backcrosses).

Species-specific genetic markers overcome these limitations by allowing hybrid identification for both sexes and multiple life history stages, and distinguishing hybrid lines from backcrosses. In this thesis, I developed and validated of a 20-SNP panel, showing that it accurately and reliably detects admixed individuals, and provides a metric to characterize the process of hybridization.

By distinguishing backcrosses from hybrid lines and distinguishing early stage hybrids from late stage hybrids, hybrids can be separated into distinct groups. This allows prediction of the relative proportions of cysts in a local cyst bank, as well as the reliable frequency of species-specific alleles in a local gene pool. The second thesis chapter characterizes genetically admixed populations across the natural range of *B. sandiegonensis* in the United States of America based on the extent of habitat disturbance and genetic introgression. Results suggest that hybridization at low levels is found across the native range of *B. sandiegonensis*. Low levels of hybridization (facilitated by the passive dispersal of encysted embryos) may be a part of the evolutionary history of this group, although it is not so frequent as to dissolve species boundaries. In contrast, anthropogenic activities can facilitate higher levels of admixture, until basins are comprised of mostly non-native *B. lindahli* and *B. lindahli* backcrosses.

By providing a framework to classify populations based on the frequency of hybridization, researchers and habitat managers can better categorize the extent of hybridization across habitat types, quantify the relative contribution of natives and non-natives alleles to the local gene pool, and, perhaps, predict the short-term trajectory of the temporal process of hybridization. The extent of hybridization, and variation in patterns of allelic introgression is low in undisturbed pools (i.e. undisturbed coastal vernal pools and inland playas).

In disturbed basins that resemble near-functional vernal pools, the gene pool and cyst bank remain dominated by *B. sandiegonensis*. Road ruts and similar artificial basins are often dominated by *B. lindahli* or hybrids. However, there is high genetic variation among road ruts, and no general pattern or process is evident. This could be due to different outcomes when a road is built through an existing vernal pool landscape, periodic runoff from adjacent vernal pools, abiotic selective pressures and/or different cyst bank hatching dynamics.

Future Directions

Studying the hybridization process in *Branchinecta* fairy shrimp presents challenging opportunities for further investigation of the process of evolution. The cyst bank creates overlapping generations, violating the assumption of some basic theoretical models. Beyond the relative abundance of each species in the cyst bank, recruitment dynamics are driven by the frequency of species-specific alleles.

Patterns of hybridization are often site-specific, suggesting that the underlying processes may not be uniform. Future studies should investigate the origins of non-native *B. lindahli* inhabiting road ruts in historical coastal vernal pool habitats. A reduced representation library approach (such as restriction associated digest sequencing) in association with climate modeling could further our understanding of how recombination occurs in the genome. These data could also provide estimates of gene flow, relatedness, and projected distributions of hybrids in native coastal vernal pools. In order to accurately investigate patterns of hybridization, a nuclear phylogeny that includes the entire *Branchinecta* genus will also be necessary. Aspects of this genus' diversified reproductive bet hedging strategy can only be studied through advancements in reliable short- and long-term laboratory culture techniques. Finally, a clear understanding is needed of how the local conditions (i.e., water temperature, vegetation diversity, inundations period) affect the relative success of subsequent recruitment. Investigating patterns of hybridization with respect to the inherent fluctuations characteristic of temporary wetland ecosystems will further our understanding of this relatively under investigated system.

Conservation recommendations

Hybridization presents one of the most challenging problems for protecting and managing species under the Endangered Species Act because it can cause the extinction of distinct genetic, phenotypic, and/or evolutionary units (Taylor et al. 2006; Doremus 2010; Keller 2014; Bohling 2015).

Because *B. lindahli* develops faster and hatches at warmer temperatures, it seems poised to rapidly colonize road ruts, disproportionately transform the cyst bank, and persist through even sub-optimal inundation events. If future patterns of rainfall become sporadic and the frequency of premature drying events increases, climate change may promote the replacement of *B. sandiegonensis* by *B. lindahli* and hybrids in marginal habitats in coastal southern California. As a function of the continued introduction of *B. lindahli* into coastal vernal pool habitats, the number of hybridizing populations is likely to increase. Conservation efforts should not only focus on the survey of more *B. sandiegonensis* populations for intact genetic integrity habitat, but also limit the incursion of vehicular traffic onto or adjacent to functional vernal pool habitat. Restoration efforts including pool mitigation or complete restoration can also benefit from this work by choosing inoculum that has been verified to be genetically non-admixed. However, certain road ruts represent an extreme type of disturbance, and these artificial populations do not resemble native coastal vernal pools in terms of the diversity and ecological roles of native species. An aggressive strategy that eradicates the most compromised basins may be needed to limit the overall spread of hybridization and *B. lindahli* alleles into adjacent undisturbed vernal pools. Once *B. lindahli* becomes established in colonized habitats, it will be difficult to keep this evolutionary successful generalist from disproportionately contributing to the remaining local cyst bank. With the help of continued efforts to protect habitat, limit the large-scale introduction of non-natives, and short distance dispersal of hybrids, *B. sandiegonensis* may be able to persist indefinitely.