PHYLOGEOGRAPHY OF *LEPTASTERIAS* SPECIES RELATIVE TO AN ESTUARINE OUTFLOW IN THE PACIFIC NORTHWEST

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by

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San Francisco, California

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CERTIFICATION OF APPROVAL

I certify that I have read *Phylogeography of* Leptasterias *Species Relative to an Estuarine Outflow in the Pacific Northwest* by Jeyna Kim Perez, and that in my opinion this work meets the criteria for approving a thesis submitted in partial fulfillment of the requirement for the degree Master of Science in Biology: Ecology, Evolution, and Conservation Biology at San Francisco State University.

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PHYLOGEOGRAPHY OF *LEPTASTERIAS* SPECIES RELATIVE TO AN ESTUARINE OUTFLOW IN THE PACIFIC NORTHWEST

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Comparing the phylogeography of sympatric species complexes can provide evidence of shared responses to historic events. *Leptasterias* is a genus of brooding, low-dispersing sea stars comprising several cryptic species complexes found from California to Alaska. Assessing their response to environmental events may elucidate processes underlying their diversity. Prior phylogenetic work around the San Francisco Bay, California suggests an outflow-associated pattern of *Leptasterias* clade distributions. For comparison, we examined *Leptasterias* phylogeography in the San Juan Islands (SJI), Washington to assess the potential effects of low-salinity outflow from the Fraser River. Mitochondrial cytochrome oxidase 1 (COI) barcoding (n=268) confirmed three sympatric cryptic clades of *Leptasterias* in the SJI discussed in Foltz 2008: *L. aequalis* A (*L. pusilla* complex), *L. aequalis* B (*L. aequalis* complex), and *L. hexactis* C (*L. hexactis* complex). Fine-scale sampling in the SJI showed an association between *Leptasterias* spp. COI clade frequency distribution and habitat exposure to waves and estuarine outflow. Selective forces from stressors such as low-salinity plumes or wave exposure could be maintaining the clade distributions seen in this island archipelago. In California, clades

within the *L. pusilla* complex dominated bay-proximal sites more frequently exposed to low-salinity plumes out of the San Francisco Bay. Similarly in the SJI, the *L. pusilla* complex dominated sites more frequently exposed to low-salinity plumes from the Fraser River, while *L. aequalis* and *L. hexactis* dominated more wave-exposed, marine sites. This study suggests that estuarine sources may influence spatial genetic variation among *Leptasterias* populations. This combined regional comparison of the distribution of cryptic *Leptasterias* lineages relative to large sources of estuarine outflow confirms some effect of low-salinity plumes and wave exposure. Spatial and temporal patterns seen related to estuarine features in California poses concern for *Leptasterias* populations in the SJI, and further regional work in the context of local habitat is needed to expand upon these findings.

I certify that the Abstract is a correct representation of the content of this thesis.

Chair, Thesis Committee

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Date

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INTRODUCTION

Speciation within and among marine invertebrate populations can occur via mechanisms of reproductive isolation, which include differences in reproductive strategies, environmental tolerance, and presence of geographic barriers (Avise et al. 1987, Palumbi 1994). Differences in larval development mode, and consequently dispersal potential, may affect ecological and evolutionary processes on populations (Hart et al. 2003, Hart et al. 2017, Hart & Marko 2010, Hellberg 1996, Kelly & Palumbi 2010, Lee & Boulding 2009, Strathmann 1986). Hydrodynamic processes can have a significant impact on patterns of genetic variation in marine invertebrates. Currents, salinity, and temperature can act as gene flow barriers and create selective pressures on populations (Bashevkin et al. 2016, Byrne 2011, Kelly & Palumbi 2010, Palumbi 1994, Pia et al. 2012, Shirley & Stickle 1982). Variability in species' tolerance to such pressures combined with standing genetic variation can result in divergence (Waters et al. 2004). Studies aimed at understanding the dynamics between these mechanisms may provide a better understanding of localized adaptation and the ability to project future community dynamics in a changing climate.

Differences in reproductive strategies could result in different levels of genetic structure between species (Hart et al. 2003, Hart et al. 2017, Hellberg 1996, Kelly & Palumbi 2010, Lee & Boulding 2009, Strathmann 1986). Planktotrophic larvae have a high dispersal potential and rely heavily on ocean currents for food availability and transport. Lecithotrophic larvae typically have a lower dispersal potential than planktotrophic larvae, and rely on the fitness of the mother and the quality of the local environment. Lecithotrophic taxa (brooders) tend to have low gene flow between populations, a high potential for local adaptation, and experience more frequent speciation and extinction events compared to organisms with pelagic larvae (Barbosa et al. 2013, Hart et al. 2003, Hart et al. 2017, Strathmann 1986, Yamada 1989). Brooding taxa can be an ideal system to assess adaptive potential and how environmental and biological processes cause diversification. A brooding sea star species complex native to the Pacific Coast can be used as a model to assess causes of diversification.

The *Leptasterias* genus comprises brooding sea stars found in rocky intertidal and subtidal habitats from California to Alaska (Chia 1968, Foltz et al. 2008, Kwast et al. 1990). They are small in size, ranging from 4 to 6 cm in diameter, and are significant predators on multiple intertidal taxa (Chia 1966, Menge 1975). The *Leptasterias* genus has several cryptic lineages that have largely been unresolved in its taxonomy. Groupings

by morphological or behavioral characters have underestimated the number of lineages present along the coast (Fisher 1930, Chia 1966), most likely due to low levels of interspecific morphological variation, character inconsistencies among individuals, and potential hybrids (Foltz et al. 2008, Foltz 1997, Hrincevich et al. 2000). Molecular methods have since been employed to clarify lineages within *Leptasterias* (Flowers & Foltz 2001, Foltz et al. 2008, Kwast et al. 1990). Due to the life history and low dispersal capabilities of *Leptasterias* spp., they are likely to have high levels of population structure and low gene flow between populations. Assessments of *Leptasterias* spp. in relation to local conditions could provide insights into the extent of population stability, and what factors in particular could be putting populations at risk of extirpation.

Recent phylogeographic studies have found intriguing patterns of *Leptasterias* clade distributions around San Francisco Bay (SFB), California. There are two mitochondrial clade complexes, *L. aequalis* and *L. pusilla*, around SFB. Clade Y within the *L. pusilla* complex appeared dominant in populations around the SFB mouth while clade K (*L. aequalis* complex) dominated sites north and south of SFB populations, suggesting that clade Y could have adapted to the variably warm, low-salinity, and potentially contaminated conditions of the SFB outflow (Melroy 2016, Melroy et al. 2017). Conversely, the SFB effluent could have been a selective pressure on bay-proximal sites, creating the phylogeographic pattern seen.

Echinoderms are generally intolerant to low-salinity, and estuarine outflow can be detrimental to sea star physiology and dispersal (Bashevkin et al. 2016, George 1999, George & Walker 2007, Roller & Stickle 1985). While there are other estuarine conditions (including temperature, pollutants, pH) that could affect sea stars, low-salinity is immediately detrimental to sea star osmoregulation and can be lethal to developing larvae, and could cause drastic shifts in abundance and distribution (Bashevkin et al. 2016, Byrne 2011, Held & Harley 2009, Pia et al. 2012, Roller & Stickle 1993). There is some laboratory evidence suggesting *Leptasterias* species have varied salinity tolerance (Braun et al. 2016, Shirley & Stickle 1982), however it is unclear if it is genetically mediated or individual plasticity. Chronic unfavorable environment could result in overall smaller stars and lower fecundity (George 1996, George 1999, Shirley & Stickle 1982). Other conditions of the intertidal, including wave exposure and niche competition with larger stars such as *Pisaster ochraceus*, could also mediate *Leptasterias* reproductive success and size (Menge 1974). Environmental conditions could have significant impacts on the phylogeography of the *Leptasterias* species complex. An additional stressor to local sea stars has been sea star wasting disease (SSWD), an epidemic that decimated over 20 species of sea stars along the Pacific Coast (Eisenlord et al. 2015, Hewson et al.

2014). It was initially believed that smaller stars such as *Leptasterias* spp. were resistant to the disease - populations of surviving *Leptasterias* spp. that co-occurred with infected ochre stars (*Pisaster ochraceus*) in Central California suggested species-level resistance to SSWD. However, *Leptasterias* spp. soon became infected as well, and over time SFB-proximal *Leptasterias* populations vanished (Cohen unpub). This shared response among sympatric *Leptasterias* clades near the bay suggests that estuarine outflow and the SSWD epidemic, in addition to differential adaptive potential among *Leptasterias* spp., could drastically shape phylogeographic patterns.

This study focuses on *Leptasterias* populations relative to another source of estuarine outflow located in the San Juan Islands, Washington (SJI), expanding upon Melroy et al. 2017. The SJI region is a transition zone among several marine taxa, including *Leptasterias*, making it an ideal site for fine-scale examination of local effects on sympatric species (Foltz et al. 2008, Foltz et al. 1996, Marko 2004). Several studies have examined *Leptasterias* relative to wave exposure in the SJI (George 1996, 1999), however morphological inconsistencies and hybridization within and among species made confounded identification. In the SJI, *Leptasterias* has been synonymized as one specie, *Leptasterias hexactis*, but was later found that there were three coexisting species: *Leptasterias aequalis*, *Leptasterias pusilla,* and *Leptasterias hexactis* (Kwast et al. 1990, Foltz et al. 1996, 2008). Additionally, a fourth species historically described as *Leptasterias epichlora* (Brandt 1835) was misidentified and rejected (Fisher 1930), and subsequent literature was misleading in characterizing this group. Resolving current genetic species in the SJI in comparison with previous work would confirm local patterns found in the past.

The San Juan archipelago is located in the Strait of Georgia, north of Puget Sound in Washington. This region is influenced by the Fraser River, one of the largest sources of freshwater outflow in the Pacific Northwest. The Fraser River drains about 25% of British Columbia, and about 75% of its total freshwater outflow is discharged into the Strait of Georgia north of the SJI (Thomson 1981). The freshwater plume from the Fraser can affect surface water salinity over many kilometers, extending into the SJI (Sutherland et al. 2011, Thomson 1981). The Fraser River outflow is much larger in magnitude compared with the San Francisco Bay outflow, of which 80% is diverted for agriculture and urbanization, resulting in more polluted and smaller mean annual outflow compared to the Fraser River (Emmett et al. 2000, Roden 1967). With such striking phylogeographic patterns observed in *Leptasterias* populations around SFB, it is possible that outflow-associated patterns could be seen in the SJI as well.

Additionally, chronic SSWD has been documented throughout the San Juan archipelago (Multi-Agency Rocky Intertidal Network (MARINe)), creating an opportunity for a temporal comparison of how the disease has shaped *Leptasterias* distributions. At the onset of the SSWD epidemic, *Leptasterias* species in central California displayed a delayed response to the disease in comparison to heavily impacted, larger star species such as *Pisaster ochraceus* (CS Cohen, per obs). Standing genetic variation in *Leptasterias* could have enhanced its ability to initially resist SSWD, but as populations declined differential survival became apparent among different localities, similar to sea stars such as *Pisaster ochraceus* (Bates et al. 2009, Eisenlord et al. 2015, Hewson et al. 2014, Menge et al. 2016). Locally-adapted, low-dispersing *Leptasterias* populations could have underlying genetic variation that could enhance or reduce their sensitivity to SSWD and resulting selective sweeps could have altered population composition in the SJI (Schiebelhut et al. 2018). Post-epidemic analyses of populational genetic variation could reveal beneficial alleles that aided in survival against SSWD.

Eukaryotic elongation factor 1-α subunit (EF1-α) is a nuclear protein-coding gene that is highly conserved among many taxa, and its intron regions have been useful in species-level phylogeographic studies (Foltz et al. 2007, Maroni 1993, Cho et al. 1995). However it became increasingly apparent that multiple functional copies of EF1-α occur across the nuclear genome in several invertebrate species (Nahavandi et al. 2012). The intron-exon structure of EF1-α has been used to assess beneficial functional alleles and to identify phylogeographic structure (Duda & Palumbi 1999). EF1-α has been utilized in *Leptasterias* phylogeny, and Intron 4 of the EF1-α locus displayed size polymorphisms, which could indicate a duplication of this locus (Foltz et al. 2007, 2008). The maintenance of one or several copies of EF1-α in the *Leptasterias* nuclear genome could be a signal of fluctuating population structure, or selection. In the ochre star (*Pisaster ochraceus*), there appeared to be an indirect functional relationship between EF1-α alleles and SSWD resistance, though without statistical significance (Pankey & Wares 2009, Wares & Schiebelhut 2016). Among EF1-α genotypes in a limited sample size, heterozygotes seemed to have a signal for higher oxygen consumption and a modest response to elevated temperature, which could lead to lower incidence of SSWD (Chandler & 2017, Wares 2019). EF1-α functional fingerprinting of *Leptasterias* could be used to detect structure in populations and genotypic response to SSWD, relative to low-salinity in the SJI.

A current assessment is needed in the San Juan archipelago to elucidate the biogeography of *Leptasterias* relative to the Fraser River estuarine plume. In particular, which mitochondrial clades appear adapted to chronic low-salinity conditions and which

populations may be at risk of decline. In this study, sites were categorized as "low-salinity" or "marine" based on proximity and exposure to the Fraser River plume using simulations from the MoSSea model (University of Washington, Sutherland et al. 2011) and LiveOcean (UW Coastal Modeling Group). "Low-salinity" sites were characterized as having greater salinity fluctuations due to exposure to the Fraser River plume and wave-protected. "Marine" sites were characterized as having less exposure to the Fraser River plume and more wave-exposed.

Temporal comparisons of museum samples provide further insight into genetic changes in this region due to the SSWD epidemic. An assessment of the EF1-α locus, a nuclear locus that could possibly detect population structure in response to selective sweeps, could lead to predictions of the impact of the SSWD epidemic on a genetic level within the *Leptasterias* species complex. Multilocus sequence data was used to: 1) characterize temporal and spatial variation of mitochondrial *Leptasterias* clades in the SJI, and 2) test for variation in a SSWD-associated locus related to geography and *Leptasterias* species. Limited data on *Leptasterias* individual morphology, size, injury, and microhabitat were gathered to supplement data on putative species, low-salinity exposure, and SSWD.

METHODS

I. Sample Collection and DNA Extraction

One hundred and eighty-nine *Leptasterias* individuals were collected from 17 intertidal locations throughout the San Juan Islands from 2014 to 2016 for contemporary sampling (Table 1). Ray tissue samples were collected from individuals and stored in 95% ethanol.

Fifty-five historical *Leptasterias* individuals collected from 1937 to 1998 were obtained from the Invertebrate Zoology collections at the California Academy of Sciences (CAS). These historical samples were collected from sites in the Puget Sound and Strait of Juan de Fuca in Washington (Table 1). Forty-seven *Leptasterias* samples collected from 1909 to 2003 throughout the Puget Sound were obtained from the Smithsonian National Museum of Natural History (NMNH). Tube feet were sampled from historical individuals, stored in 95% ethanol, and transported to the EOS Center. Tube feet were transferred into milliQ water and left on a shaker for two days to remove excess ethanol

prior to DNA extraction. All DNA extractions were carried out using NucleoSpin Tissue Columns (Macherey-Nagel Inc., Bethlehem, PA, USA).

II. Amplification of COI locus

Forward primer COILF, and reverse primer COILR (Melroy 2016) were used in this study to amplify a 536 base pair region comprised a portion of cytochrome oxidase subunit I (378bp), tRNA-Arg (67bp) and ND4L (89bp) genes (henceforth referred to as COI for simplicity). For contemporary samples, PCR reactions were carried out with the following components: 5-500 ng DNA template, 0.2 μM of each primer, 1X PE II Buffer, 1 mM dNTPs, 2.5 mM $MgCl_2$, 1.25 μg BSA, 1 unit of Taq DNA polymerase (New England Biolabs, NEB, Ipswich, MA, USA) and milliQ water up to the final 25 ul volume. Thermal cycling conditions were: initial denaturation at 96°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 46°C for 30 seconds, extension at 72°C for one minute for a total of 35 cycles and final extension at 72°C for 5 minutes. For historical samples, thermal cycling conditions included a total of 40 cycles.

III. Amplification of EF1-α Intron 4 locus and Amplicon Scoring

EF1-α Intron 4 forward (Int4F) and reverse (Int4R) primers were obtained from Foltz 2007. PCR reactions were carried out in a 40μL reaction volume with the following components: 5-100 ng DNA template, 1.5μL of each primer, 5μL of 1X PE II Buffer, 5 μL of 25 mM $MgCl_2$, 15μL of 0.5 mM total dNTPS, 10μL of milliQ water, and 1μL of Taq DNA polymerase (New England Biolabs, NEB, Ipswich, MA, USA). Touchdown thermal cycling conditions were: two cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for one minute, and extension at 72°C for one minute, followed by nine cycles with the annealing temperature reduced 1°C per cycle to 55°C, ending with 20 cycles at an annealing temperature of 55°C and a 7 minute final extension at 72°C. PCR products were assessed by gel electrophoresis using a 2.5% agarose gel stained with ethidium bromide and run for 100 minutes at 140V.

IV. Sequencing Reactions

PCR templates were cleaned of residual dNTPs and primers with a SAP/EXO reaction (Affymetrix, Santa Clara, CA, USA). Cycle sequencing reactions were carried out using Big Dye v.3.1 (Applied Biosystems, ABI, Foster City, USA) using the following protocols: initial denature at 96°C for 1 minute, denature 96°C for 10 seconds, annealing at 50°C for 30 seconds, extension at 60°C for 5 minutes, for a total of 30 cycles. Cycle sequencing reactions were carried out in the reverse direction for COI and in the forward direction for EF1-α INT4, for a total of 40 cycles. Cycle sequencing products were sequenced using an ABI 3130 genetic analyzer located at the EOS Center.

V. COI Phylogenetic Analysis

All COI sequences were compared to NCBI published sequences and Melroy (2016) using Geneious v. 11.0.5 (Kearse et al. 2012) to delimit haplotypes. Ten repetitions of the MUSCLE algorithm were used to generate an alignment that was edited by eye. The COI alignment was translated in Mesquite v. 3.6 (Maddison & Maddison 2018) to validate the correct reading frame. All sequences in this study will be submitted to NCBI and accession numbers will be published.

Leptasterias camschatica was used as an outgroup for all phylogenetic analyses based on previous phylogeny (Foltz et al. 2008, Melroy 2016). Maximum likelihood (ML) analyses were performed on contemporary and historic COI haplotypes in MEGA v. 7.0.26 (Tamura et al. 2011). The automated model selection feature was used to choose the most appropriate nucleotide substitution model using the Akaike Information Criterion (AIC; Huelsenbeck & Crandall 1997) and the model GTR+I+G was selected. Bootstrap analyses were performed using a neighbor-joining tree as the starting tree for a heuristic search with 1000 replicates.

MrModeltest v.2.3 (Nylander 2004) was used in PAUP v. 4 (Swofford 2002) to determine the Bayesian model of nucleotide evolution, and the GTR+I model was selected using the AIC Criterion. A phylogenetic tree was constructed in MrBayes v. 3.3.2 (Huelsenbeck & Ronquist 2001, Ronquist et al. 2012) using Bayesian Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) estimation. The analysis was run for 2,000,000 generations with sampling every 500 generations using 4 heated chains. Analyses started with a random starting tree. Trees sampled before reaching a split standard deviation frequency of 0.02 were discarded as the burn-in. The remaining trees were used to construct a 50% majority-rule consensus tree with Bayesian Posterior

Probabilities (BPP) as nodal support. Trees were visualized in FigTree v1.4.0 (Rambaut, 2009).

VI. Population Genetic Analysis

L. pusilla and *L. aequalis* were combined in several population genetic analyses due to their species hybridization potential and abundance across sites (Foltz et al. 2008). The best nucleotide substitution model was obtained from jmodeltest (Guindon & Gascuel 2003, Darriba et al. 2012) and HKY+I+G was concordant with AIC and BIC scores. MEGA v. 7.0.26 (Tamura et al. 2011) was used to generate intraspecific and interspecific genetic distances for COI using the T93 model, which was closest to the jmodeltest output.

DnaSP v5.10 (Rozas et al. 2003) was used to calculate genetic diversity indices including haplotype diversity (h) and nucleotide diversity (π) for each population, as well as neutrality statistics Tajima's D and Fu and Li's F and D. Small populations (DE, SI, HI, SK) geographically close to Anacortes were combined into a single group ANA for these analyses. Fu's Fs neutrality statistics and pairwise comparisons (F_{ST} and Φ_{ST}) were calculated to evaluate genetic differentiation between localities using Arlequin v3.5 (Excoffier et al. 2005). A minimum spanning network of haplotype frequencies was constructed using DnaSP and PopART (Leigh & Bryant 2015) to assess geographic correlation to lineages. An Analysis of Molecular Variance (AMOVA) was done to assess variation in *Leptasterias* spp. between marine and low-salinity exposed sites, among islands, and among populations in the SJI.

Mismatch distributions were generated in Arlequin and DnaSP for *L. pusilla*, *L. aequalis*, and *L. hexactis* COI haplotypes. Multimodal curves are usually indicative of stable populations while unimodal curves are indicative of a population expansion event (Rogers & Harpending 1992), and significance is measured by Harpending's raggedness index and sum of squared differences. Nonsynonymous and synonymous substitution ratios (dN/dS) for the three species were generated in MEGA, and a one-way test for positive selection (dN>dS) was conducted on all species using the Nei-Gojobori model (Nei & Gojobori, 1986).

VII. EF1-α INT4 Variation

22 EF1-α INT4 forward sequences (Table 1) were aligned in Geneious v. 11.0.5 using the MUSCLE algorithm and trimmed to the same length, 518bp. EF1-α INT4F sequences were analyzed by eye to assess locations and frequencies of single nucleotide polymorphisms (SNPs) and indels. Based on the alignment, comparisons were made between the two amplicons, between *L. hexactis* and *L. pusilla*, and between marine and low-salinity sites.

VIII. Morphological Analyses

Subsets of individuals were brought to Friday Harbor Laboratories to examine under a dissecting microscope. Several morphological characters such as color and spine and pedicellaria abundance and shape were used from taxonomic keys as well as literature (Foltz et al. 1996, Kwast et al. 1990, Light, Carlton, & Light 2007, M. Strathmann pers. comm.). Photos were analyzed in ImageJ (Rasband 2018) to estimate individual sea star size. The average of three disc diameter measurements was used to denote an individual's size. Star size was binned every 0.5cm, and frequency histograms were generated comparing size among species, and between marine and low-salinity sites. A two-way ANOVA was conducted to compare the effect of genetic identity or exposure characterization on size. A photo index shows *Leptasterias* sea stars grouped by clade and site (Appendix 1).

RESULTS

Of the 268 total COI sequences in this study, 230 sea stars were from contemporary sampling (of which 28 sequences were included from Melroy 2016), and 38 sea stars were from CAS historical collections (Supplemental S1). All of the NMNH samples were unsuccessful in amplification of COI. Previous haplotypes from other regions (Melroy 2016) were included in phylogenetic analyses, but excluded from population genetic analyses. A 536 base pair region of COI included 116 variable sites, of which 69 were parsimony informative. The COI region consisted of 45 haplotypes, including 8 private haplotypes in contemporary samples and 2 private haplotypes in historical samples.

I. Phylogenetic Analysis of Contemporary and Historical Samples

Variable and parsimony informative COI base position changes included 17 first position changes, 15 second position changes, and 38 third position changes. Maximum Likelihood and Bayesian phylogeny resolved the *L. aequalis/L. pusilla* complex from *L. hexactis* (Fig. 1a). When analyzing just Bayesian results, there was further nodal support grouping *L. pusilla* from *L. aequalis* (Fig. 1b). Six monophyletic clades, *L. pusilla* clades D and Z, *L. aequalis* clade B, Group 1, and *L. hexactis* clades C and G described in Foltz (2008) and Melroy (2016, 2017) were resolved with Bayesian nodal support (posterior probability >95%), but support was not significant for clade B in the Maximum Likelihood phylogeny (bootstrap values <70%). Clade Y and A in the *L. pusilla* complex did not have statistical support in either analysis.

Genetic distances within and among the three clades were calculated using the TN93 model of substitution (Table 2). The genetic distance within clade *L. aequalis* A was 0.1%, which was comparable to Melroy (2016). However, genetic distance within *L. aequalis* B (0.4%) and *L. hexactis* C (0.1%) were lower compared to previous values (0.9%, 0.4% respectively). In this study, the genetic distance between putative species *L. aequalis* and *L. pusilla* was 0.9%, falling on the lower end of previous values that ranged from 0.9 - 2.9%. The distance between *L. hexactis* from the other two putative species ranged from 4.1 - 4.9%, which was higher than previous values ranging from 3.8 - 4.3% (Melroy, 2016).

II. Population Genetic Analyses

Analysis of *Leptasterias* populations from the San Juan Island archipelago (excluding 79 reference haplotypes from Melroy (2016) used above in phylogenetic analyses) revealed 16 COI haplotypes, of which there were 9 private haplotypes in contemporary sampling and 2 in historical sampling. Of the 9 private contemporary haplotypes, 4 belonged to the *L. pusilla* clade, 3 to *L. aequalis*, and 2 to *L. hexactis.* Both of the historical private haplotypes belonged to the *L. pusilla* clade. Haplotype and clade distributions throughout the San Juan Island archipelago showed a gradient that followed inferred outflow exposure (Fig. 2 -3). In marine sites, *L. hexactis* clade C dominated, while clade A within *L. pusilla* dominated sites characterized as more exposed to estuarine outflow.

The minimum spanning network showed 16 haplotypes that formed three major clusters concordant with the COI clades found in the archipelago (Fig. 4). The *L. aequalis* and *L.*

pusilla haplotype clusters differed by two mutations, and both differed from *L. hexactis* by 13-15 mutations. Median joining and TCS networks were tested and maintained this topology. The *L. aequalis* and *L. pusilla* complexes contained many low-frequency haplotypes. Within *L. pusilla*, one historical *Leptasterias* individual (from Clallam Bay, collected 1998) genotyped as clade D, which was a common clade found in California (Melroy 2016). The network showed a distributional pattern of haplotypes throughout the San Juan Island archipelago related to outflow exposure. The haplotypes within *L. pusilla* and *L. aequalis* mostly contained individuals from low-salinity-exposed locales, and those in *L. hexactis* were mostly comprised of individuals from marine locales.

Haplotype diversity for *Leptasterias aequalis* and *Leptasterias pusilla* populations ranged from 0.182 to 0.767 for COI (Table 3). Nucleotide diversity indices were low for all populations, ranging from 0.00049 to 0.008. The lowest nucleotide diversity values occurred in marine sites, and the highest nucleotide diversity values occurred in sites considered exposed to low salinity variation, concordant with the phylogeographic pattern noted above.

Intraspecies haplotype diversity was calculated for all populations for *L. hexactis, L. pusilla,* and *L. aequalis* and were 0.172, 0.649, and 0.229 respectively (Table 4). Nucleotide diversity for *L. aequalis* was 0.072%, which was significantly lower compared to previous values ranging from 0.44 - 0.57% (Foltz et al. 2008, n=8 *L. aequalis* Clade B). Nucleotide diversity for *L. pusilla* was 0.36%, which fell within the range of previous values (Foltz et al. 2008, n=3 *L. pusilla* Clade A). *L. hexactis* had lower nucleotide diversity (0.049%) compared to the other two species (Foltz et al. 2008, n=2 *L. hexactis*).

Leptasterias aequalis and *Leptasterias pusilla* pairwise comparisons (F_{ST} and Φ_{ST}) showed significantly high genetic differentiation among most localities (Table 6). Φ_{ST} values ranged from 0.71 to 0.98 between populations on the western side of San Juan Island to those on either Orcas Island or the mainland, indicating islands as barriers to gene flow, which also corresponds with the exposure gradient. Lower Φ_{ST} and F_{ST} values showed some gene flow amongst low-salinity populations on San Juan Island. Marine populations had significant connectivity with nearly zero Φ_{ST} values. Clallam Bay, the outermost population in the Strait of Juan de Fuca, had high genetic differentiation compared to most populations, but had the most connectivity with Eagle Cove, which suggests possible dispersal from marine sites into these locales. *Leptasterias* hexactis pairwise comparisons (F_{ST} and Φ_{ST}) showed relative connectivity among marine locales, except for Deadman Bay. Deadman Bay showed fixation (F_{ST} = 1.00) against all

population pairs. Of the 15 samples there, six belonged to one private haplotype, which could explain this value.

The Tamura & Nei model was used in 10,000 permutations for AMOVA analyses (Table 5). With no regional groupings including all species, 53.67% of the variation observed was explained among populations, and 46.33% within populations. When comparing all species between low-salinity and marine sites, 62.63% of the observed variation was explained between the two groups and was the strongest explanation of variation among AMOVA hypotheses. When *L. aequalis* and *L. pusilla* were analyzed separately, exposure remained the highest cause of observed variation at 53%. Island connectivity including all three clades was tested beginning with five groups, which was not significant among islands and showed more variation within populations (43.8%). When *L. aequalis* and *L. pusilla* were tested separately they also showed more within population variation than among islands (45.8%).

Across most *L. pusilla* and *L. aequalis* populations, Tajima's D, Fu and Li's F and Fu and Li's D statistics had no significant values (Table 3). Reuben Tarte showed significant negative Tajima's D, Fu and Li's F and D statistics, and, when assessed with a positive Fu's Fs value, suggests that a population expansion or purifying selection occurred at this site. When populations were divided into COI clades, *L. pusilla* had significant negative Tajima's D, Fu and Li's D and F, and Fs values, possibly indicating a population expansion or purifying selection (Table 4).

The mismatch distribution analysis for *L. pusilla* showed multiple curves, however it followed the hypothesis for recent population expansion. The raggedness index (r=0.37, p-value=0.27) and sum of squared differences (SSD = 0.12, p-value=0.09) for *L. pusilla* were not significant, and the hypothesis for a recent population expansion could not be rejected (Fig. 5a). The mismatch distributions for *L. aequalis* and *L. hexactis* showed unimodal curves (Figs. 5b and 5c) and Harpending's raggedness indices were not significantly different from the expected unimodal curve (*L. aequalis:* Harpendings raggedness index r=0.39, p-value=0.93; *L. hexactis*: Harpendings raggedness value r=0.60, p-value=0.85), so the hypothesis for a recent expansion could not be rejected for *L. aequalis* and *L. hexactis.*

Codon-based z-tests of selection for *L. pusilla, L. aequalis*, and *L.hexactis* showed that all species had no significant signal of positive selection (dN>dS, p>0.05). *L. pusilla* and *L. hexactis* had a higher number of synonymous substitutions than nonsynonymous mutations, while *L. aequalis* had a higher number of nonsynonymous mutations (Table

9). However, *L. pusilla* had a significant signal of purifying selection (dN<dS, p=0.02), which is consistent with negative Tajima's D, Fu and Li's D and F statistics.

III. EF1-α INT4 Variation

Amplification of two EF1-α INT4 loci suggests its duplication in the *Leptasterias* genus. EF1-α INT4 primers amplified either a 1000 base pair or 2000 base pair fragment in each sample, but never both in one sample. Within a 518 base pair region of EF1-α INT4 sequence, a 181 base pair region containing multiple indels distinguished between the loci. Outside of this 183bp region, the two loci differed by 8 polymorphic sites. The 1kb locus was found in 17 individuals, which were identified as *L. hexactis* (n=12) and *L. pusilla* (n=5) and were collected from both exposure characterizations. The 1kb locus was fairly conserved among sequences, and there was one polymorphic site within the 1kb locus. The 2kb locus was found in 5 individuals, which were identified as *L. pusilla* (n=4) and *L. aequalis* (n=1) and found only in Friday Harbor, a low-salinity locale. The 2kb locus had two polymorphic sites among sequences. *L. pusilla* individuals (n=9) retained either the 1kb or 2kb EF1-α INT4 locus, but not both simultaneously. *L. hexactis* individuals (n=11) possessed only the 1kb EF1-α INT4 locus.

IV. Historical Samples

Of the historical samples collected from CAS and NMNH, only 38 successfully amplified for COI from CAS. All of the NMNH samples were unsuccessful in amplification, perhaps due to degraded tissue (though samples were from 2003). Historical samples from Anacortes, Friday Harbor, and Clallam Bay shared haplotypes with contemporary samples, and two private haplotypes were revealed from Clallam Bay and Friday Harbor (Fig. 3). Historical sampling revealed the three COI clades *L. pusilla, L. aequalis,* and *L. hexactis*, with one individual from Clallam Bay delineating into clade D within *L. pusilla*, which was the only sample aligning with that clade in this study. The clade distribution of historical samples followed patterns seen among contemporary samples, with marine sites comprised mostly of *L. hexactis* (CB, Fig 2.) and low-salinity sites comprised mostly of *L. pusilla* (FHL, ANA, Fig. 2).

V. Morphological Observations

The photos of 133 stars were analyzed in ImageJ to obtain average disc diameters. A Shapiro-Wilks test of normality was done by species. An analysis of variance showed that the effect of species on size was significant (two-way anova, $F_{2,129} = 5.86$, $p = 0.0036$). Larger stars were identified as *L. hexactis* while smaller stars identified as *L. pusilla* and *L. aequalis* (Fig. 6b). Between marine and low-salinity sites, an analysis of variance showed that exposure did not impact size of individuals (two-way anova, $F_{1,129} = 0.155$, $p = 0.694$) and the majority of stars were between 1 - 2.5cm in size (Fig. 6a).

Injury, symptoms of disease (white lesions), and arm regeneration were noted among collected stars (Table 8a). Rates of these instances were low and similar between marine sites (6.61% of total individuals) and low-salinity sites (5.06% of total). Among the three *Leptasterias* mitochondrial clades (Table 8b), about half of the injuries observed were found in *L. pusilla* (51.7%).

Photos from stars collected in August 2016 had the best resolution and were used to assess color patterns (Appendix 1). Subjectively, it seemed that *L. pusilla* stars were characteristically dull green or brown with no contrasting mottling patterns, whereas *L. aequalis* and *L. hexactis* had more variation in mottling and contrasting colors, such as olive green, dark brown, tan, dark red, and burnt orange. The chevron pattern noted by Kwast (1990) was seen along the arms of *L. aequalis* specimens. Pink colored variants of *Leptasterias* spp. were not found on collections, though they have been noted in the SJI before. Data gathered on spine and pedicellariae characteristics was limited and did not have enough diagnostic power for this study. Subjectively, aboral spines formed patterns of rosettes, radial rows down the arms, or inconsistent patterns among individuals. Number of pedicellariae on each spine along the adambulacral groove varied from three to ten among individuals.

DISCUSSION

Fine-scale sampling of *Leptasterias* spp. throughout a small island archipelago revealed a distributional pattern of three sympatric mitochondrial species related to exposure to a low-salinity estuarine outflow. Strong genetic differentiation among *Leptasterias* clades and populations from one side of the archipelago to the other suggests that there is a phylogeographic barrier, potentially from salinity and wave exposure. The strong disjunct in clade distribution between exposure characterization of sites suggests that *L. pusilla* is more adapted to protected habitats that experience greater salinity fluctuation, and *L. hexactis* is more adapted to wave-exposed, more marine habitats. Several studies have

noted this exposure difference on San Juan Island and its potential correlation with sea star haplotype distribution, size, and reproductive success (Bingham et al. 2000, Chia 1966, George 1996, George 1999, Foltz et al.1996, Foltz et al. 2008), yet fine scale work on sea star phylogeography in this region had yet to be done relative to the Fraser River outflow. Spatial and temporal comparisons of these three *Leptasterias* complexes in the context of a large estuarine plume after an epidemic (SSWD) showed high structure in a small, complex region.

I. Phylogeographic Patterns of Three Leptasterias *spp. Complexes*

Similar phylogenetic studies on brooding asteroids (*Patiriella* sp.) revealed cryptic species, which suggested an underrepresented amount of diversity (Hart et al. 2003, Hart et al. 2017). In this study we anticipated similar levels of cryptic diversity for brooding *Leptasterias*. Populations in the San Juan Island archipelago consisted of primarily three COI clades: *L. aequalis* A (*L. pusilla* complex), *L. aequalis* B (*L. aequalis* complex), and *L. hexactis* C (*L. hexactis* complex). Mitochondrial clades in the SJI corresponded with representative GenBank sequences generated by Foltz et al. (2008) and Melroy et al. (2017). While bootstrap support values were weak for separating the *L. aequalis* from the *L. pusilla* complex, the significant posterior probability grouping of the *L. pusilla* complex from the rest of the complex leave us confident that three putative species continue to exist in the SJI. In addition, the 16 COI haplotypes formed three major clusters correlated with the phylogeny. The *L. aequalis* and *L. pusilla* haplotypes differed in as few as two mutations, so several networks were tested (TCS, median joining, minimum spanning). No cyclical mutations among the three clades of haplotypes were revealed, indicating no homoplasy in COI in these *Leptasterias* spp. A novel clade revealed in Melroy (2016), Clade Z, had support with both analyses. Clade Y, revealed in Melroy et al. 2017, did not have significant support. When third codon positions were excluded in ML analyses, a few haplotypes were regrouped within complexes, but overall topology among the three complexes remained unchanged. Bootstrap values were reduced when third position changes were excluded, indicating lack of saturation of mutations at this position, and so all sites were included in analyses (Fig. 1). Distributional patterns of clades and haplotypes through the archipelago suggest exposure as a phylogeographic barrier, which is alluded to in previous literature (Foltz et al. 1996, George 1994). With greater sampling depth in this study, this pattern is confirmed, and appears to be maintained over time.

II. Population Genetic Analyses and Tidal Currents

Among *Leptasterias* species in the San Juan archipelago, greater genetic distances were found between geographically distant populations, even at distances < 1km, indicating strong partitioning by salinity and wave exposure. Among the more abundant *L. pusilla* and *L. aequalis* clades, populations from the western edge of San Juan Island showed high genetic differentiation from populations on the northern side of Orcas Island (F_{ST} and Φ_{ST} values >0.6, AMOVA 62% variation explained by exposure). Within a finer scale on San Juan Island, *L. pusilla* and *L. aequalis* showed significant differentiation between the western and eastern sides of the island (F_{ST} and Φ_{ST} values = 0.09 to 0.76), most likely maintained by strong currents. Tidal currents are bisected along the western edge of San Juan Island, with the strongest flow occurring along the southwestern edge of the island and slower flow going around Cattle Point and into the archipelago (Thomson 1981). Along with salinity tolerance, *Leptasterias* species could be differentially adapted to wave exposure. Smaller, low-salinity tolerant *L. pusilla* and *L. aequalis* individuals could be more easily detached from substrate in wave-exposed habitats, and so could be limited to low flow habitats internal of the archipelago. The larger, low-salinity intolerant *L. hexactis* individuals (Fig. 6b) could be more adapted to high energy wave-exposed habitats, and thus found more abundant on the western coast of San Juan Island. *L. hexactis* along the marine side of San Juan Island surprisingly had no differentiation between sites (F_{ST} and Φ_{ST} values = 0). In stark contrast, the population at Deadman Bay showed an F_{ST} value of 1 against nearly all population pairs, most likely due to the higher frequency of a single private hapolotype at this location.

Populations within marine or low-salinity exposure characterization exhibited some levels of gene flow, most likely due to close proximity and the migration of adults (F_{ST} and Φ_{ST} values < 0.1). Among low-salinity sites, there were signals of connectivity between *Leptasterias* populations, suggesting that currents potentially aided in dispersal. Tidal streams on the northeastern end of the archipelago is bisected by the northern end of Orcas Island, and strong flows head into the eastern edge of San Juan Island and around Cypress and Blakely Islands near Anacortes. Populations from northern Orcas Island shared haplotypes with those on the mainland near Anacortes and showed low F_{ST} values (<0.1). On Lopez Island, the one population in MacKaye Harbor shared connectivity with eastern San Juan Island populations Friday Harbor, Reuben Tarte, and Griffin Bay (F_{ST} values <0.1). Lack of island separation among the more widely dispersed populations of *L. pusilla* and *L. aequalis* suggest that some level of gene flow is

occurring within the archipelago. Despite the low dispersal capability of *Leptasterias* spp., it is possible that individuals are crossing between islands, either by crawling or potentially drifting on debris on currents (Highsmith 1985).

III. Population Expansion

Population expansion events are characterized by high haplotype diversity, low nucleotide diversity, and significant negative Tajima's D and Fu and Li's statistics (Aris-Brosou & Excoffier 1996). *L. aequalis* and *L. hexactis* showed negative neutrality indices, but were not statistically significant (Table 4). However mismatch distribution analyses showed distributions in *L. aequalis* and *L. hexactis* consistent with the hypothesis of population expansion. *L. pusilla* showed significant negative Tajima's D and Fu and Li's statistics, and failure to reject the null hypothesis in mismatch distribution analysis showed concordance with population expansion (Table 4). The multiple curves in *L. pusilla* distribution could be due to multiple factors. Population substructuring and mutation rate heterogeneity may account for the pattern seen in *L. pusilla* (Aris-Brosou & Excoffier 1996, Maltagliati et al. 2010). Multiple haplogroups consisting of unique haplotypes (Fig. 4) could be contributing to the four distinct peaks in the distribution curve.

Differential selection among these haplogroups and variance in reproductive success are other factors that could have contributed to the demographic history seen within *L. pusilla* in the SJI. Invertebrates with non-pelagic larvae are suggested to have higher nonsynonymous mutations concordant with positive selection (Foltz 2003, Foltz et al. 2004). A codon-based z-test was significant for rejecting the null hypothesis of neutrality in favor of the purifying selection hypothesis for *L. pusilla*, which is concordant with the significant neutrality indices described. Glacial recessions and advances and marine epidemics have historically shaped marine invertebrate populations in the Pacific Northwest, and could have been selective pressures on *L. pusilla* (Maltagliati et al. 2010). Demographic decline and slow recovery in response to such events could be an explanation for the demographic history of *Leptasterias pusilla* seen in the San Juan archipelago.

IV. Pleistocene Refugia

Washington has historically been an area of phylogenetic disjunctions and of range overlap in several marine taxa (Jacobs et al. 2004, Marko 2004). During the Pleistocene, deglaciation of the Cordilleran ice sheet in the Puget Sound region left opportunities for marine taxa to expand into the Puget Sound. TMRCA divergence times between *L. hexactis* and *L. aequalis* date divergence to 2.5 My, which corresponds to Pleistocene glacial cycles (Foltz et al. 2008). In higher latitudes, taxa with few haplotypes in a large geographic area is consistent with range expansion from refugia (Marko 2004). Melroy (2016) found 58 COI haplotypes across central California, Washington, and Alaska, whereas only 16 COI haplotypes were found in the San Juan archipelago. The low haplotypic diversity and distribution in the SJI suggests *Leptasterias* populations expanded into this region from the outer coast.

SJI *L. hexactis* haplotypes are shared amongst populations on the outer north coast near Vancouver. The *L. pusilla* and *L. aequalis* complex shared haplotypes more similar to those found in Oregon and California. Fluctuating glacial cycles could have resulted in recolonization and selection events in *Leptasterias* history in the SJI. Maintenance of current population structure between opposite sides of the SJI suggest that a combination of the low dispersal capability of *Leptasterias* larvae, island geography, and the unique oceanographic characteristics of the archipelago could have maintained differentiation between one side of the SJI and the other, resulting in the current phylogeographic pattern. *Leptasterias* populations could have preferentially adapted to their current locales, or wave exposure and salinity gradients could have acted as selective pressures maintaining the differentiation. However an assessment of variation at other loci could reveal functional reasons for the maintained pattern of *L. hexactis* in marine sites, and *L. pusilla* in low-salinity sites.

V. Size-polymorphism Variation in EF1-α INT4 Indicates Duplication in Leptasterias *spp.*

The use of EF1-α INT4 was initially intended to identify functional variation among *Leptasterias* species associated with disease resilience (Pankey & Wares 2009, Wares & Schiebelhut 2016). However, following a correction on genotypic correlation with SSWD incidence (Wares 2019), the intron was instead used in this study to assess intronic variation with respect to COI species and exposure to estimate phylogeographic patterns in the nuclear genome.

The two loci in this study show substantial length and sequence differences that indicate at least one duplication event of the intron in the *Leptasterias* nuclear genome. The incidence of the 1kb locus within *L. hexactis* and *L. pusilla* suggests that the duplication of Intron 4 most likely occurred before their divergence. The presence of another locus, the 2kb locus, solely in *L. pusilla* in a low-salinity site warrants further examination of the variation of Intron 4. Variation in this nuclear locus could reveal its utilization to detect population structure between the two species, between exposures. However, this data set is small and requires more sequences of Intron 4 to validate this observation.

VI. Morphological Variation

The history of characterizing *Leptasterias* species by morphology and genotype has been notably confounded in the Puget Sound. Sea star morphotypes described in the Puget Sound were once synonomized as *L. hexactis* (Chia 1966), but are currently described as three separate species: *L. pusilla* (Fisher 1930), *L. aequalis* (Stimpson 1862), *L. hexactis* (Stimpson 1862). A fourth species that was historically described in Puget Sound, *Leptasterias epichlora* (Brandt 1835), was rejected by Fisher (1930) and subsequent literature was misleading in characterizing this group. Inconsistency in delineating *Leptasterias* species has been due to cryptic morphology of several haplotypes and hybridization (Foltz et al. 2008, Foltz et al. 1996, Hrincevich et al. 2000). While some morphological characters were examined among a subset of sea stars, the primary focus in this study was on potential differences in size and injury frequency among marine and low-salinity sites.

Difference in size could be due to many variables including prey availability, reproductive success, environmental pressures, competitive exclusion, and genetically mediated differences. Several studies have noted exposure differences on either side of San Juan Island contributing to reproductive and size differences (George 1996). Sea star size has been correlated with food productivity in a habitat, stress from wave-exposure, and niche competition with larger stars such as *Pisaster* (Bingham et al. 2000, Menge 1972, 1974). In reduced salinity conditions, larvae could develop abnormally, resulting in either mortality or reduced adult fitness (George 1999, George & Walker 2007, Pia et al. 2012). We hypothesized that low-salinity conditions would result in smaller stars, and wave-exposed sites would have larger, more robust stars adapted to such conditions. Larger stars would have more tube feet and a greater area for adhesion, which is vital in heavy wave-exposed environments. The one-way ANOVA comparing size to salinity

characterization did not support this hypothesis, however, there was a correlation between size and COI species, suggesting genetic mediation of size (Fig. 6). Given the phylogeographic pattern, it appears that the smaller *L. pusilla* stars are more adapted to low-salinity, protected sites. The more robust *L. hexactis* stars have larger bodies, making them better suited to more wave-exposed, marine sites.

There were few field observations of wasting disease, and field observations were often unreliable in the seasonal conditions. Signs of lesions and body wall decay were not detectable until stars were brought under examination in the lab. Signs of arm autotomy, lesions, and body wall openings were all synonymised as "injuries," though not necessarily attributed to SSWD, since identifiable symptoms were indeterminable during collections. Marine locales had the highest incidence of injury, and were highest in *L. pusilla* stars (Table 8). Previous work around Anacortes found higher rates of arm autotomy and damage (30-40%) among *Leptasterias* individuals, due to physical damage from intertidal debris or predation by crab species (Bingham et al. 2000). Collection sites on the west side of San Juan Island featured habitats that were more cliff-like, with large logs and debris washed up along shores. Collection sites within the archipelago were mostly comprised of cobblestone and beaches. Injury in *Leptasterias* at these locations could be attributed to impact from debris, but no observations were made on crab predation. Incidentally, the highest number of injured stars came from Friday Harbor (n=8), and three of the four 2kb EF1-α INT4 loci were found in these individuals. Symptoms in these three stars consisted of arm autotomy and superficial lesions. Further comparisons between EF1-α INT4 variation and injury symptoms could elucidate potential patterns between the 2kb EF1-α INT4 locus, habitat, and suspected wasting symptoms.

VII. Comparisons to California: Estuarine Outflow Could Differentially Impact Populations

The rapid and drastic changes seen in *Leptasterias* clade distributions around the San Francisco Bay in Central California prompted this endeavor to examine patterns relative to another source of estuarine outflow influencing the San Juan Island archipelago. The clear disjunction seen around SFB associated with low-salinity outflow was also seen through the SJI archipelago, although it is complicated by island geography. Nonetheless, a clear separation between sites characterized as low salinity and marine-exposed exists in the archipelago.

In Central California, Clade K within the *L. aequalis* complex was more widespread and appeared more frequently at sites further from the San Francisco Bay. Clade B from the same *L. aequalis* complex was also found more widespread across the SJI, and was found more frequently at marine sites than low-salinity sites. Clade Y within the *L. pusilla* complex dominated locations near the SF Bay outflow in California, and Clade A from the same *L. pusilla* complex also dominated low-salinity sites in the San Juan archipelago. Given the recent extirpations of *L. pusilla* in Central California, populations in Washington could also be at similar risk. Signals of demographic change and purifying selection in *L. pusilla*, as well as its tendency for small body size and higher instances of injury could put *L. pusilla* at risk in the SJI.

However, over the 18 year period sampled in this study, clade composition overall has remained stable in the SJI, alluding to adaptive success of *Leptasterias* spp. to various environmental factors and disease epidemics. Resilience of these clades over time, with high abundance noted in contemporary sampling, bodes optimism in the face of potential extirpation, contrary to patterns seen in these complexes in California. While this study showed no significant species turnover throughout the SJI like in Central California, future scenarios of climate change could quickly change patterns. Rising sea levels, greater storm events, and increased estuarine outflow from snow melt could all impact the SJI region (Morrison, Quick, & Foreman, 2002). Continued monitoring of low-dispersing species facing these threats is needed to project future community dynamics.

REFERENCES

Aris-Brosou, S., & Excoffier, L. (1996). The impact of population expansion and mutation rate heterogeneity on DNA sequence polymorphism. Molecular Biology and Evolution, 13(3), 494–504. https://doi.org/10.1093/oxfordjournals.molbev.a025610

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., … Saunders, N. C. (1987). Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. Annual Review of Ecology and Systematics, 18(1), 489–522. https://doi.org/10.1146/annurev.es.18.110187.002421

Barbosa, S. S., Klanten, S. O., Puritz, J. B., Toonen, R. J., & Byrne, M. (2013). Very fine-scale population genetic structure of sympatric asterinid sea stars with benthic and pelagic larvae: Influence of mating system and dispersal potential. Biological Journal of the Linnean Society, 108(4), 821–833. https://doi.org/10.1111/bij.12006

Bashevkin, S. M., Lee, D., Driver, P., Carrington, E., & George, S. B. (2016). Prior exposure to low salinity affects the vertical distribution of Pisaster ochraceus (Echinodermata: Asteroidea) larvae in haloclines. Marine Ecology Progress Series, 542, 123–140. https://doi.org/10.3354/meps11563

Bates, A. E., Hilton, B. J., & Harley, C. D. G. (2009). Effects of temperature, season and locality on wasting disease in the keystone predatory sea star Pisaster ochraceus. Diseases of Aquatic Organisms, 86(3), 245–251. https://doi.org/10.3354/dao02125

Bingham, B. L., Burr, J., & Head, H. W. (2000). Causes and consequences of arm damage in the sea star Leptasterias hexactis. Canadian Journal of Zoology, 78, 596–605. https://doi.org/10.1139/z99-250

Braun,C., Fisher, R., Bucholz, J., D'Avignon, M., Jaffe, N., Park, S., Langhans, M., Barnette, S.R., Salesky, M., Gong, Z., Villegas, P., Weinberg, R., Chow, B., Eberl, R., and Cohen, C.S. (2016). Leptasterias spp. and Sea Star Wasting Disease: A Temperature and Salinity Challenge Experiment. Poster presentation. Western Society of Naturalists Annual Meeting. Monterey, CA November 10-13.

Byrne, M. (2011). Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. Oceanography and Marine Biology: An Annual Review, 49, 1–42. https://doi.org/10.1016/j.marenvres.2011.10.00

Chandler, V. K., & Wares, J. P. (2017). RNA expression and disease tolerance are associated with a "keystone mutation" in the ochre sea star Pisaster ochraceus . PeerJ, 5, e3696. https://doi.org/10.7717/peerj.3696

Chia, F. (1966). Systematics of the Siz-Rayed Sea Star, Leptasterias, in the Vicinity of San Juan Island, Washington, 15(4), 300–306.

Chia, F. (1968). Some observations on the development and cyclic changes of the oöcytes in a brooding starfish, Leptasterias hexactis. Journal of Zoology, 154(4), 453–461. https://doi.org/10.1111/j.1469-7998.1968.tb01677.x

Cho, S., Mitchell, A., Regier, J.C., Mitter, C., Poole, R.W., Friedlander, T.P., and Zhao, S. (1995). A Highly Conserved Nuclear Gene for Low-Level Phylogenetics: Elongation Factor -1a Recovers Morphology-based Tree for Heliothine Moths. Molecular Biology and Evolution, 12(4), 650–656.

Darriba D., Taboada, G.L., Doallo, R., and Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9(8), 772.

Duda, T. F., & Palumbi, S. R. (1999). Population structure of the black tiger prawn, Penaeus monodon, among western Indian Ocean and western Pacific populations. Marine Biology, 134(4), 705–710. https://doi.org/10.1007/s002270050586

Eisenlord, M. E., Groner, M. L., Yoshioka, R. M., Elliott, J., Maynard, J., Fradkin, S., … Harvell, C. D. (2015). Ochre star mortality during the 2014 wasting disease epizootic: role of population size structure and temperature. Philosophical Transactions B, 1–11. https://doi.org/10.1098/rstb.2015.0212

Emmett, R., Llansó, R., Newton, J., Thom, R., Hornberger, M., Morgan, C., … Llanso, R. (2000). Geographic Signatures of North American West Coast Estuaries. Estuaries, 23(6), 765. https://doi.org/10.2307/1352998

Excoffier, L., Laval, G., Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1, 47.

Flowers, J. M., & Foltz, D. W. (2001). Reconciling molecular systematics and traditional taxonomy in a species-rich clade of sea stars (Leptasterias subgenus Hexasterias). Marine Biology, 139(3), 475–483. https://doi.org/10.1007/s002270100595

Foltz, D. W., Bolton, M. T., Kelley, S. P., Kelley, B. D., & Nguyen, A. T. (2007). Combined mitochondrial and nuclear sequences support the monophyly of forcipulatacean sea stars. Molecular Phylogenetics and Evolution, 43(2), 627–634. https://doi.org/10.1016/j.ympev.2006.10.012

Foltz, D. W., Nguyen, A. T., Kiger, J. R., & Mah, C. L. (2008). Pleistocene speciation of sister taxa in a North Pacific clade of brooding sea stars (Leptasterias). Marine Biology, 154(3), 593–602. https://doi.org/10.1007/s00227-008-0952-9

Foltz, D W, Stickle, W. B., Campagnaro, E. L., & Himel, A. E. (1996). Mitochondrial DNA polymorphisms reveal additional genetic heterogeneity within the Leptasterias hexactis (Echinodermata: Asteroidea) species complex. Marine Biology, 125, 569–578.

Foltz, David W. (1997). Hybridization frequency is negatively correlated with divergence time of mitochondrial DNA haplotypes in a sea star (Leptasterias spp.) species complex. Evolution, 51(1), 283–288. https://doi.org/10.1668/0003-1569

Foltz, David W. (2003). Invertebrate Species with Nonpelagic Larvae Have Elevated Levels of Nonsynonymous Substitutions and Reduced Nucleotide Diversities. Journal of Molecular Evolution, 57(6), 607–612. https://doi.org/10.1007/s00239-003-2495-5

Foltz, David W., Hrincevich, A. W., & Rocha-Olivares, A. (2004). Apparent selection intensity for the cytochrome oxidase subunit i gene varies with mode of reproduction in echinoderms. Genetica, 122(2), 115–125. https://doi.org/10.1023/B:GENE.0000041002.97173.1e

George, S. B. (1994). Variation in Reproductive Investment, 109(1951), 95–98.

George, S.B. (1996). Echinoderm egg and larval quality as a function of adult nutritional state. Oceanologica Acta, 19(3-4), 297-308.

George, S. B. (1999). Egg quality, larval growth and phenotypic plasticity in a forcipulate seastar. Journal of Experimental Marine Biology and Ecology, 237(2), 203–224. https://doi.org/10.1016/S0022-0981(98)00205-6

George, S. B., & Walker, D. (2007). Short-term fluctuation in salinity promotes rapid larval development and metamorphosis in Dendraster excentricus. Journal of Experimental Marine Biology and Ecology, 349(1), 113–130. https://doi.org/10.1016/j.jembe.2007.05.010

Guindon, S. and Gascuel, O. (2003). A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood". Systematic Biology 52: 696-704.

Hart, M. W., Byrne, M., & Johnson, S. L. (2003). Patiriella pseudoexigua(Asteroidea: Asterinidae): a cryptic species complex revealed by molecular and embryological analyses. Journal of the Marine Biological Association of the UK, 83(05), 1109–1116.

Hart, M. W., Byrne, M., & Smith, M. J. (2017). Molecular Phylogenetic Analysis of Life-History Evolution in Asterinid Starfish Author (s): Michael W . Hart , Maria Byrne and Michael J . Smith Published by : Society for the Study of Evolution Stable URL : http://www.jstor.org/stable/2411007 JSTOR is , 51(6), 1848–1861.

Hart, M. W., & Marko, P. B. (2010). It's about time: Divergence, demography, and the evolution of developmental modes in marine invertebrates. Integrative and Comparative Biology, 50(4), 643–661. https://doi.org/10.1093/icb/icq068

Held, M. B. E., & Harley, C. D. G. (2009). Responses to low salinity by the sea star Pisaster ochraceus from high- and low-salinity populations. Invertebrate Biology, 128(4), 381–390. https://doi.org/10.1111/j.1744-7410.2009.00175.x

Hellberg, M. E. (1996). Dependence of Gene Flow on Geographic Distance in Two Solitary Corals with Different Larval Dispersal Capabilities Author (s): Michael E . Hellberg Published by : Society for the Study of Evolution Stable URL : http://www.jstor.org/stable/2410657. Society, 50(3), 1167–1175.

Hewson, I., Button, J. B., Gudenkauf, B. M., Miner, B., Newton, A. L., Gaydos, J. K., … Harvell, C. D. (2014). Densovirus associated with sea-star wasting disease and mass mortality. Proceedings of the National Academy of Sciences, 111(48), 17278–17283. https://doi.org/10.1073/pnas.1416625111

Highsmith, R. (1985). Floating and algal rafting as potential dispersal mechanisms in brooding invertebrates. Marine Ecology Progress Series, 25, 169–179. https://doi.org/10.3354/meps025169

Hrincevich, A.W., Rocha-Olivares, A., & Foltz, D. W. (2000). Phylogenetic Analysis of Molecular Lineages in a Species-Rich Subgenus of Sea Stars (Leptasterias Subgenus Hexasterias). American Zoologist, 40(3), 365–374. https://doi.org/10.1668/0003-1569(2000)040[0365:PAOMLI]2.0.CO;2

Huelsenbeck J.P., Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics, 17, 754-755.

Jacobs, D. K., Haney, T. A., & Louie, K. D. (2004). Genes, Diversity, and Geologic Process on the Pacific Coast. Annual Review of Earth and Planetary Sciences, 32(1), 601–652. https://doi.org/10.1146/annurev.earth.32.092203.122436

Kearse M, Moir R, Wilson A et al. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28, 1647-1649.

Kelly, R. P., & Palumbi, S. R. (2010). Genetic structure among 50 species of the northeastern pacific rocky intertidal community. PLoS ONE, 5(1). https://doi.org/10.1371/journal.pone.0008594

Kwast, K. E., Foltz, D. W., & Stickle, W. B. (1990). Population genetics and systematics of the Leptasterias hexactis (Echinodermata: Asteroidea) species complex. Marine Biology, 105(3), 477–489. https://doi.org/10.1007/BF01316319

Lee, H. J., & Boulding, E. G. (2009). Spatial and temporal population genetic structure of four northeastern Pacific littorinid gastropods: The effect of mode of larval development on variation at one mitochondrial and two nuclear DNA markers. Molecular Ecology, 18(10), 2166–2184. https://doi.org/10.1111/j.1365-294X.2009.04169.x

Leigh, J.W. & Bryant, D. (2015). PopART: Full-feature software for haplotype network construction. Methods Ecol Evol 6(9):1110–1116.

Light, S. F., Carlton, J. T., & Light, S. F. (2007). The Light and Smith manual : intertidal invertebrates from central California to Oregon. Retrieved from https://www.ucpress.edu/book/9780520239395/the-light-and-smith-manual

Maddison, W. P. & Maddison D.R. (2018). Mesquite: a modular system for evolutionary analysis. Version 3.6 http://www.mesquiteproject.org

Maltagliati, F., Di Giuseppe, G., Barbieri, M., Castelli, A., & Dini, F. (2010). Phylogeography and genetic structure of the edible sea urchin Paracentrotus lividus (Echinodermata: Echinoidea) inferred from the mitochondrial cytochrome b gene. Biological Journal of the Linnean Society, 100(4), 910–923. https://doi.org/10.1111/j.1095-8312.2010.01482.x

Marko, P. B. (2004). "What's larvae got to do with it?" Disparate patterns of post-glacial population structure in two benthic marine gastropods with identical dispersal potential. Molecular Ecology, 13(3), 597–611. https://doi.org/10.1046/j.1365-294X.2004.02096.x

Maroni, G. (1993). An Atlas of Drosophila Genes : Sequences and Molecular Features. Retrieved June 6, 2019, from http://books.google.com/books?id=fSHbUGXsZq4C&printsec=frontcover&dq=intitle:An+atlas+of+Drosophila+genes+sequences+and+molecular+features&hl=&cd=1&source=gbs_api

Melroy, L.M. (2016). Temporal and spatial variation in population structure of a direct-developing cryptic species complex, Leptasterias spp. (Master's thesis). San Francisco State University, San Francisco.

Melroy, L. M., Smith, R. J., & Cohen, C. S. (2017). Phylogeography of direct-developing sea stars in the genus Leptasterias in relation to San Francisco Bay outflow in central California. Marine Biology, 164(7), 1–14. https://doi.org/10.1007/s00227-017-3184-z

Menge, B. A. (1972). Foraging Strategy of a Starfish in Relation to Actual Prey Availability and Environmental Predictability, 42(1), 25–50.

Menge, B. A. (1974). Effect of Wave Action and Competition on Brooding and Reproductive Effort in the Seastar , Leptasterias hexactis. Ecology, 55(1), 84–93. https://doi.org/10.2307/1934620

Menge, B. A. (1975). Brood of Broadcast? The Adaptive Significance of Different Reproductive Strategies in the Two Intertidal Sea Stars Leptasterias hexactis and Pisaster ochraceus. Marine Biology, 31, 87–100.

Menge, B. A., Cerny-Chipman, E. B., Johnson, A., Sullivan, J., Gravem, S., & Chan, F. (2016). Sea Star Wasting Disease in the Keystone Predator Pisaster ochraceus in Oregon: Insights into Differential Population Impacts, Recovery, Predation Rate, and Temperature Effects from Long-Term Research. PloS One, 11(5), e0153994. https://doi.org/10.1371/journal.pone.0153994

Morrison, J., Quick, M. C., & Foreman, M. G. G. (2002). Climate change in the Fraser River watershed: Flow and temperature projections. Journal of Hydrology, 263(1–4), 230–244. https://doi.org/10.1016/S0022-1694(02)00065-3

Nahavandi, N., Ketmaier, V., & Tiedemann, R. (2012). Intron structure of the elongation factor 1-alpha gene in the ponto-caspian amphipod pontogammarus maeoticus (Sowinsky, 1894) and its phylogeographic utility. Journal of Crustacean Biology, 32(3), 425–433. https://doi.org/10.1163/193724012X626584

Nei, M., & Gojobori, T. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Molecular Biology and Evolution. https://doi.org/10.1093/oxfordjournals.molbev.a040410

Nylander, J.A.A. (2004). MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Palumbi, S. R. (1994). Genetic divergence, reproductive isolation, and marine speciation. Annual Review of Ecology and Systematics, 25, 547–572. https://doi.org/10.1146/annurev.es.25.110194.002555

Pankey, M. S., & Wares, J. P. (2009). Overdominant maintenance of diversity in the sea star Pisaster ochraceus. Journal of Evolutionary Biology, 22(1), 80–87. https://doi.org/10.1111/j.1420-9101.2008.01623.x

Pia, T. S., Johnson, T., & George, S. B. (2012). Salinity-induced morphological changes in Pisaster ochraceus (Echinodermata: Asteroidea) larvae. Journal of Plankton Research, 34(7), 590–601. https://doi.org/10.1093/plankt/fbs032

Rambaut, A. (2009). FigTree. Retrieved from http://tree.bio.ed.ac.uk/software/figtree/.

Rasband, W.S. (1996-2018). ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/

Roden, G. I. (1967). On river discharge into the northeastern Pacific Ocean and the Bering Sea. Journal of Geophysical Research, 72(22), 5613. https://doi.org/10.1029/JZ072i022p05613

Rogers, A.R. & Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. Molecular Biology and Evolution, 9(3), 552–569.

Roller, R. A., & Stickle, W. B. (1985). Effects of salinity on larval tolerance and early developmental rates of four species of echinoderms. Canadian Journal of Zoology, 63(7), 1531–1538. https://doi.org/10.1139/z85-227

Roller, R. A., & Stickle, W. B. (1993). Effects of temperature and salinity acclimation of adults on larval survival , physiology , and early development of Lytechinus variegatus (Echinodermata: Echinoidea). Marine Biology, 116, 583–591.

Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X., & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics, 19, 2496-2497.

Schiebelhut, L. M., Puritz, J. B., & Dawson, M. N. (2018). Decimation by sea star wasting disease and rapid genetic change in a keystone species, Pisaster ochraceus . Proceedings of the National Academy of Sciences, 115(27), 7069–7074. https://doi.org/10.1073/pnas.1800285115

Shirley, T. C., & Stickle, W. B. (1982). Responses of Leptasterias hexactis (Echinodermata: Asteroidea) to low salinity - I. Survival, activity, feeding, growth and absorption efficiency. Marine Biology. https://doi.org/10.1007/BF00396894

Strathmann, R. R. (1986). What controls the type of larval development? Summary statement for the evolution session. Bulletin of Marine Science, 39(2), 616–622.

Sutherland, D. A., MacCready, P., Banas, N. S., & Smedstad, L. F. (2011). A Model Study of the Salish Sea Estuarine Circulation * . Journal of Physical Oceanography, 41(6), 1125–1143. https://doi.org/10.1175/2011jpo4540.1

Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tamura K., Peterson D., Peterson N., et al. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution, 28, 2731-2739.

Thomson, R. E. (1981). Oceanography of the British Columbia Coast. Canadian special publications of fisheries and aquatic sciences (Vol. 56). Retrieved from http://www.dfo-mpo.gc.ca/Library/487.pdf

Wares, J. P. (2019). Re-evaluation of homozygous lethality for EF1A indel polymorphism in Pisaster ochraceus. PeerJ, 1–19.

Wares, J. P., & Schiebelhut, L. M. (2016). What doesn't kill them makes them stronger: an association between elongation factor 1- α overdominance in the sea star Pisaster ochraceus and "sea star wasting disease." PeerJ, 4, e1876. https://doi.org/10.7717/peerj.1876

Waters, J. M., O'Loughlin, P. M., & Roy, M. S. (2004). Cladogenesis in a starfish species complex from southern Australia: Evidence for vicariant speciation? Molecular Phylogenetics and Evolution, 32(1), 236–245. https://doi.org/10.1016/j.ympev.2003.11.014

Yamada, S. B. (1989). Are direct developers more locally adapted than planktonic developers? Marine Biology, 103(3), 403–411. https://doi.org/10.1007/BF00397275

Table 1 – Number of samples, year(s) sampled, and exposure characterization for all sites.

Location		Site Code	Lat, Long	Exposure	Year	COI		EF1-α INT4	
Contemporary sites	*Locale*					n	Nh (Pr)	n	
Deception Pass (subtidal)	Anacortes	DE	48.41, -122.65	Low-salinity	2016	2	2		
Friday Harbor	San Juan Island	FHL	48.55, -123.01	Low-salinity	2016	21	6 (2)	5	
Hat Island	Padilla Bay	HI	48.52, -122.54	Low-salinity	2016	2	1		
Lonesome Cove	San Juan Island	LCO	48.62, -123.11	Low-salinity	2016	19	3		
Camp Orkila	Orcas Island	OCO	48.70, -122.95	Low-salinity	2016	22	3		
Point Doughty	Orcas Island	OPD	48.70, -122.94	Low-salinity	2016	11	2		
Reuben Tarte	San Juan Island	RET	48.61, -123.10	Low-salinity	2015, 2016	5,19	5 (1)	3	
Saddlebag Island	Padilla Bay	SI	48.53, -122.55	Low-salinity	2016	2	2 (1)		
Skyline (subtidal)	Anacortes	SK	48.49, -122.69	Low-salinity	2016	1	1		
Shannon Point Beach	Anacortes	SPB	48.50, -122.67	Low-salinity	2016	9	3	3	
Griffin Bay	San Juan Island	GB	48.51, -123.02	Low-salinity	2014, 2015	9, 19	5 (2)		
MacKaye Harbor	Lopez Island	LMH	48.44, -122.86	Low-salinity	2016	12	3 (1)		
Cattle Point	San Juan Island	CP	48.45, -122.96	Marine	2016	16	4		
Deadman Bay	San Juan Island	DBS	48.51, -123.14	Marine	2016	15	2 (1)		
Eagle Cove	San Juan Island	EAC	48.46, -123.03	Marine	2016	13	2		
False Bay	San Juan Island	FBS	48.48, -123.07	Marine	2015, 2016	4, 20	3	10	
Smugglers Cove	San Juan Island	SMC	48.56, -123.18	Marine	2016	9	2	1	
Historic sites	*Locale*		*CAS ID*			N	Nh (Pr)		
Friday Harbor	San Juan Island	FHL	193795	Low-salinity	1998	4	3 (1)		
Anacortes	Anacortes	ANA	193798	Low-salinity	1998	15	3		
Clallam Bay	Sekiu	CB	166537, 193796	Marine	1991, 1998	19	3 (1)		

Figure 1 – a) COI haplotype Maximum Likelihood phylogeny. Black circles indicate nodal support: left shading indicates Baysian posterior probabilities >95%, and left shading indicates bootstrap support of >70%. *Leptasterias camtschatica* is a sister taxa to these three clades and is used as an outgroup. Colors indicate COI clades identified in Foltz 2008 and Melroy et al. 2016, 2017. b) Collapsed Bayesian tree to the right indicates three species complexes with Bayesian posterior probabilities.

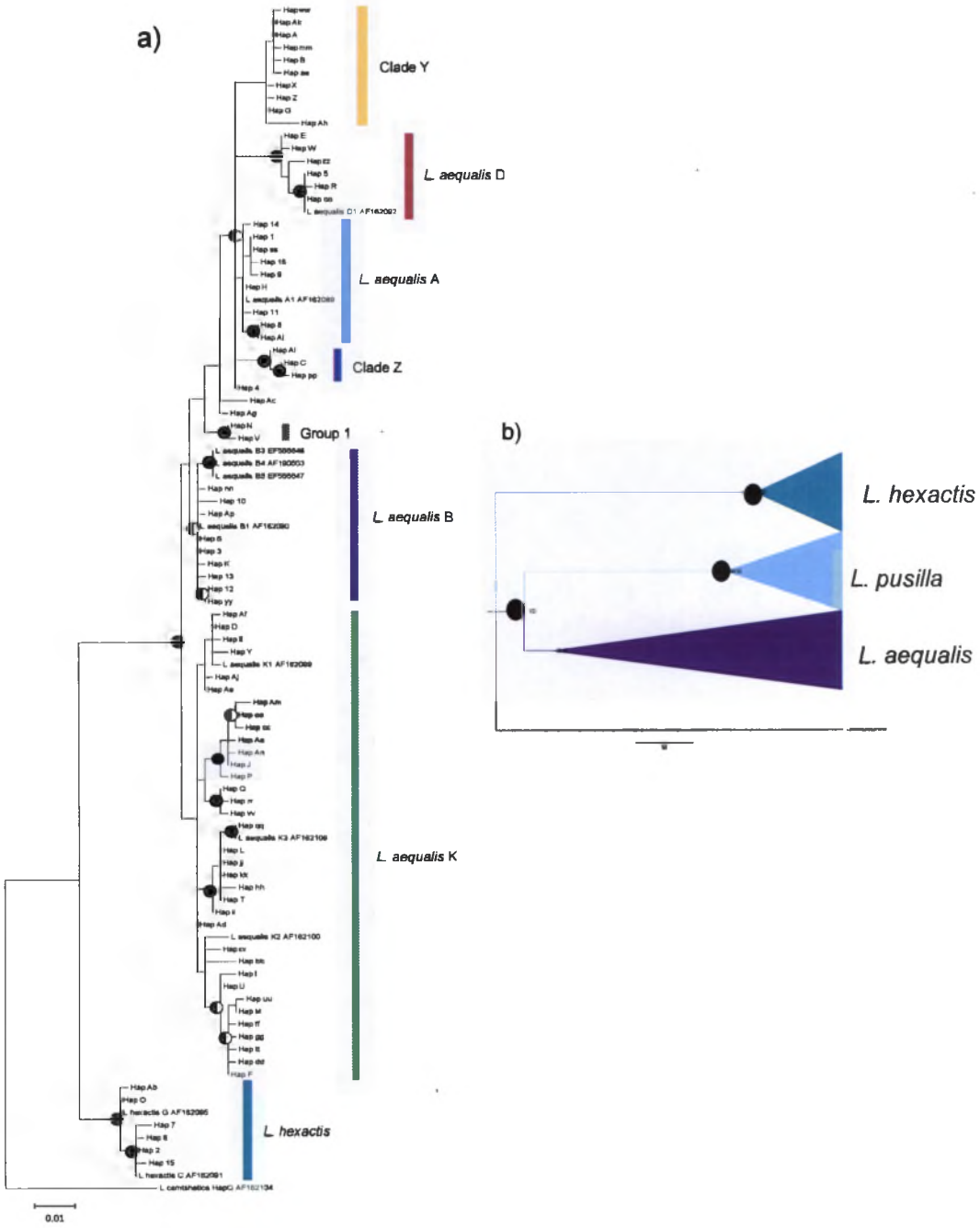


Table 2 – TN93 genetic distances within and among COI clades plus standard error by bootstrap.

	Within COI clades	L. aequalis	L. pusilla	L. hexactis
L. aequalis	0.001±0.000	-	-	-
L. pusilla	0.004±0.002	0.009±0.004	-	-
L. hexactis	0.001±0.000	0.041±0.011	0.049±0.012	-

Table 3 – Molecular diversity indices and neutrality statistics for *L. aequalis* and *L. pusilla* by location. Bold values indicate Bonferroni corrected significance of $p < 0.0125$. *Smaller populations near Anacortes (DE, SK, SI, HI) were grouped for analyses.

Exposure	Site	n	Hd ± SE	π ± SE	Tajima's D	Fu and Li's D	Fu and Li's F	Fu's Fs
Low-salinity	***ANA**	*17*	0.419±0.141	0.003±0.001	**-0.881**	0.594	0.219	16.554
Low-salinity	*FHL*	*25*	0.767±0.068	0.004±0.001	**-0.849**	**-0.838**	**-0.98**	25.721
Low-salinity	GB	28	0.648±0.089	0.008±0.001	1.300	1.009	1.284	36.737
Low-salinity	LCO	17	0.382±0.113	0.002±0.000	0.730	0.895	0.973	32.027
Low-salinity	LMH	12	0.621±0.118	0.002±0.000	0.22	0.972	0.885	23.382
Low-salinity	OCO	22	0.593±0.085	0.002±0.000	1.318	0.992	1.247	34.252
Low-salinity	OPD	11	0.182±0.144	0.001±0.001	-1.6	-1.874	-2.031	7.524
Low-salinity	RET	23	0.447±0.118	0.005±0.004	**-2.31**	**-3.259**	**-3.47**	31.252
Low-salinity	SPB	8	0.250±0.180	0.00049±0.00035	-1.055	-1.126	-1.203	16.693
Marine	CP	5	0.400±0.237	0.002±0.001	-0.972	-0.972	-0.954	12.178
Marine	DBS	9	0.500±0.128	0.001±0.000	0.986	0.84	0.962	0
Marine	FBS	7	0.476±0.171	0.002±0.001	0.687	1.178	1.145	17.557
Marine	SMC	5	0.600±0.175	0.001±0.000	1.225	1.225	1.157	0

Table 4 – Haplotype and nucleotide (as percentage plus standard error) diversity indices and neutrality statistics by COI Clade. Bold values indicate Bonferroni corrected significance of $p < 0.0125$.

COI Clade	n	Nh	Hd ± SE	π ± SE	Tajima's D	Fu and Li's D	Fu and Li's F	Fu's Fs
L. aequalis	41	4	0.229±0.085	0.072±0.030	-1.643	-1.114	-1.483	-2.253
L. pusilla	151	9	0.649±0.025	0.36±0.066	**-2.168**	**-7.212**	**-6.2038**	-1.148
L. hexactis	76	3	0.172±0.056	0.049±0.016	-0.932	-1.00426	-1.14552	-1.44

Table 5 – AMOVA analyses using Tamura & Nei distances for 10,000 permutations.

Hypothesis	Source of variation	Variance components	Percentage of variation	Fixation index	*P*
No spatial/exposure groups	Among populations	3.3	53.67		
	Within populations	2.85	46.33	F_{ST} =0.68	<0.001
All *Leptasterias* clades by exposure	Between low-salinity and marine sites	5.49	61.63	F_{CT}=0.62	<0.001
	Among populations within groups	0.57	6.41	F_{SC}=0.17	<0.001
	Within populations	2.85	31.96	F_{ST}=0.68	<0.001
All *Leptasterias clades* by island geography (five groups)	Among islands	0.93	14.33	F_{CT} =0.14	0.14
	Among populations within islands	2.74	42.05	F_{SC} =0.49	<0.001
	Within populations	2.85	43.62	F_{ST} =0.56	<0.001
L. pusilla and *L. aequalis* clades by exposure	Between low-salinity and marine sites	1.46	53.37	F_{CT} =0.53	<0.001
	Among populations within groups	0.397	14.48	F_{SC} =0.31	<0.001
	Within populations	0.882	32.15	F_{ST} =0.68	<0.001
L. pusilla and *L. aequalis* clades by island geography (five groups)	Among islands	0.58	30.18	F_{CT} =0.30	0.027
	Among populations within islands	0.46	24.06	F_{SC} =0.34	<0.001
	Within populations	0.881	45.76	F_{ST} =0.54	<0.001

Figure 2 – Clade frequencies for contemporary and historic samples by site in the San Juan Islands. Years are noted for samples collected prior to 2016. Colors correspond with clades in Figure 2, and size of circles represents relative sample size. The top left insert indicates direction of low-salinity plume and marine influx into the San Juan Island archipelago, with blue indicating low-salinity-exposed, and orange as marine-exposed.

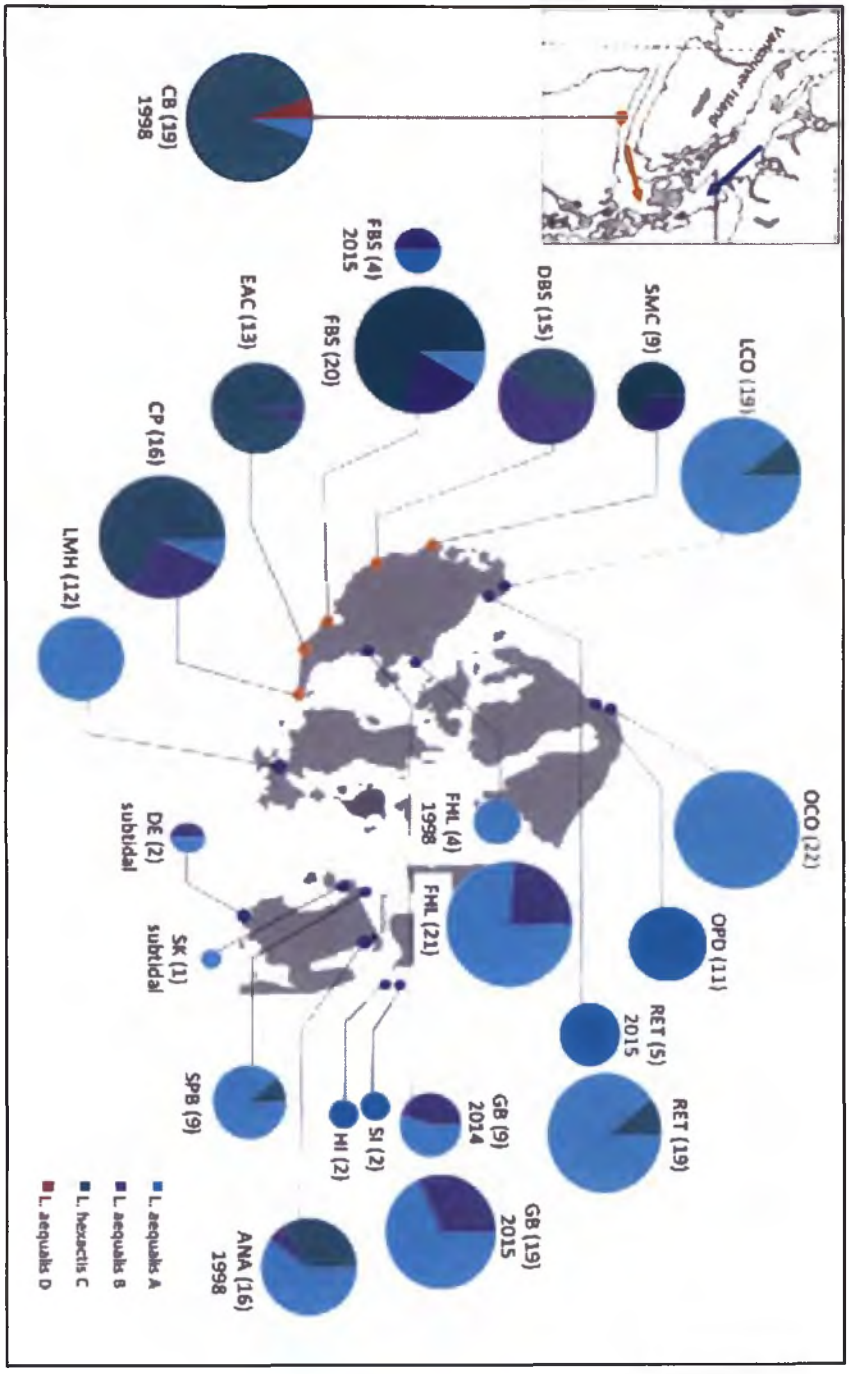


Figure 3 – Haplotype frequencies for contemporary and historic samples by site. Colors represent different haplotypes, with black representing private haplotypes. Numbers in black wedges indicate number of private haplotypes found within the site. The top left insert indicates direction of estuarine plume and marine influx into the San Juan Island archipelago, with blue indicating low-salinity-exposed, and orange as marine-exposed.

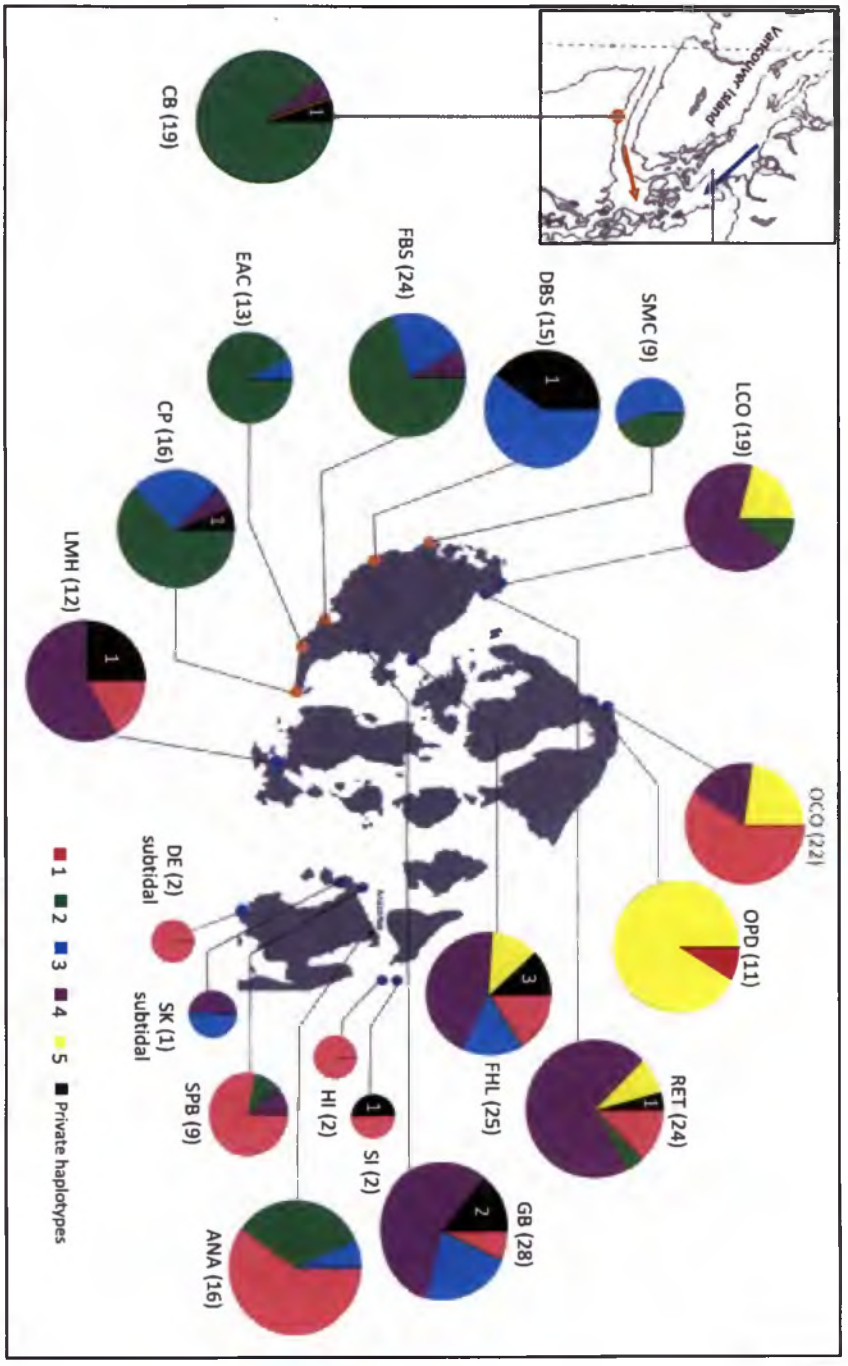


Figure 4 – Minimum spanning network of 16 COI haplotypes. Black circles represent mutational steps. Darker shades are populations more low-salinity exposed, and lighter shades are populations more marine exposed.

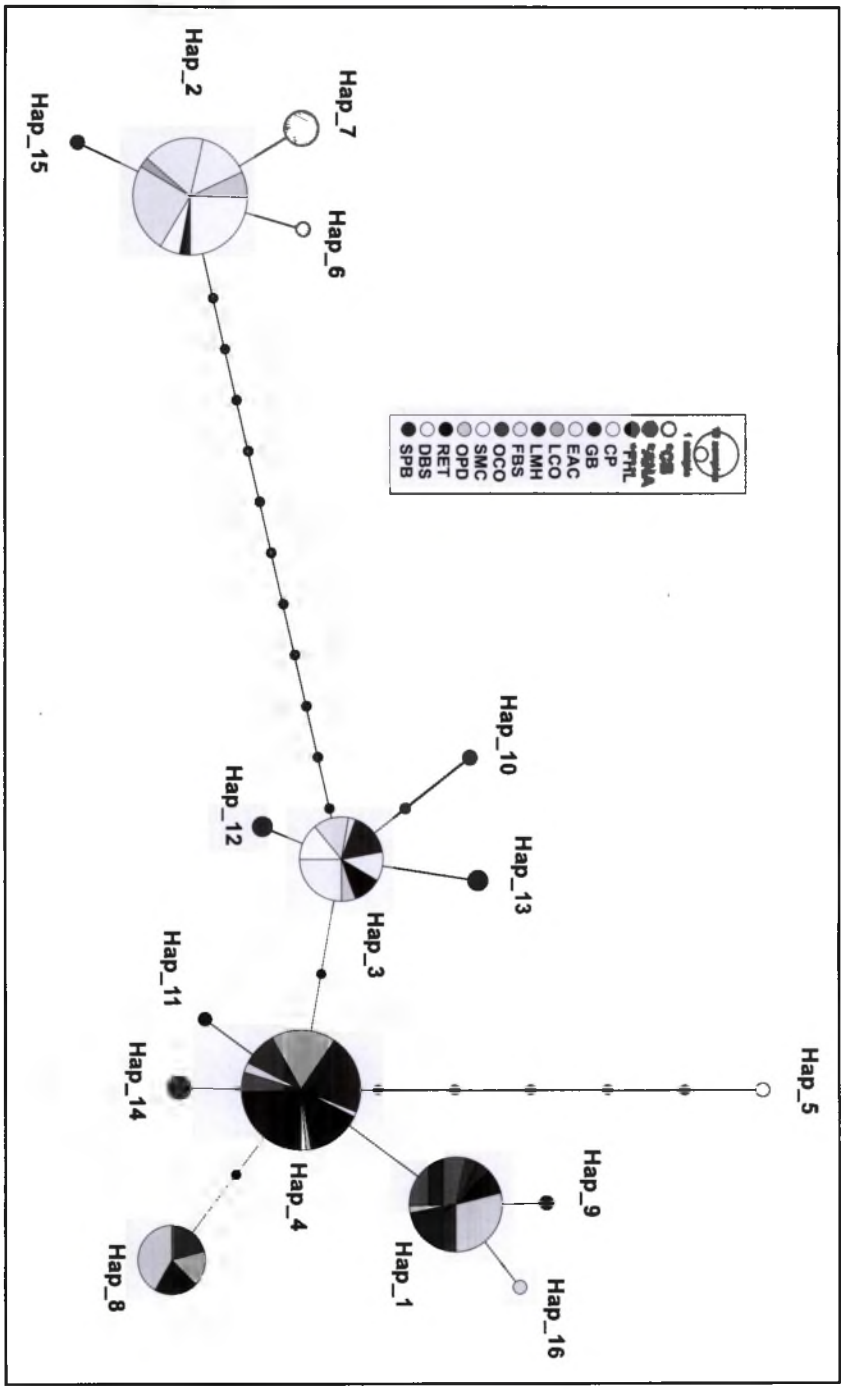


Table 6 – *L. aequalis* and *L. pusilla* Φ_{ST} values below diagonal, F_{ST} values above diagonal. Orange denotes marine sites, blue for low-salinity sites. Bold values are significant at $p < 0.05$.

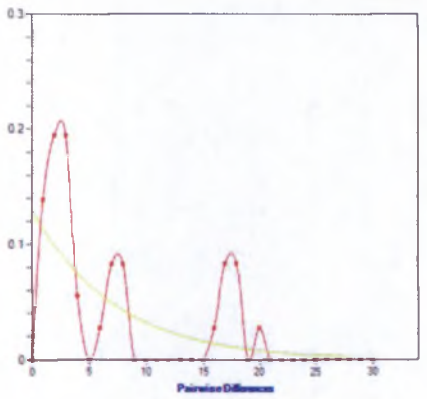
	ANA	SPB	CP	DBS	EAC	FBS	SMC	FHL	RET	GB	LCO	OPD	OCO	LMH	CB
ANA	-	-0.048	**0.538**	**0.703**	0.525	**0.511**	**0.562**	**0.274**	**0.493**	**0.392**	**0.580**	**0.656**	0.055	**0.390**	0.467
SPB	-0.046	-	0.683	**0.883**	0.750	**0.630**	**0.708**	**0.297**	**0.523**	**0.422**	**0.630**	**0.771**	0.046	**0.414**	0.578
CP	**0.714**	**0.919**	-	0.126	-1.000	-0.183	-0.220	**0.193**	**0.492**	**0.226**	**0.542**	**0.747**	**0.445**	**0.390**	0.372
DBS	**0.820**	**0.982**	0.126	-	0.000	0.214	0.072	**0.427**	**0.699**	**0.475**	**0.758**	**0.900**	**0.614**	**0.654**	**0.866**
EAC	0.720	0.962	-1.000	0.000	-	-0.667	-1.000	0.087	0.553	0.192	0.618	0.818	0.407	0.379	0.000
FBS	**0.691**	**0.888**	-0.183	0.214	-0.667	-	-0.137	**0.148**	**0.421**	0.165	**0.466**	**0.699**	**0.418**	**0.329**	0.291
SMC	**0.735**	**0.927**	-0.220	0.072	-1.000	-0.137	-	**0.237**	**0.530**	**0.274**	**0.580**	**0.763**	**0.474**	**0.437**	0.461
FHL	**0.165**	**0.304**	**0.434**	**0.602**	0.402	**0.396**	**0.468**	-	0.056	0.003	**0.085**	**0.394**	**0.147**	0.026	-0.065
RET	0.035	**0.175**	**0.434**	**0.598**	0.386	**0.403**	**0.468**	-0.041	-	0.046	-0.002	**0.618**	**0.325**	0.032	0.076
GB	**0.465**	**0.618**	0.196	**0.434**	0.115	0.142	0.242	**0.133**	**0.172**	-	**0.087**	**0.528**	**0.280**	0.040	-0.024
LCO	**0.215**	**0.549**	**0.728**	**0.856**	0.777	**0.681**	**0.750**	-0.021	0.005	**0.298**	-	**0.621**	**0.388**	0.103	0.145
OPD	**0.588**	**0.832**	**0.918**	**0.969**	0.944	**0.897**	**0.925**	**0.478**	**0.406**	**0.713**	**0.583**	-	**0.427**	**0.585**	**0.698**
OCO	0.051	0.078	**0.812**	**0.883**	**0.834**	**0.790**	**0.825**	**0.240**	**0.118**	**0.569**	**0.246**	**0.512**	-	**0.239**	0.255
LMH	0.121	**0.474**	**0.794**	**0.907**	0.838	**0.752**	**0.813**	0.058	-0.005	**0.383**	**0.131**	**0.723**	**0.225**	-	-0.026
CB	0.446	0.686	0.657	**0.881**	0.099	0.660	0.711	0.329	0.251	**0.520**	0.560	**0.757**	**0.580**	0.551	-

Table 7 – *L. hexactis* Φ_{ST} values below diagonal, F_{ST} values above diagonal. Orange denotes marine sites, blue for low-salinity sites. Bold values are significant at $p < 0.05$.

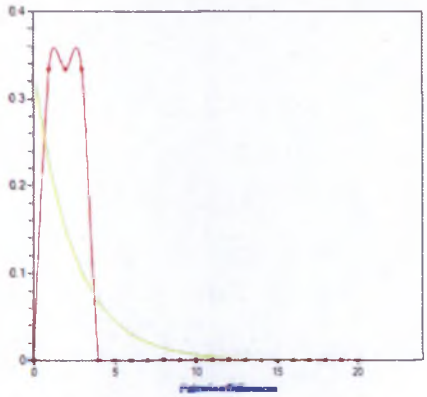
	ANA	CB	CP	DBS	EAC	FBS	LCO	RET	SMC	SPB
ANA	-	0.000	-0.089	**1.000**	0.000	0.000	0.000	0.000	0.000	0.000
CB	0.000	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
CP	-0.089	0.042	-	**0.943**	0.008	0.042	-0.325	-1.000	-0.125	-1.000
DBS	**1.000**	**1.000**	**0.943**	-	**1.000**	**1.000**	**1.000**	1.000	**1.000**	1.000
EAC	0.000	0.000	0.008	**1.000**	-	0.000	0.000	0.000	0.000	0.000
FBS	0.000	0.000	0.042	**1.000**	0.000	-	0.000	0.000	0.000	0.000
LCO	0.000	0.000	-0.325	**1.000**	0.000	0.000	-	0.000	0.000	0.000
RET	0.000	0.000	-1.000	1.000	0.000	0.000	0.000	-	0.000	0.000
SMC	0.000	0.000	-0.125	**1.000**	0.000	0.000	0.000	0.000	-	0.000
SPB	0.000	0.000	-1.000	1.000	0.000	0.000	0.000	0.000	0.000	-

Figure 5 – Mismatch distribution of pairwise distances for COI haplotypes for: a) *Leptasterias pusilla* (Harpendings raggedness index r=0.37, p-value=0.27, SSD = 0.12, p-value=0.09), b) *Leptasterias aequalis* (Harpendings raggedness index r=0.39, p-value=0.93), and c) *Leptasterias hexactis* (Harpendings raggedness value r=0.60, p-value=0.85) (computed in DnaSP v.5.10 and Arlequin v.3.5).

a)



b)



c)

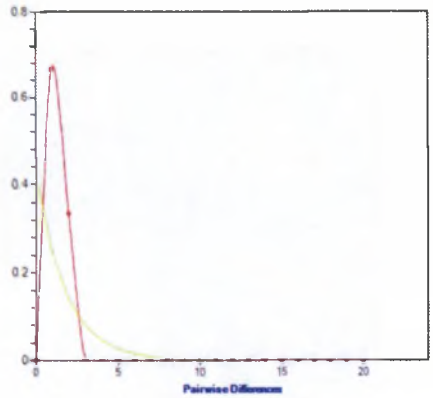
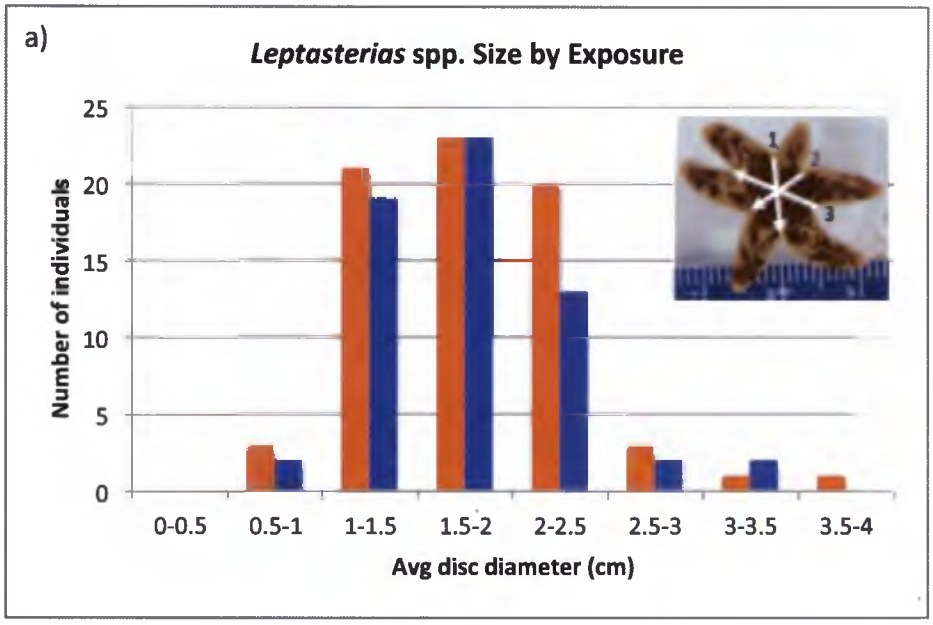


Figure 6 – Frequency histogram of *Leptasterias* individuals by size (average disc diameter), a) comparing marine and low-salinity sites (two-way ANOVA, $F_{1,129} = 0.155$, $p = 0.694$) and b) comparing COI clades (two-way ANOVA, $F_{2,129} = 5.86$, $p = 0.0036$). Figure 6 c) shows a box plot of all variables considered. Insert shows an example of three disc diameter measurements that were taken to obtain sea star size.



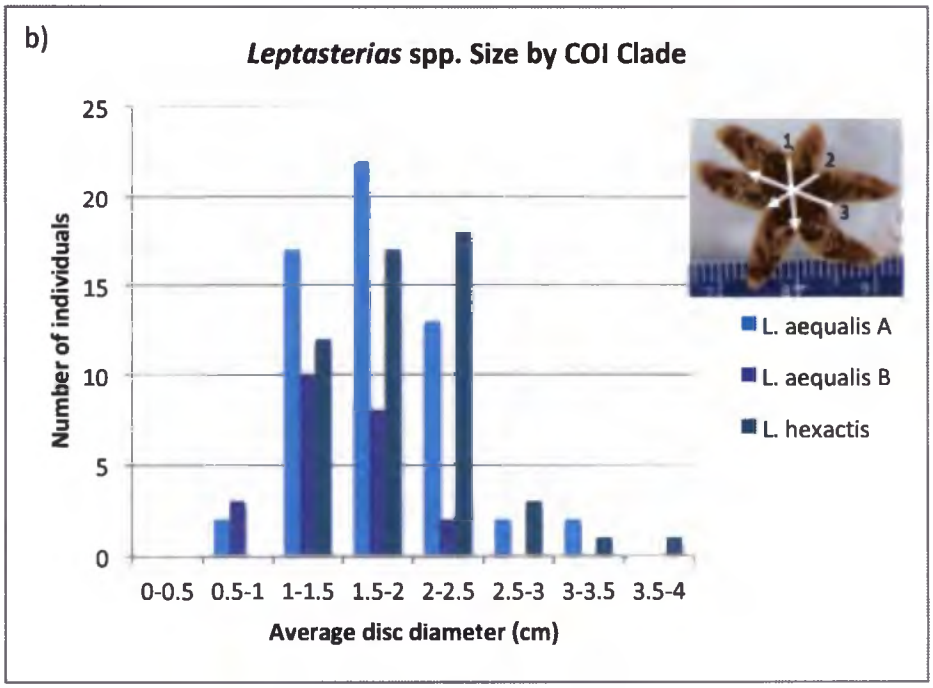


Figure 6 – Frequency histogram of *Leptasterias* individuals by size (average disc diameter), a) comparing marine and low-salinity sites (two-way anova, 0.155, p = 0.694) and b) comparing COI clades (one-way anova, $F_{2,129} = 5.86$, p = 0.0036). Figure 6 c) shows a box plot of all variables considered (size is in cm).

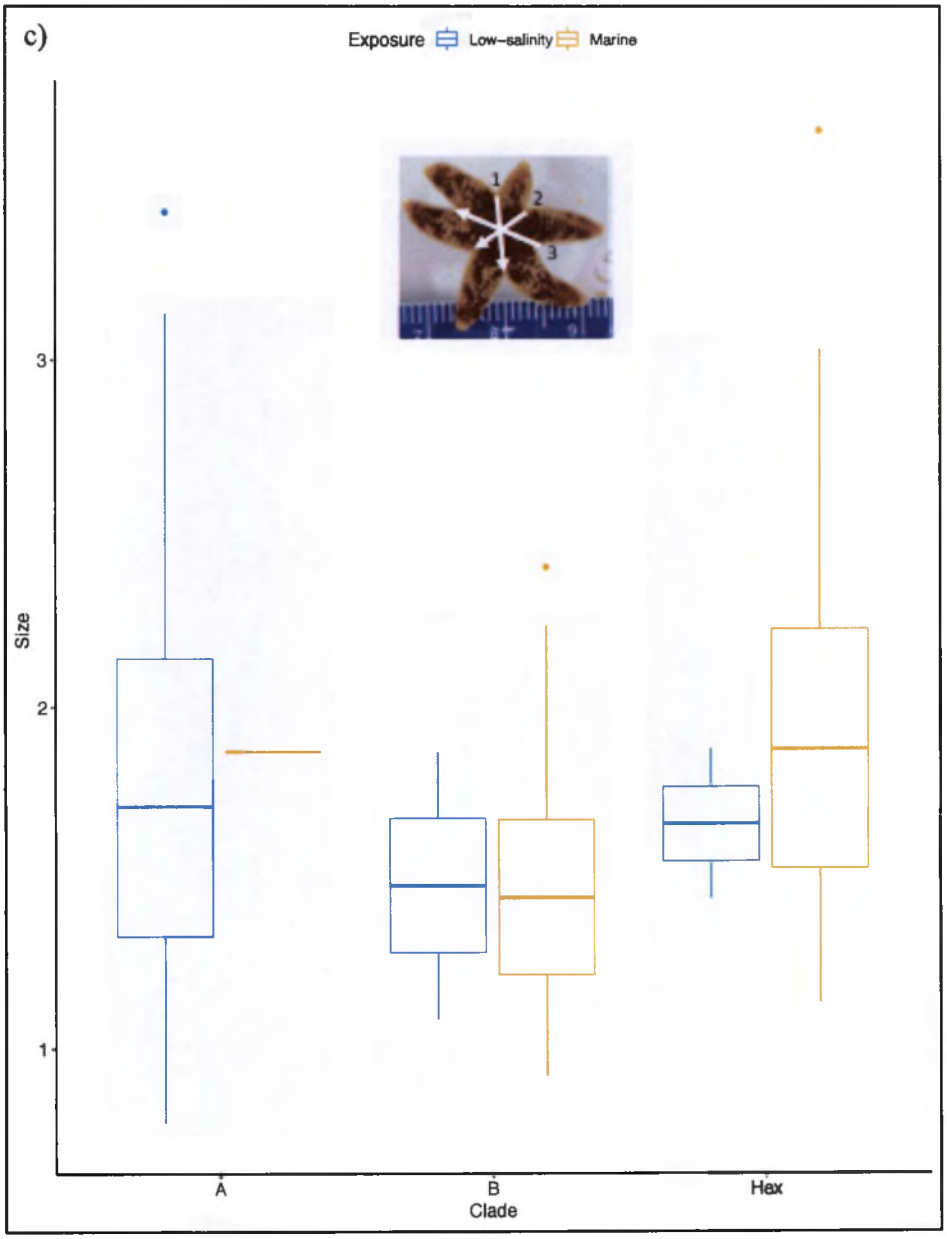


Figure 7 – MUSCLE alignment of 22 EF1-α INT4 forward sequences highlighting differences between 1000bp and 2000bp amplicons. Color bars on right indicate a) COI species corresponding with colors from Fig 2, and b) exposure characterization, orange as marine and blue as low salinity.

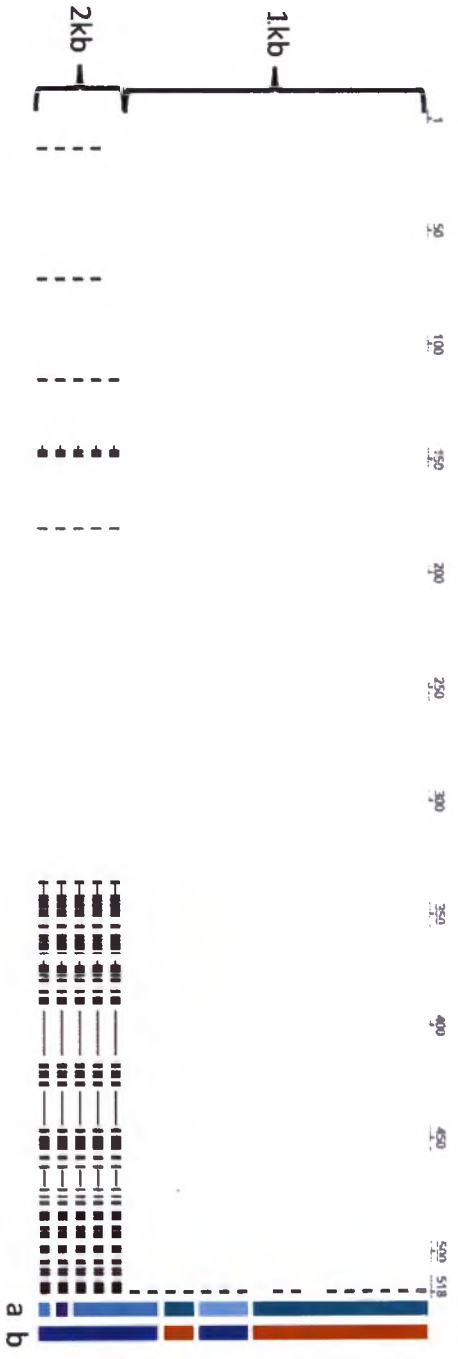


Table 8a – Injury observations from contemporary field sampling by site and exposure.

Site Code	# injured individuals	total # stars collected in field	% injured individuals observed per site	% injured individuals observed overall
CP	4	20	20.0%	13%
DBS	3	38	7.9%	10%
EAC	2	26	7.7%	7%
FBS	1	21	4.8%	3%
SMC	3	12	25.0%	10%
Marine Total	17	117	14.5%	6.61%
FHL	8	34	23.5%	27%
LCO	3	35	8.6%	10%
LMH	4	28	14.3%	13%
OPD	1	26	3.8%	3%
RET	1	17	5.9%	3%
Low-salinity Total	13	140	9.3%	5.06%

Table 8b – Proportion of injured individuals among COI clades.

COI clade	#injured individuals	% injured individuals observed overall
L. pusilla	15	51.7%
L. aequalis	8	27.6%
L. hexactis	7	24.1%

Table 9 – Codon-based Test of Positive Selection for combined coding regions COI (378bp) and ND4L (90bp). There was no signal of positive selection among species. The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) in favor of the alternative hypothesis (positive selection, dN > dS) (in the Probability column) is shown. dS and dN are the numbers of synonymous and nonsynonymous substitutions per site, respectively. The variance of the difference was computed using the Nei-Gojobori method for 1000 replicates. All positions containing gaps and missing data were eliminated.

COI species	Probability*	Test statistic (dN-dS)
L. pusilla	1.000	-2.185
L. hexactis	1.000	-0.930
L. aequalis	0.487	0.034
*rejecting the null in favor of the alternative hypothesis (dN>dS), significance is p<0.05		

Appendix 1 – Catalogue of *Leptasterias* spp. photos taken in August 2016 at Friday Harbor Labs, organized by clade and site. Star ID and ruler (mm) are included in photos. Photos with a yellow border indicate stars showing injury symptoms and included in Tables 8a and 8b.

Leptasterias aequalis A

Friday Harbor (FHL)

Leptasterias aequalis A

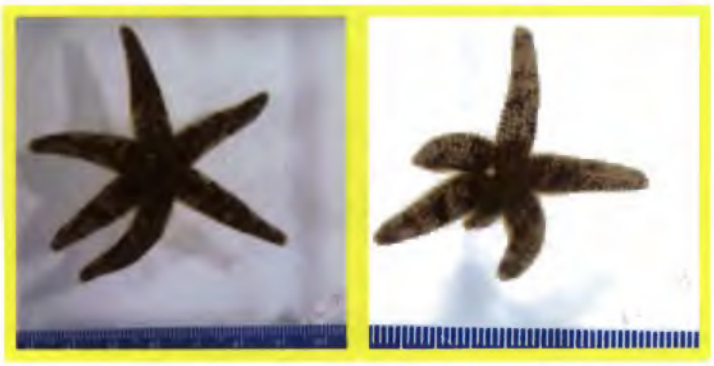
Friday Harbor (FHL)

Leptasterias aequalis A

Lonesome Cove (LCO)

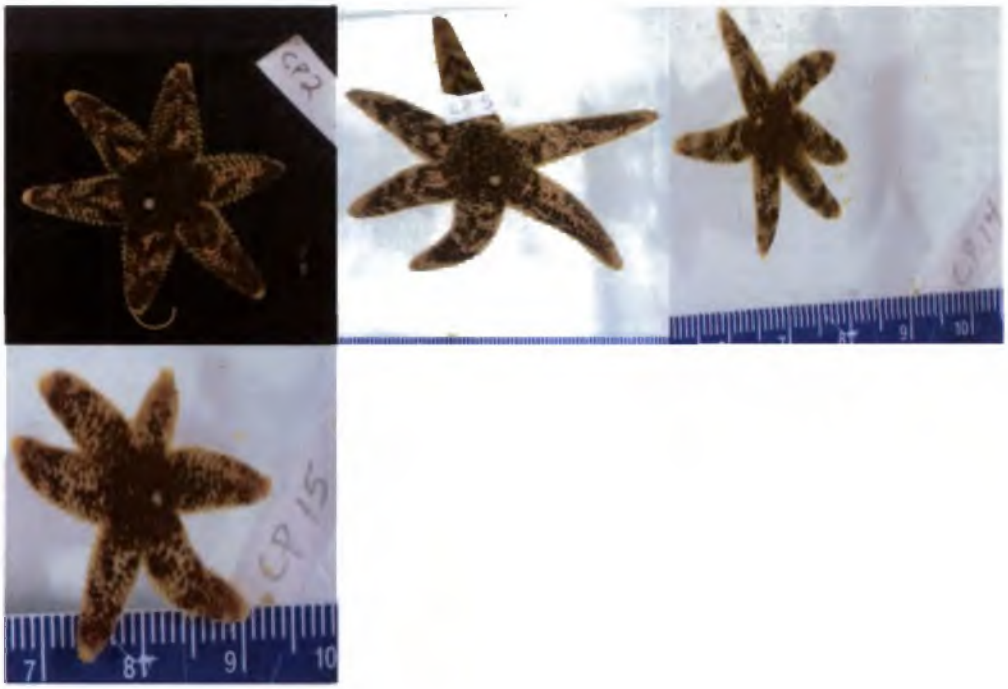
Leptasterias aequalis B

Friday Harbor (FHL)



Leptasterias aequalis B

Cattle Point (CP)

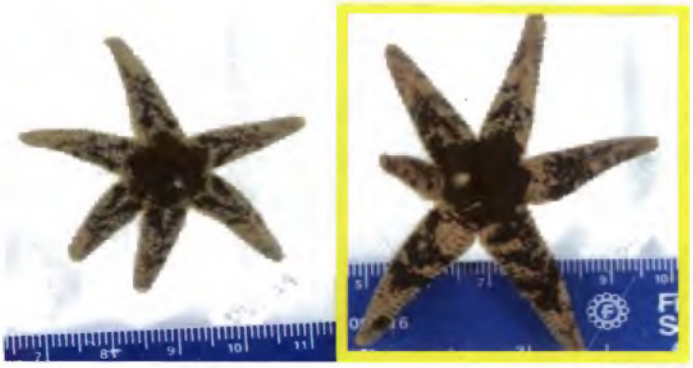


Leptasterias aequalis B

Deadman Bay (DBS)

Leptasterias aequalis B

Deadman Bay (DBS)



Leptasterias aequalis B

Eagle Cove (EAC)

Leptasterias hexactis

Cattle Point (CP)

Leptasterias hexactis

Cattle Point (CP)

Leptasterias hexactis

Deadman Bay (DBS)

Leptasterias hexactis

Deadman Bay (DBS)

Leptasterias hexactis

False Bay (FBS)