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Little evidence for genetic variation associated with susceptibility to sea star wasting syndrome in the keystone species *Pisaster ochraceus*

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Abstract

The keystone species *Pisaster ochraceus* suffered mass mortalities along the northeast Pacific Ocean from Sea Star Wasting Syndrome (SSWS) outbreaks in 2013–2016. SSWS causation remains of debate, leading to concerns as to whether outbreaks will continue to impact this species. Considering the apparent link between ocean temperature and SSWS, the future of this species and intertidal communities remains uncertain. Surveys of co-occurring apparently normal and wasting *P. ochraceus* along the central Oregon coast in 2016 allowed us to address whether variation in disease status showed genetic variation that may be associated with differences in susceptibility to SSWS. We performed restriction site-associated DNA sequencing (2bRAD-seq) to genotype ~72,000 single nucleotide polymorphism (SNP) loci across apparently normal and wasting sea stars. Locus-specific analyses of differentiation (F_{ST}) between disease-status groups revealed no signal of genetic differences separating the two groups. Using a multivariate approach, we observed weak separation between the groups, but identified 18 SNP loci showing highest discriminatory power between the groups and scanned the genome annotation for linked genes. A total of 34 protein-coding genes were found to be located within 15 kb (measured by linkage disequilibrium decay) of at least one of the 18 SNPs, and 30 of these genes had homologies to annotated protein databases. Our results suggest that the likelihood of developing SSWS symptoms does not have a strong genetic basis. The few genomic regions highlighted had only modest levels of differentiation, but the genes associated with these regions may form the basis for functional studies aiming to understand disease progression.

KEYWORDS
DAPC, keystone species, marine disease, sea star wasting syndrome (SSWS)

1 | INTRODUCTION

Rising sea water temperatures, due to climate change, are becoming increasingly stressful to marine ecosystems. As a result, marine diseases have become more prevalent in the last few decades (Harvell et al., 2002, 2004). Disease outbreaks can have detrimental downstream effects on marine species due to changes in community structure and age distribution (Behringer et al., 2018; Burge et al., 2014). Many marine taxa have suffered intense population declines as a result of the increasing prevalence of diseases (Burge et al.,

2014; Harvell et al., 2004). These declines can result in reduced variation due to population bottlenecks and genetic drift, and possibly from strong directional selection associated with tolerance or resistance to disease (Nei et al., 1975; Zenger et al., 2003).

The Sea Star Wasting Syndrome (SSWS) epidemic event that began in 2013 is believed to be the largest marine wildlife disease on record (Gravem et al., 2021; Harvell et al., 2019). SSWS affected over 20 species of sea stars from Baja California, Mexico, to the Gulf of Alaska, USA (Hewson et al., 2014, 2018), and severely reduced population sizes of several sea star species (Gravem et al., 2021; Harvell et al., 2019; Hewson et al., 2014; Menge et al., 2016; Miner et al., 2018; Montecino-Latorre et al., 2016). Similar SSWS symptoms have been observed in British Columbia in 2008 (Bates et al., 2009), along the US east coast (Bucci et al., 2017), the South Pacific (Pratchett, 1999; Zann et al., 1990), Australia and Yellow Sea (Hewson et al., 2019). While the viral candidate sea star-associated densovirus (SSaDV) has been debunked (Jackson et al., 2020), other possible causative or exacerbating agents remain unknown, with hypotheses including pathogen(s) (Lloyd & Pespeni, 2018), inconsistent aetiology stress responses between locations, species and environment (Hewson et al., 2018), microbial dysbiosis (Lloyd & Pespeni, 2018), and microbial-driven depletion of oxygen at the animal–water interface (Aquino et al., 2021). There is also mixed evidence for whether anomalously warm waters linked to global warming initiated the outbreak (Aalto et al., 2020; Eisenlord et al., 2016; Menge et al., 2016, 2016b; Miner et al., 2018; Tracy et al., 2020). Regardless, it is clear that the disease is exacerbated in warmer conditions (Bates et al., 2009; Eckert et al., 1999; Eisenlord et al., 2016; Kohl et al., 2016), and that severe population reductions occurred in warmer southern regions (Gravem et al., 2021; Harvell et al., 2019; Miner et al., 2018). The interplay between climate change and disease is a growing threat to wildlife species, especially when it causes rapid and extreme population declines. What is still unclear is whether tolerance or resistance to some of these diseases has a genetic basis that may allow populations to adapt if outbreaks continue to occur.

The keystone species *Pisaster ochraceus* was severely affected by SSWS over much of its range. In Oregon, their populations declined by 50%–94% (Menge et al., 2016). Because *P. ochraceus* aids in maintaining fast-growing *Mytilus californianus* populations from overgrowing intertidal zones (Paine, 1966, 1969, 1974), declines in their populations have resulted in trophic cascades and regime shifts in intertidal regions (Burt et al., 2018; Miner et al., 2018; Schultz et al., 2016). Loss of *P. ochraceus* due to SSWS could have detrimental impacts on coastal ecosystems. It is likely that SSWS has exerted strong selection on *P. ochraceus* populations. Recent genetic studies on this and other affected sea star species suggest a genetic component to variation in SSWS susceptibility. Individuals with SSWS symptoms showed elevated expression levels in genes associated with immune response and tissue remodelling (Fuess et al., 2015; Gudenkauf & Hewson, 2015; Ruiz-Ramos et al., 2020). In addition, Schiebelhut et al. (2018) observed allele frequency shifts before and after peak SSWS outbreaks in California populations of *P. ochraceus*. More specifically, they detected changes in restriction site-associated DNA sequencing (RAD-seq) haplotype frequencies between pre-SSWS adults and post-SSWS adults, as well as between pre-SSWS adults and recruits in the populations after SSWS. These changes occurred in few loci, but were consistent across independent geographical samples (Schiebelhut et al., 2018). However, because Schiebelhut et al. (2018) genotyped only apparently normal individuals (asymptomatic), it is still unclear whether the allele shifts were caused by the disease itself or by other co-occurring factors.

Here, we build upon the work of Schiebelhut et al., (2018) by investigating genomic differences between wasting individuals (i.e., presenting with SSWS) and grossly, or apparently normal individuals from the same localities during an outbreak of SSWS. We examine genetic variation in 200 *P. ochraceus* individuals collected in central Oregon in 2016, 2 years following the initial spring 2014 SSWS outbreak in Oregon (Menge et al., 2016). At this time, both apparently normal and wasting sea stars were common at each of the six Oregon sites sampled. We reasoned that, by being found on the same transects as wasting individuals and hence probably exposed to similar conditions, sea stars found to be apparently normal may carry genetic variants associated with resistance or tolerance to SSWS. By combining field surveys of natural disease prevalence with high-throughput single nucleotide polymorphism (SNP) genotyping, we assess the contribution of sea star genetic variation to SSWS occurrence. Our data set is the result of a unique opportunity to compare apparently normal and wasting individuals from the same time and place during the SSWS epidemic.

2 | MATERIALS AND METHODS

2.1 | Field surveys and tissue sampling

Field surveys aimed at quantifying the prevalence of SSWS were conducted between April and July 2016 in the low intertidal zone at five sites in central Oregon: Fogarty Creek (44.8386, −124.0588), Boiler Bay (44.8303, −124.0608), Yachats Beach (44.3114, −124.1086), Strawberry Hill (44.2492, −124.1154) and Tokatee Klootchman (44.2037, −124.1170). These surveys were conducted using 5 × 2-m belt transects (5–10 transects per site). Animals were collected by hand at low tide. Arm length was recorded (centre to longest arm) for each animal, and only adults (with >3 cm arm length) were scored for disease status (Menge et al., 2016). We recorded visual disease symptoms based on the six-level ranking protocol, as per Menge et al. (2016); these included, in order of severity: twisting arms (1), deflated (2), lesions (3), lost arms (4), losing grip on rocks (5), and disintegrating or "melting" (6). Animals were considered apparently normal (rank of 0) if none of these symptoms existed.

We returned to each of these sites to collect tissue for genetic analysis (Table S1). We also collected tissue from apparently normal and wasting individuals at a sixth site (Smelt Sands, 44.3212, −124.1081, on August 16, 2016), but we did not conduct transect surveys there. From each individual, tube feet (~5–10) were collected using scrubbed and sterilized forceps, then stored in 1.5-ml microcentrifuge tubes

containing 1 ml of 95% ethanol. All samples were stored on ice and then at −20°C until ready for DNA isolation. In total, we collected tissue from 410 sea stars, 92 of which were wasting.

2.2 | Library preparation and sequencing

For genotyping, we included only individuals with the highest wasting scores from each site, which ranged from ranks 3 to 6 (Table S1). In total, 82 individuals were included in the wasting group and 112 in the apparently normal group. DNA was extracted using the E.Z.N.A Tissue DNA kit (Omega Biotek) and quantified using a fluorescence method (Quant-iT dsDNA Assay Kit, ThermoFisher). We used the 2bRAD protocol for genotyping SNPs (Wang et al., 2012), following the original published protocol, but using the enzyme AlfI. We also used adaptors with "NN" overhangs to target 100% of restriction sites. Multiplexed individuals were pooled at approximately equimolar amounts (after quantification via quantitative PCR [polymerase chain reaction]) and sequenced across five lanes of an Illumina HiSeq 3000 as 50-bp single reads, at the Center for Genome Research and Biocomputing at Oregon State University.

2.3 | Data filtering

Adaptors were trimmed and low-quality reads were filtered (phred score <30) using publicly available scripts (https://github.com/Eli-Meyer/2brad_utilities/). Because of the cleavage pattern of the AlfI enzyme, 2bRAD DNA inserts are 34–36 bp in length. Therefore, after adaptor and quality trimming, we filtered out any reads that were shorter than 34 bp to reduce chances of mismapping. Cleaned reads were mapped to the reference *Pisaster ochraceus* genome (NCBI accession GCA_010994315.1) using SHRIMP (Rumble et al., 2009), reporting the top three maximum hits per read. We used STACKS version 1.35 (Catchen et al., 2011, 2013) to call genotypes using default parameters, with samples coded by disease status group (wasting/apparently normal). Additional filtering parameters used included: selection of a single (first) SNP per stack, removal of loci that were genotyped in only one group, minimum minor allele frequency (MAF) set to 0.025, a minimum minor allele count set to 4, and only loci represented in at least 50% of samples were retained. Using VCFTOOLS (Danecek et al., 2011), we retained only biallelic loci and only genotypes with a minimum coverage of six reads. Finally, we used PLINK (Purcell et al., 2007) to remove individuals that were missing more than 50% of loci. This filtering pipeline retained a data set with 133 individuals (74 normal and 59 wasting) and 71,784 SNP loci, which we will refer to as the "full data set."

2.4 | Population structure

Before comparing genotypic variation between sea stars varying in disease status, we assessed whether significant population genetic structure exists among the sampled sites. We used the Bayesian clustering approach in STRUCTURE (Pritchard et al., 2000) with the data set of 133 individuals, but we further filtered it to reduce computational burden. For this, we filtered loci that were genotyped in at least 90% of individuals, using VCFTOOLS, retaining 4,626 loci. STRUCTURE runs included 100,000 burn-in and 100,000 Markov chain Monte Carlo (MCMC) sampling replicates, assuming admixture and with sampling locality used as prior information. We ran three values of K to assess whether clustering occurring at the specific site ($K = 6$) or at the subregion level ($K = 3$) were more likely than a large panmictic population ($K = 1$). To confirm that parameters converged, each value of K was run three separate times and likelihoods and clustering patterns were compared across runs. This clustering analysis suggested genetic variation was not structured among sampling sites (Figure S1). We therefore considered our samples as coming from a single population in the subsequent analyses.

2.5 | Analyses of genetic variation

We used two types of approaches for estimating differentiation between disease status groups. We estimated Weir & Cockerham's F_{ST} (Weir & Cockerham, 1984) using GPAT++ (Shapiro et al., 2013), with significance levels adjusted using the false discovery rate (FDR) in R. To test for F_{ST} outliers that may indicate selection, we used BAYESCAN version 2.1 (Foll & Gaggiotti, 2008) with the following settings: prior odds of the neutral model of 10, burn-in of 50,000 replicates, a thinning interval of 10, 20 pilot runs for 5,000 iterations, and recorded output of 5,000 iterations. Significance for Bayescan F_{ST} outliers was assessed at a q-value FDR of 0.01.

We also used a multivariate approach to test for differentiation between grossly normal and wasting sea stars. A discriminant analysis of principal components (DAPC) (Jombart et al., 2010) was used, implemented in the R package "adegenet" (Jombart, 2015) and identified outlier loci based on their loadings associated with the discriminant function separating the two groups.

2.6 | Regions linked to discriminant loci

To identify functional candidates that may be associated with disease status, we scanned for protein-coding genes linked to outlier SNPs. We first determined the appropriate genomic window size for scanning around each SNP by estimating linkage disequilibrium (LD) between pairs of SNPs. The r^2 was calculated using VCFTOOLS between pairs of SNPs within 100,000-bp windows. Average r^2 was calculated in bins of 100-bp increments, and were plotted against physical distance. This plot showed that LD decays rapidly up to 15 kb, then continues to decrease but at lower rates (Figure S2). We hence scanned a 30-kb window centred at each outlier SNP (15 kb on either side) by overlaying the protein-coding genome annotation from Ruiz-Ramos et al. (2020) onto the genome assembly, using the Integrative Genomics Viewer (Robinson et al., 2011).

3 | RESULTS

3.1 | Field observations

A total of 3,670 *Pisaster ochraceus* were counted in belt transects across five sites, with the incidence of SSWS ranging from 5.2% to 10.0% (Table 1). In three of the sites (Boiler Bay, Yachats Bay and Strawberry Hill), all surveyed wasting sea stars showed either symptoms of lesions or arm loss, while at Tokatee Klootchman, 25% showed the more advanced symptom of grip loss. Fogarty Creek harboured sea stars with the most advanced stage of SSWS; all wasting individuals surveyed were disintegrating (Table 1). Metadata associated with the 410 individuals from which we collected tissue sample can be found in Table S1.

3.2 | Sequencing and genotyping

Illumina sequencing yielded ~1.6 billion reads. After removing low-quality reads, 1.4 billion reads remained, with an average of 6.9 million reads per sample. A total of 259,407 RAD stacks passed sample and population filters, surveying a total of 9,526,221 bases (~2.3% of the 401.9-Mb genome) and with mean per-sample coverage of 38.1×. As mentioned above, 71,784 RAD stacks contained at least one polymorphic site, and a single random SNP was retained per stack in the full data set (Table S2). Moreover, given our estimated LD block of 15 kb, this full SNP set allowed for an average of 2.5 SNPs sampled per block. This level of coverage suggests our data set may provide sufficient power to detect genomic regions associated with phenotypic differences, if these exist and have a strong genetic component (Lowry et al., 2017a, 2017b).

3.3 | Analyses of genomic variation

Genomic differentiation between grossly normal and wasting sea stars was very low based on F_{ST} estimates. Across the final SNP data set (71,784), Weir & Cockerham's F_{ST} had a median value of 0.00314, and nearly 45% of loci had $F_{ST} = 0$ (Table S2). Moreover, while 362 loci had moderate F_{ST} values (≥0.1), no locus showed significant differentiation after FDR adjustments; the lowest adjusted *p*-value was 0.172 (Figure 1). Outlier tests with F_{ST} using BAYESCAN also showed no evidence of selection in any locus in our data set (Figure S3).

DAPC analyses showed modest separation between apparently normal and wasting groups (Figure 2a). Based on loading values from the DAPC, we identified 18 SNPs across 10 chromosomes contributing most to the differentiation between the two groups (Figure 2b; Table S3). Allele frequency differences across these loci ranged from 0.014 to 0.253 (Table S3). Using a 30-kb window centred at each of these SNP positions, we detected 34 protein-coding genes predicted by the genome annotation from Ruiz-Ramos et al. (2020). BLAST searches of these protein sequences against the Uniprot/Swissprot databased revealed that 30 of them have predicted products with known functional annotation (Table S4). Chromosomes 3 and 8 harboured the most genes linked to these SNPs (seven genes each); in chromosome 8, one SNP was linked to three genes, and the others to two each (Figure 3).

We assessed whether the 18 SNP loci identified as outliers from our DAPC analyses overlapped with haplotypes from Schiebelhut et al. (2018) that were the most discriminatory between post- and pre-SSWS adults in their samples from California. For this, we compared the 30-kb ranges encompassing our SNP outliers to the haplotype coordinates from Schiebelhut et al. (2018). We detected no overlap in the two sets of outliers, with the shortest distance detected as ~257 kb (Table S5).

4 | DISCUSSION

The recent outbreaks of SSWS at multiple coastal sites caused severe population declines in several sea star species. Mitigation techniques for addressing outbreaks when the causative agent(s) is unknown should run in parallel with studies attempting to determine the cause (Groner et al., 2016). With rising sea water temperatures resulting in a higher prevalence of marine diseases (Harvell et al., 2002; Tracy et al., 2019), we are likely to see similar scenarios of mass mortality outbreaks impacting marine species more frequently and having little time to address management or conservation plans. Assessing the potential for natural population resilience is a critical

TABLE 1 Summary of field surveys of prevalence of SSWS in *Pisaster ochraceus* in the central Oregon coast

Site	Mean total observed	% Apparently Normal	% Wasting	% Lesions	% Arm(s) lost	% Losing grip	% Disintegrating
Fogarty Creek	484	93.9	6.1	0	0	0	100
Boiler Bay	316	90.0	10.0	28.6	71.4	0	0
Yachats Beach	360	93.5	6.5	77.8	22.2	0	0
Strawberry Hill	495	94.8	5.2	72.3	27.7	0	0
Tokatee Klootchman	180	90.3	9.7	50	25	25	0

Note: Surveys are based on five to 10 belt transects (5 × 2 m) conducted in the low intertidal zone in April and in July 2016. Values are the averages between the field surveys. Symptom categories for grossly wasting individuals are based on the ranking proposed by Menge et al. (2016).

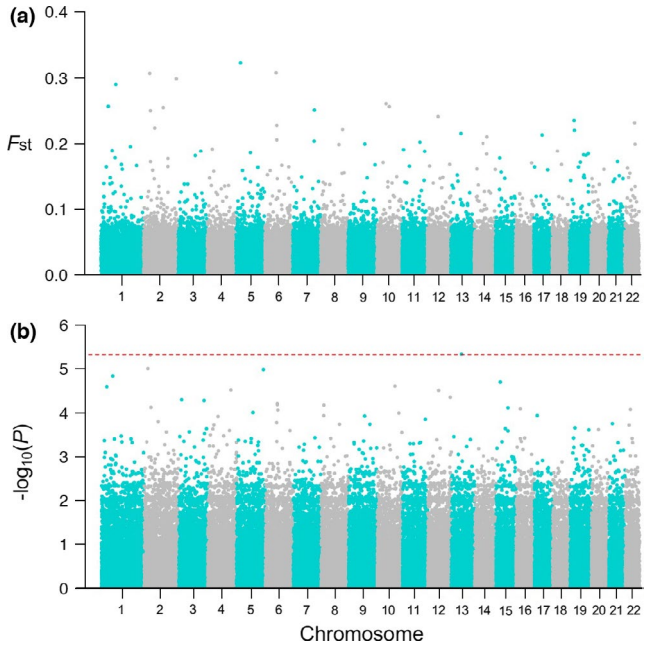

FIGURE 1 Tests of differentiation across SNP loci (n = 71,784) between wasting and apparently normal *Pisaster ochraceus* from central Oregon. (a) Weir & Cockerham's F_{ST}. (b) The p-values for differentiation at each locus. Red dashed line marks the lowest FDR-adjusted value of 0.172

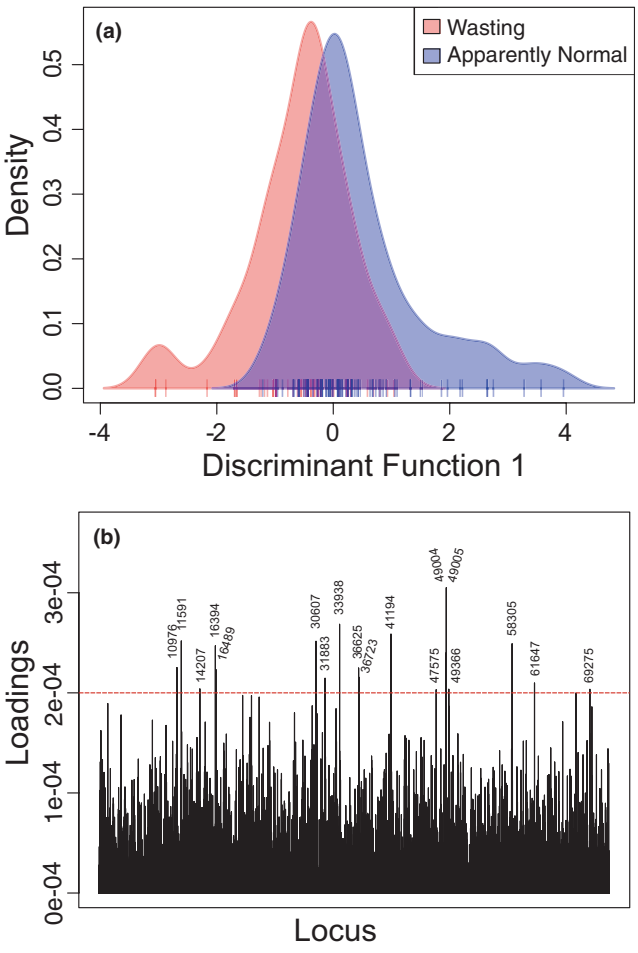

FIGURE 2 Discriminant analysis of principal components (DAPC) comparing apparently normal (n = 74) to wasting (n = 59) sea stars. (a) Distribution of scores for each group. Tick marks on the x-axis represent individual sea stars. (b) Loadings of loci associated with highest between-group variance. Loci above the dashed red line were considered outliers and examined further. Numbers above outlier peaks are locus IDs, which can be found in Tables S2 and S3

step in predicting the long-term fate of affected species and of the communities they in turn influence. For example, selectively rearing disease-resistant oysters in hatcheries has been a useful tool in avoiding disease outbreaks that are decimating wild populations (Agnew et al., 2020; Dégremont et al., 2015). While many marine species are not amenable for selective breeding, examining genomic variation in natural populations can address whether these species have the genetic makeup for adaptation to marine diseases on their own.

In this study, we took advantage of the co-occurrence of wasting and apparently normal individuals of *Pisaster ochraceus* in central Oregon to scan for genomic regions that potentially predict individual SSWS status. After genotyping nearly 72,000 SNP loci across 133 individuals, we found no strong patterns of differentiation between wasting and apparently normal individuals. Loci with elevated F_{ST} were not clearly concentrated as peaks in any genomic region, and no single locus showed a statistically significant level of allele frequency differences. Using a multivariate approach as a complement to the locus-specific F_{ST} analyses, 18 SNP loci stood out as contributing to genomic differentiation between the two groups of individuals based on disease status. Overall, we argue that a genetic basis for SSWS resilience in *P. ochraceus* is probably weak, but we identified a list of genomic regions and functional candidates that may serve as a basis for studies of gene expression, physiology or comparative genomics during future SSWS outbreaks.

While the proximate cause(s) of SSWS at the individual level are still unknown, recent experimental studies are consistent with a pathogen agent. For example, individuals that were wasting in the laboratory showed physiological and gene expression responses that suggest innate immunity, cytokine-like systems and tissue remodelling (Fuess et al., 2015; Gudenkauf & Hewson, 2015; Ruiz-Ramos et al., 2020). Our findings showed no evidence for a strong genetic component to SSWS tolerance or resistance, but the weakly associated loci we identified may have small but cumulative effects, which is expected for a polygenic trait. This trait may hence require much higher powered studies for detecting associated loci with more precision (Gagnaire & Gaggiotti, 2016).

Schielbelhut et al. (2018) detected allelic shifts in grossly normal *P. ochraceus* adults and juveniles before and after the SSWS outbreak in California. They found three loci putatively under selection and reported on 100 discriminatory haplotypes between time periods. Interestingly, the 18 SNP loci we detected as most

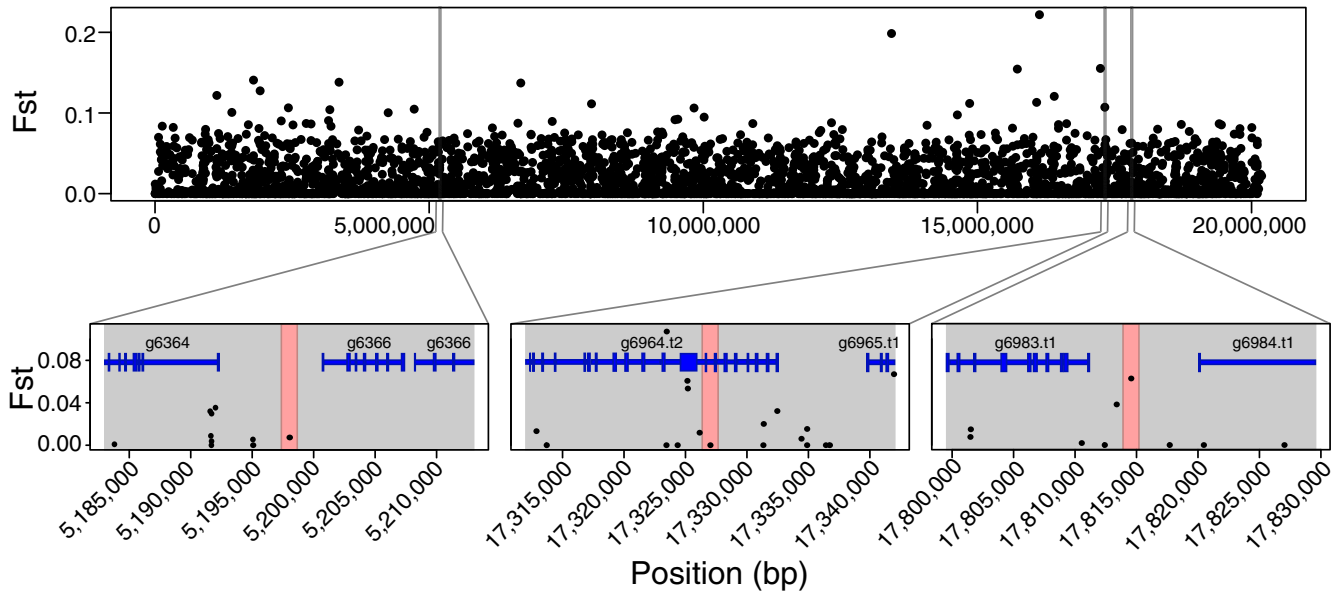

FIGURE 3 Landscape of F_{ST} and protein-coding genes surrounding outlier SNPs along chromosome 8. Thin grey bars on the top panel indicate positions of the outlier SNPs (positions 5,198,014, 17,327,004 and 17,814,546 bp). Insets on the bottom panel depict a closeup with a 30-kb window centred at each outlier SNP (highlighted in red). Blue boxes cover the coordinates of protein-coding genes, with thin vertical lines depicting exons

discriminatory in our samples did not occur within 30 kb of those reported by Schielbelhut et al. (2018), and most were between 250 kb and 2 Mb apart. Lack of overlap in these genomic regions is perhaps not surprising given the multitude of differences between the studies, such as year of sampling, the health status of sea stars and geographical location. For instance, SSWS is known to be associated with spikes in sea water temperature (Bates et al., 2009; Eckert et al., 1999; Eisenlord et al., 2016; Kohl et al., 2016), and daily deviations from annual sea water temperatures in Oregon were more prevalent in 2013/2014 than in California (Miner et al., 2018). Such relevant environmental differences between California and Oregon may hence also cause different selective pressures. Moreover, the reduced-representation nature of RADseq and the use of different restriction enzymes suggest that a lack of overlap between different study outlier markers is not indicative of a lack of biological relevance.

The scarcity of SSWS-associated loci detected is also possibly a result of reduced coverage from the inherent nature of RADseq methods (Lowry et al., 2017a). While RADseq is an efficient and cost-effect method for producing thousands of SNPs along the entire genome, these markers remain sparse. Despite the limitations, many RADseq studies have found loci attributed to adaptive selection when coverage is adequate (Epstein et al., 2016; Lowry et al., 2017a; McKinney et al., 2017). Marker density aimed at detecting phenotype–genotype associations is recommended to be high relative to LD in the target species (Lowry et al., 2017a). Based on this metric, our RADseq effort in this study adequately covers the full genome, with on average 2.5 SNPs found per every 15-kb linkage block. Therefore, we argue that our results are not due to low marker density, but perhaps may be improved by genotyping a higher number of individuals.

Our study joins that of Schielbelhut et al. (2018) and Ruiz-Ramos et al. (2020) in assessing genomic variation in the keystone species *P. ochraceus* and highlighting the importance of understanding the causes and responses to devastating SSWS outbreaks. While current patterns remain obscure, the accumulation of putative functional genomic regions will serve as invaluable resources for continued field and laboratory studies. In addition to physiological and transcriptomic experiments, we suggest the need for a concerted effort to sample large numbers of wasting and apparently unaffected individuals across several geographical regions, and ideally using low-coverage whole-genome sequencing for substantially increased power (Lou et al., 2021).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

S.G. and F.B. conducted field sampling. A.B. undertook sample preparation with help from F.B. A.B. and F.B. performed processing and

analysis of data. A.B. wrote the manuscript with assistance from F.B. and S.G.

DATA AVAILABILITY STATEMENT

Illumina sequence reads are deposited in the NCBI Sequence Read Archive (SRA), in accessions SRR13611638–SRR13611837.

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