TITLE

Ecological load and balancing selection in circumboreal barnacles

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Abstract

Acorn barnacle adults experience environmental heterogeneity at various spatial scales of their circumboreal habitat, raising the question of how adaptation to high environmental variability is maintained in the face of strong juvenile dispersal and mortality. Here we show that 4% of genes in the barnacle genome experience balancing selection across the entire range of the species. Many of these genes harbor mutations maintained across 2 million years of evolution between the Pacific and Atlantic oceans. These genes are involved in ion regulation, pain reception, and heat tolerance, functions which are essential in highly variable ecosystems. The data also reveal complex population structure within and between basins, driven by the trans-Arctic interchange and the last glaciation. Divergence between Atlantic and Pacific populations is high, foreshadowing the onset of allopatric speciation, and suggesting that balancing selection is strong enough to maintain functional variation for millions of years in the face of complex demography.

Introduction

The relationship between genetic variation and adaptation to heterogeneous environments remains a central conundrum in evolutionary biology (Botero, et al. 2015). Classical models of molecular evolution predict that populations should be locally adapted to maximize fitness (Williams 1966). However, species inhabiting highly heterogeneous environments violate this expectation: if gene flow is high in relation to the scale of environmental heterogeneity, species will harbor variation that is beneficial in one condition but deleterious in another (Gillespie 1973), and the resulting ecological load (i.e., the fitness difference between the best and the average genotype across the range of environments where offspring may settle) will prevent local adaptation. Conversely, if gene flow is low, favored alleles will become locally fixed and species should display low levels of genetic variation. Paradoxically, many natural populations living in variable environments possess high dispersal capabilities, and harbor more variation than expected under classical models (Metz and Palumbi 1996; Mackay, et al. 2012; Messer and Petrov 2013; Bergland, et al. 2014). This disconnect between nature and theory has motivated the hypothesis that balancing selection, a process where selection

favors multiple beneficial alleles at a given locus, is at play to maintain adaptations in these habitats (Levene 1953; Hedrick 2006).

The northern acorn barnacle (*Semibalanus balanoides*) is a model system to study adaptations to ecological variability. This barnacle is a self-incompatible, simultaneous hermaphrodite which outcross only with adjacent individuals. Adult barnacles are fully sessile and occupy broad swaths of intertidal shores in both the North Pacific and North Atlantic oceans. These habitats experience high levels of cyclical and stochastic ecological heterogeneity which impose strong selection at multiple spatial scales: microhabitats (intertidal shores), mesohabitats (bays and estuaries) and macrohabitats (continental seaboards) (Schmidt, et al. 2008; Nunez, et al. 2020). Barnacle larvae, on the other hand, engage in extensive pelagic dispersal by ocean currents, and may settle in habitats completely different from those of their parents (Flowerdew 1983). This contrast between strong adult selection and high juvenile dispersal prevents local adaptation. In addition, *S. balanoides* has a complex demography. It originated in the Pacific, and colonized the Atlantic during the many waves of the trans-Arctic interchange (1-3 mya) (Vermeij 1991). Like most circumboreal species, it was subjected to drastic range shifts due to the Pleistocene glacial cycles (Wares and Cunningham 2001; Flight, et al. 2012), and more recently due to anthropogenic climate change (Jones, et al. 2012). As such, S. *balanoides* is a premier system to study how adaptive genetic variation is maintained over broad spatial and evolutionary scales, in the face of ecological load.

Three decades of work have shown that balancing selection, via marginal overdominance (a case where the harmonic mean fitness of heterozygous genotypes must be larger than that of either homozygote) (Levene 1953), maintains adaptive variation at the metabolic gene Mannose-6-phopate isomerase (***Mpi***) in barnacles across the entire North Atlantic basin(Schmidt and Rand 1999; Dufresne, et al. 2002; Rand, et al. 2002; Veliz, et al. 2004; Nunez, et al. 2020). These findings motivate two questions which are addressed in this paper. First, how pervasive are balanced polymorphisms in the barnacle genome? And, second, what genes are targets of balancing selection? To investigate functional polymorphism in *S. balanoides*, we quantified genomic variation in North Pacific and North Atlantic populations (**Figs. 1A-1C**). In the Pacific, we analyzed samples from British Columbia, Canada (**WCAN**) as well as a sample of the sister taxon *Semibalanus cariosus*. In the Atlantic, we analyzed samples from Maine (**ME**), Rhode

Island (**RI**), Iceland (**ICE**), Norway (**NOR**), and the United Kingdom (**UK**). For all populations, we sequenced multiple libraries including: a single individual barnacle genome to ~50X coverage, pools of 20-38 individuals per population (i.e., pool-seq (Schlotterer, et al. 2014)), as well as ~600 bp amplicons from the mitochondrial (**mtDNA**) *COX I* gene (including previously published *COX I* data(Wares and Cunningham 2001)). We mapped these datasets to our newly assembled *S. balanoides* genome (**SI Appendix 1**) and characterized genetic diversity across all populations (**SI Appendix 2**). We first present our findings in the context of the barnacle's phylogeography and demographic history. This is pivotal to understand the historical conditions which can contribute to ecological load. Then, we characterize the pervasiveness of balancing selection across the genome, as well as the age of balanced polymorphisms and their putative functional significance in highly heterogeneous environments.

Results

Standing variation across oceans. Our pool-seq panels discovered ~3M high quality single nucleotide polymorphisms (**SNPs**) across populations at common allele frequencies (>5%). When linkage is removed at 500 bp, the SNP panel thins to ~690,000. Principal component analysis (PCA), on the LD-thinned SNPs, shows that variation is strongly subdivided by ocean basins (**Fig. 1D**). PC 1 captures 74% of the variation, and partitions populations across basins. PC 2 (8.5% var.) partitions Atlantic populations into 2 discrete east-west clusters. The western cluster contains ME, RI, ICE, and the eastern cluster contains UK and NOR. These clusters are supported by the abundance of mtDNA haplotypes within and between ocean basins (**Fig. 1D inset; Table S1**)(Wares and Cunningham 2001; Flight, et al. 2012; Nunez, et al. 2018). The large divergence between oceans is also captured in levels of nucleotide diversity ($\boldsymbol{\pi}$; a metric of standing genetic variation). Surprisingly, North Atlantic populations harbor more genetic variation (π = 1.05%) than their Pacific, ancestral, conspecifics (π = 0.55%; **Fig. 1E; Fig. S1**). We also estimated the Tajimas' D statistic (***D***), a measure of the excess (D<0), or deficit (D>0), of rare alleles in populations. These data indicate that all North Atlantic populations, especially NOR, have negatively skewed genome-wide values of D (**Figs. 1E, S2**).

Historical phylogeography and structure. We reconstructed changes of historical effective population sizes (N_e) with the multiple sequentially Markovian coalescent model (MSMC) using individual whole genomes (Schiffels and Durbin 2014). Our results provide evidence for different phylogeographic trajectories in response to the events of the glaciations (**Figs. 1G, 1H**). For instance, the Eastern Cluster and the Western Cluster populations shared a common demography throughout the Pleistocene (**Fig. 1G**) but diverged in recent geological time. Namely, Eastern populations (especially NOR) experienced striking increases in N_e in the recent past (**Fig. 1I**), likely following the asynchronous deglaciation of the Fennoscandian ice sheet (Ruddiman and Mcintyre 1981; Patton, et al. 2017). Western populations, on the other hand, experienced a demographic contraction which started during the last glacial period and ended during the last glacial maxima (~20 kya; **Fig. 1J**)(Brochmann, et al. 2003; Maggs, et al. 2008; Flight, et al. 2012).

We estimated gene flow by computing f_3 statistics (Reich, et al. 2009) for all possible combinations of target, source 1, and source 2 populations, using individual whole genomes (**Fig. S3; Table S2**). Our analysis finds no evidence of recent gene flow across oceans. This result is supported by two additional lines of evidence. First, a mtDNA molecular clock analysis (Drummond, et al. 2002), which suggests that Pacific and Atlantic populations have not exchanged migrants in nearly 2 million years (**SI Appendix 3**). And second, estimates of genetic differentiation (i.e., F_{ST}) which reveal large amounts of genome-wide divergence (**Fig. S4**), and foreshadows the onset of allopatric speciation across oceans. Within the North Atlantic, F_{ST} is low (likely due to shared demography until the glacial maximum) and the f_3 analysis suggest that admixture is pervasive (**Fig. S3, Table S2**). These findings are supported by additional ABBA-BABA tests for gene tree heterogeneity (Green, et al. 2010) (see **SI Appendix 4**). Overall, these findings present three important points. First, they exemplify the complex demography that underlie standing variation in natural populations. Second, they confirm that barnacles harbor high levels of genetic variation genome-wide. And third, they reveal the pervasiveness of gene flow and shared variation within ocean basins, where environmental heterogeneity is extensive across “micro” (1-3 meter) and “meso” (1-10 kilometer) scales. These conditions provide the environmental context for ecological load at the genomic scale.

Balancing selection in barnacles. Balancing selection is expected to produce molecular and phylogenetic footprints not consistent with neutrality (Fijarczyk and Babik 2015). Molecular footprints include: enrichment of old alleles (e.g., trans-species polymorphisms; **TSPs**), elevated genetic variation (high π), deficit of rare alleles ($D > 0$), excess SNPs at medium allele frequencies, reduced divergence around the balanced locus (low F_{ST}), as well as the accumulation of non-synonymous variation in the vicinity of balanced polymorphisms, a phenomenon known as sheltered load (Uyenoyama 2005). Likewise, balancing selection will produce a phylogenetic signal composed of diverged clades, corresponding to the balanced haplotypes. Deeply diverged clades will occur when balancing selection has maintained variation over long evolutionary times (i.e., ancestral balancing selection(Fijarczyk and Babik 2015)). A joint analysis of our Pacific, Atlantic, and outgroup (*S. cariosus*) datasets reveal 11,917 cosmopolitan SNPs (i.e., SNPs that segregate in both oceans) which are also TSPs (**Dataset S1**). TSPs, genome-wide, occur in 0.14% coding regions, 0.21% in introns, 0.02 % in promoters, 0.01% in 5'UTRs, and < 0.01% in 3'UTRs. The remainder TSPs occur in 0.09% of intergenic regions. An enrichment analysis which compares the abundance of TSPs, of each genomic class, relative to all discovered SNPs, reveals that TSPs are significantly over-enriched in coding loci (**Fig. 2A**), and 4,415 segregate at high frequencies in all populations (TSPs with heterozygosity [H_E] > 0.30; **Fig. S5**). These patterns of variation could be the result of neutral processes such as recurrent mutation (homoplasy) across all populations of either species. However, the enrichment of cosmopolitan, nonsynonymous, TSPs at common frequencies is not consistent with neutrality. Under a model of strict neutrality, segregating mutations are eventually lost in populations after speciation (Clark 1997). Moreover, coding regions are subjected to purifying selection which removes deleterious and mildly deleterious nonsynonymous variants (Hartl and Clark 1997).

We compared patterns of genetic variation in exons bearing TSPs and other exons. When accounting for exon length, we observe consistently elevated values of D and π for TSP-bearing exons relative to other exons (**Figs. 2B and 2C**; **S6**). Except for the ME vs. RI comparison (**Fig. S7**), TSP-bearing exons have consistently low F_{ST} values (**Fig. 2D**). To quantify sheltered load, we compared the ratio of H_E values at nonsynonymous (**NS**) and synonymous (**S**) mutations in TSP-bearing and other exons. Our results show that medium sized TSP-bearing exons (~500 bp) harbor an excess of non-synonymous NS H_E

(**Fig. 2E**). Notably, we observed that differences between TSP-bearing and other exons become less apparent as exons get longer. This regionalization of the signal occurs due to the small linkage blocks in the species (Nunez, et al. 2020). We observe 1,107 TSPs that cause nonsynonymous changes and occur in 312 genes with high confidence annotations (4%; **Dataset S2**). Consistent with our expectation of balancing selection, site frequency spectrum (**SFS**) analyses show that these 312 genes harbor an excess of SNPs at medium allele frequencies relative to other annotated genes (**Fig. 2F**).

Age of balanced polymorphisms. To determine the age of balanced polymorphisms, we ran topological tests on the allele trees for each TSP region across the 312 candidate genes. We built trees using phased haplotypes for each TSP-bearing region for all single individual genomes. We used these allele trees to compute the cophenetic distance (**CPD**) between tips. We classified allele trees as having or lacking highly diverged alleles based on the relative mean CPD between haplotypes from the same population vs. from different populations ($CPD_{w\text{-}b}$; **see supplementary methods**). The analysis reveals that of the 312 allele trees, 150 carry a significant signature of ancestral balancing selection ($CDP_{w\text{-}b} > 0$, Bonferroni $P < 1x10^{-9}$; **Fig. 2G; Dataset S2**). This suggests maintenance of diverged haplotypes for more than 2 million years, with extreme cases in which haplotypes are shared across species (8-10 million years)(Perez-Losada, et al. 2008; Herrera, et al. 2015). The remaining genes with $CDP_{w\text{-}b} < 0$ may either represent cases where the balanced alleles are younger, or oversampling of homozygous individuals for any given marker.

Targets of selection. We partitioned our dataset among genes with positive and negative $CPD_{w\text{-}d}$ allele trees and conducted gene ontology (GO) enrichment analyses. The 150 genes with positive $CPD_{w\text{-}d}$ trees show enrichment for terms related to "ion channel regulation", including genes involved in environmental sensing, and circadian rhythm regulation (**Table S3**). We show examples for 3 candidate genes under ancestral balancing selection involved in environmental sensing: 1) the painless gene (*Pain*; g1606; **Fig. 3A**), which is involved in nociception (i.e., pain reception), as well as detection of heat and mechanical stimuli (Tracey, et al. 2003; Xu, et al. 2006); 2) the Pyrexia gene (*Pyx*; g3472; **Fig. 3B**), which is involved in negative geotaxis, and responses to heat (Lee, et al. 2005); and 3) the shaker cognate w gene (*Shaw*; g3310; **Fig. 3C**), which is involved in regulation of circadian rhythm (Hodge and Stanewsky 2008; Buhl, et al. 2016). These

three examples showcase canonical footprints of balancing selection around the TSP, concomitant with a bimodal allele tree. Among genes with negative $CPD_{w\text{-}d}$ we observe enriched functions for "anatomical structure formation" including genes coding for motor proteins and muscle genes (**Table S4**). In all cases, we used RNA-seq data from ME individuals to confirm that these loci are expressed in adult barnacles.

Discussion

In intertidal barnacles, the dichotomy of strong adult selection and high offspring dispersal means that any allele that is beneficial to parental fitness in one generation may be neutral or deleterious in the next (Gillespie 1973). This leads to a fundamental question in evolutionary biology: how are adaptations maintained in the face of extreme ecological variability? In this paper, we provide evidence that balancing selection is widespread across the barnacle genome, with 4% of annotated genes harboring functional balanced polymorphisms. Notably, these polymorphisms occur in genes with important functions for life in variable environments, and many have been maintained for at least 2 million years despite a complex phylogeographic history (Wares and Cunningham 2001; Flight and Rand 2012). Naturally, the heterogeneous nature of the rocky intertidal imposes a segregation 'cost' for these balanced polymorphisms, as they occur in individuals that, due to high dispersal, recruit in sub-optimal habitats for any given genetic makeup. This ecological load, defined as $L_e = (W_{max} - \bar{W})/ W_{max}$ (where $\bar{W}$ is mean fitness, and W_{max} is optimal fitness, across all habitats), will be substantial, as demonstrated by comprehensive recruitment studies in natural habitats (Bertness 1989; Bertness, et al. 1992; Pineda, et al. 2006). For example, at initial settlement, barnacle density can be as high as 76 individuals per cm^2, but at maturity, it can be as low as 0.15 individuals per cm^2 (0.2% survival)(Pineda, et al. 2006). This mass mortality is habitat- and genotype-dependent (Schmidt and Rand 2001). This is the type of 'fitness cost' envisioned in the Levene model of balancing selection (Levene 1953). As such, our data suggests that the problem of ecological load is a defining condition of the barnacle life cycle. And, more generally, it argues in favor of balancing selection, via marginal overdominance, as the fundamental process underlying maintenance of adaptation in variable environments.

Is pervasive balancing selection plausible in nature? Under classical models of population genetics, when loci are considered to be independent of each other, the additive effects of widespread balanced polymorphism results in unbearable amounts of fitness variance and genetic death (Kimura and Crow 1964; Lewontin and Hubby 1966). However, if balanced loci have interactive effects (e.g., epistasis), multiple polymorphisms could be maintained with minimum effects on the distribution of fitness variance (King 1967; Milkman 1967; Sved, et al. 1967; Wittmann, et al. 2017). Based on this theoretical framework, multiple models have been developed to describe the conditions that favor the long-term maintenance of functional variation in spatially varying environments (Gillespie 1973; Hedrick, et al. 1976). Moreover, polymorphisms will be less likely to be lost if there is a large number of ecological niches available, if there is migration among niches, and if individuals are proactive in choosing niches where their fitness is maximized (Hedrick, et al. 1976). We argue that barnacles satisfy these conditions to some degree.

First, while it is useful to summarize intertidal heterogeneity in the form of discrete microhabitats (Schmidt, et al. 2000), individual barnacles experience the rocky shore as a complex tapestry of interactive stressors at three spatial levels. At microhabitats scales, the upper and lower tidal zones pose diametrically different ecological challenges in terms of food availability, competition, predation, and risk of desiccation (Bertness, et al. 1991; Schmidt and Rand 1999, 2001). At mesohabitat scales, open coasts vs. sheltered estuaries vary in their exposure to wave action, upwelling dynamics, and biotic interactions (Sanford and Menge 2001; Dufresne, et al. 2002; Veliz, et al. 2004). These, in turn, modify micro-level stressors. Lastly, at macrohabitat scales, topological differences across shores and latitudinal variations in tidal range produce a mosaic of thermal stress along continents (Helmuth, et al. 2002). Consequentially, what selection pressures are more important for any given barnacle will emerge from the interactions among these stress gradients. This complex landscape of selection has been captured in studies of the barnacle *Mpi* gene. Accordingly, the locus is under selection at micro-levels in the Gulf of Maine (Schmidt and Rand 1999; Schmidt, et al. 2000), at meso-levels in the gulf of St. Lawrence (Canada)(Dufresne, et al. 2002; Veliz, et al. 2004), yet it shows tepid signs of selection in the Narragansett Bay (Rhode Island)(Rand, et al. 2002; Nunez, et al. 2020). Similar complexity has also been captured in temperate populations of *Drosophila*. In

these, idiosyncratic weather effects can alter the dynamics of seasonal adaptation (Bergland, et al. 2014; Machado, et al. 2019). Second, the high dispersal capacity of the larval stage ensures constant migration between these niches across generations. Finally, barnacles also have the ability to choose preferred substrates during settlement. This occurs during the spring when barnacle larvae extensively survey microhabitats for biological, chemical and physical cues produced by previous settlers before making final commitments of where to settle (Bertness, et al. 1992). Unfortunately for the barnacle, this capacity for substrate choice does not mitigate mass mortality during late summer, which leads to strong selection for particular genotypes (Schmidt and Rand 2001). Nevertheless, these behaviors may constitute a form of adaptive plasticity, helping barnacles choose habitats where their fitness may be marginally improved. Overall, this suggests that the barnacle's life history is conducive to the maintenance of balanced polymorphisms.

What variation is under selection? Our analyses indicate that 4% (312) of all annotated genes are experiencing some form of balancing selection across the entire range of the species. This number of genes harboring ancestral polymorphisms is similar to that observed in *Arabidopsis thaliana* and its close relative *Capsella rubella* (433 genes)(Wu, et al. 2017). Similar to *Semibalanus,* these plants diverged ~8 mya, and their natural populations experience high levels of ecological heterogeneity (Bakker, et al. 2006). We must acknowledge that our number may be an underestimation driven by the nascent state of the genomic tools in *Semibalanus*. Future genome assemblies, combined with improved annotations, will undoubtedly yield a more complete picture of functional variation in the species. In addition, it will allow for a more comprehensive characterization of selection in structural variants and regulatory loci, which have been shown to be fundamental in the evolution of complex phenotypes (Wray 2007; Faria, et al. 2019). Despite these limitations, our analysis recovered a large number of genes involved in key functions for life in variable environments. These will be subjects of future validation studies. For instance, the general enrichment for ion channel genes suggests selection related to osmotic regulation (Sundell, et al. 2019). This hypothesis is highly plausible given that intertidal ecosystems experience strong salinity fluctuations, repeatedly exposing barnacles to osmotic challenges at all spatial scales. In addition, we observe targets of selection involved in environmental sensing loci (e.g., *pain*, *pyx*, and

shaw; **Fig. 3**). Similar to osmotic regulation, selection on these genes is entirely plausible given the inherent variability of intertidal habitats. An important hypothesis from the allozyme era is the idea that balancing selection would target genes at the node of metabolic fluxes (Eanes 1999; Watt and Dean 2000). In such cases, balanced variation would provide biochemical flexibility to cope with environmental heterogeneity. In the same vein, we hypothesize that balancing selection may act more often on "sensor genes" which control plastic responses to ecological variation. Testing this hypothesis is beyond the scope of this paper and would require the use of allele-specific differential expression experiments in barnacles.

Complex demography and speciation. Our demographic analyses provide clues about how historical events affected genetic variation in barnacle populations. In the Atlantic, our evidence suggests a shared demography throughout the Pleistocene, and that the modern Eastern and Western clusters formed in response to recent events of last glacial cycle. These findings highlight that the low F_{ST} values observed within the basins arise due to shared ancestry. Moreover, they also suggest that population structure persists in the presence of gene flow. As such, while larvae have the capacity to disperse for hundreds of kilometers, ocean currents (Nunez, et al. 2018) and different estuarine flushing times (Brown, et al. 2001) allow regions to retain some level of geographical structuring (Johannesson, et al. 2018; Nunez, et al. 2018). Comparisons between oceans reveal a stark pattern of genome wide divergence. This pattern is driven by the separation of Pacific and Atlantic populations following the events of the trans-Arctic interchange (Vermeij 1991). Accordingly, the negative levels of D in the north Atlantic may reflect the effect of bottlenecks during the trans-Arctic interchange. Notably, the high levels of π in the Atlantic is not concordant with predictions of common colonization models in which variation of the younger population is a subset of the ancestral population (Maggs, et al. 2008). We hypothesize this could be the result of ancient admixture due to repeated trans-Arctic invasions from the Pacific (Väinölä 2003). We recognize that ancestral admixture could generate artificial signatures of balancing selection via the mixing of highly differentiated haplotypes. However, such an occurrence would affect most genes in the genome. Our evidence shows that the signatures of balancing selection are highly localized in TSP-regions. For example, while D is elevated in TSP-regions, it is negatively skewed genome-wide. Our data does not support recent gene flow between ocean basins.

As such, after 2 million years of separation, neutral divergence appears to be driving Atlantic and Pacific populations to speciate in allopatry. A closer look to this hypothesis will require crossing individuals from both basins, and surveying offspring fitness and viability. More salient, however, is the observation of shared haplotypes between oceans in our candidate genes for balancing selection. In light of such strong background divergence, this provides evidence that balancing selection on most of these genes is strong, and that polymorphisms have been maintained for long periods of time.

Materials & Methods

Barnacle Collections. Barnacle samples were collected from Damariscotta (Maine, United States; ME), Jamestown (Rhode Island, United States, RI), Calvert Island (British Columbia, Canada; WCAN), Reykjavik (Iceland; ICE), Porthcawl (Wales, United Kingdom; UK), and Norddal (Norway; NOR). Additional samples were collected in Bergen (Norway), Tórshavn (Faroe Island), and Tjärnö (Sweden). For all samples, species identities were confirmed using Sanger sequencing of the mtDNA COX I region(Bucklin, et al. 2011). For the WCAN, RI, ME, ICE, UK, and NOR population we collected a single individual for DNA-seq, and a group of 20-40 individuals for pool-seq (**SI Appendix 2**). RNA-seq was done on four individuals from Maine. DNA-seq was done on a single individual from the sister taxa *S. cariosus*. DNA/RNA was extracted using Qiagen DNeasy/RNeasy kits. All pools and single individuals were sequenced in their own lanes of an Illumina machine by GENEWIZ LLC using 2x150 paired-end configuration.

Mapping datasets to the genome. Samples were mapped to a genome assembled *de novo* for the species (Sbal3.1; NCBI GenBank: VOPJ00000000; **SI Appendix 1**). The genome was assembled using a hybrid approach which combines PacBio reads and Illumina reads using DBG2OLC(Ye, et al. 2016) and Redundans(Pryszcz and Gabaldon 2016). Gene models were constructed using an *ab initio* method, AUGUSTUS(Stanke and Waack 2003), informed by evidence from the RNA-seq. A gene feature file (GFF) is available as **Dataset S4**. The model used for gene prediction was trained in *Drosophila melanogaster*. Genes were annotated by pairwise blast against the *Drosophila melanogaster* genome (Dmel6; NCBI GenBank: GCA_000001215.4). All annotations are available as **Dataset S5**. DNA reads from all populations were mapped

to Sbal3.1 using bwa mem(Li 2013). RNA reads were mapped using HiSat2(Kim, et al. 2015). SNPs were called using the samtools pipeline(Li, et al. 2009).

Genome analyses. Estimates of π and D were done using the popoolation-1 suite(Kofler, Orozco-terWengel, et al. 2011). Estimations of allele frequencies and F_{ST} were done using the popoolation-2 suite(Kofler, Pandey, et al. 2011). Demographic reconstructions were done using MSMC(Schiffels and Durbin 2014). The f_3 statistics were estimated using treemix(Pickrell and Pritchard 2012). Bayesian molecular clock analyses were done in BEAST2(Bouckaert, et al. 2014). ABBA/BABA statistics were calculated in *Dsuite*(Malinsky, et al. 2020). Phylogenetic inferences were done in iQtree(Chernomor, et al. 2016). GO enrichment analysis was done using GOrilla(Eden, et al. 2009) and GO terms inferred from our *Drosophila* annotation. The enrichment was assessed by comparing 2 genes list. The first composed of the genes of interest (i.e., the gene targets), the second one by all the genes annotated in Sbal3.1 (i.e., the gene universe). A detailed description of our analyses can be found in the supplementary methods section, as well as in GitHub: **https://github.com/Jcbnunez/BarnacleEcoGenomics**.

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Data deposition

Data used in this paper are available in the National Center for Biotechnology Information (NCBI), https://www.ncbi.nlm.nih.gov. Raw reads were deposited under submission id: SUB6188969. SRAs are as follows: DNAseq datasets: SRR10011798, SRR10011802, SRR10011804, SRR10011805, SRR10011807–SRR10011810, SRR10011812–SRR10011814, SRR10011819, SRR10011825; PacBio dataset: SRR10011818; RNAseq datasets: SRR10011820-SRR10011823. MtDNA sequences for the COX I genes can be acceded form the following GeneBank accessions MG925538–MG925662, MG928281–MG928323, and MT329074–MT329592. Whole mtDNAs were deposited under accessions MG010647, MG010648, MG010649, MT528636, MT528637. The barnacle genome (Sbal3.1) is available at NCBI (accession no. VOPJ00000000). A GitHub repository with code as well as with the supplementary datasets S1, S2, S3, S4, and S5, can be found at **https://github.com/Jcbnunez/BarnacleEcoGenomics.**

References

Bakker EG, Stahl EA, Toomajian C, Nordborg M, Kreitman M, Bergelson J. 2006. Distribution of genetic variation within and among local populations of Arabidopsis thaliana over its species range. Mol Ecol 15:1405-1418.

Bergland AO, Behrman EL, O'Brien KR, Schmidt PS, Petrov DA. 2014. Genomic Evidence of Rapid and Stable Adaptive Oscillations over Seasonal Time Scales in Drosophila. PLoS Genet 10:e1004775.

Bertness MD. 1989. Intraspecific Competition and Facilitation in a Northern Acorn Barnacle Population. Ecology 70:257-268.

Bertness MD, Gaines SD, Bermudez D, Sanford E. 1991. Extreme spatial variation in the growth and reproductive output of the acorn barnacle Semibalanus balanoides. Marine Ecology Progress Series 75:91-100.

Bertness MD, Gaines SD, Stephens EG, Yund PO. 1992. Components of recruitment in populations of the acorn barnacle Semibalanus balanoides (Linnaeus). Journal of Experimental Marine Biology and Ecology 156:199-215.

Botero CA, Weissing FJ, Wright J, Rubenstein DR. 2015. Evolutionary tipping points in the capacity to adapt to environmental change. Proc Natl Acad Sci U S A 112:184-189.

Bouckaert R, Heled J, Kuhnert D, Vaughan T, Wu CH, Xie D, Suchard MA, Rambaut A, Drummond AJ. 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Comput Biol 10:e1003537.

Brochmann C, Gabrielsen TM, Nordal I, Landvik JY, Elven R. 2003. Glacial survival or tabula rasa? The history of North Atlantic biota revisited. Taxon 52.

Brown AF, Kann LM, Rand DM. 2001. Gene flow versus local adaptation in the northern acorn barnacle, Semibalanus balanoides: insights from mitochondrial DNA variation. Evolution 55:1972-1979.

458 Bucklin A, Steinke D, Blanco-Bercial L. 2011. DNA Barcoding of Marine Metazoa. Annu.
459 Rev. Mar. Sci. 3:471-508.

460 Buhl E, Bradlaugh A, Ogueta M, Chen KF, Stanewsky R, Hodge JJ. 2016. Quasimodo
461 mediates daily and acute light effects on Drosophila clock neuron excitability. Proc Natl
462 Acad Sci U S A 113:13486-13491.

463 Chernomor O, von Haeseler A, Minh BQ. 2016. Terrace Aware Data Structure for
464 Phylogenomic Inference from Supermatrices. Syst Biol 65:997-1008.

465 Clark AG. 1997. Neutral behavior of shared polymorphism. Proc Natl Acad Sci U S A
466 94:7730-7734.

467 Drummond AJ, Nicholls GK, Rodrigo AG, Solomon W. 2002. Estimating mutation
468 parameters, population history and genealogy simultaneously from temporally spaced
469 sequence data. Genetics 161:1307-1320.

470 Dufresne F, Bourget E, Bernatchez L. 2002. Differential patterns of spatial divergence in
471 microsatellite and allozyme alleles: further evidence for locus-specific selection in the
472 acorn barnacle, Semibalanus balanoides? Molecular Ecology 11:113-123.

473 Eanes WF. 1999. Analysis of Selection on Enzyme Polymorphisms. Annual Review of
474 Ecology and Systematics 30:301-326.

475 Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. 2009. GOrilla: a tool for discovery
476 and visualization of enriched GO terms in ranked gene lists. Bmc Bioinformatics 10:48.

477 Faria R, Johannesson K, Butlin RK, Westram AM. 2019. Evolving Inversions. Trends
478 Ecol Evol 34:239-248.

479 Fijarczyk A, Babik W. 2015. Detecting balancing selection in genomes: limits and
480 prospects. Mol Ecol 24:3529-3545.

481 Flight PA, O'Brien MA, Schmidt PS, Rand DM. 2012. Genetic Structure and the North
482 American Postglacial Expansion of the Barnacle, Semibalanus balanoides. Journal of
483 Heredity 103:153-165.

484 Flight PA, Rand DM. 2012. Genetic variation in the acorn barnacle from allozymes to
485 population genomics. Integrative and Comparative Biology 52:418-429.

486 Flowerdew MW. 1983. Electrophoretic Investigation of Populations of the Cirripede
487 Balanus-Balanoides (L) around the North-Atlantic Seaboard. Crustaceana 45:260-278.

488 Gillespie J. 1973. Polymorphism in random environments. Theoretical population
489 biology 4:193-195.

490 Green RE, Krause J, Briggs AW, Maricic T, Stenzel U, Kircher M, Patterson N, Li H,
491 Zhai W, Fritz MH, et al. 2010. A draft sequence of the Neandertal genome. Science
492 328:710-722.

493 Hartl DL, Clark AG. 1997. Principles of population genetics: Sunderland: Sinauer
494 associates.

495 Hedrick PW. 2006. Genetic polymorphism in heterogeneous environments: The age of
496 genomics. Annual Review of Ecology Evolution and Systematics 37:67-93.

497 Hedrick PW, Ginevan ME, Ewing. EP. 1976. Genetic polymorphism in heterogeneous
498 environments. Annual Review of Ecology and Systematics 7.

499 Helmuth B, Harley CD, Halpin PM, O'Donnell M, Hofmann GE, Blanchette CA. 2002.
500 Climate change and latitudinal patterns of intertidal thermal stress. Science 298:1015-
501 1017.

502 Herrera S, Watanabe H, Shank TM. 2015. Evolutionary and biogeographical patterns of
503 barnacles from deep-sea hydrothermal vents. Mol Ecol 24:673-689.

504 Hodge JJ, Stanewsky R. 2008. Function of the Shaw potassium channel within the
505 Drosophila circadian clock. PLoS ONE 3:e2274.

506 Johannesson K, Ring AK, Johannesson KB, Renborg E, Jonsson PR, Havenhand JN.
507 2018. Oceanographic barriers to gene flow promote genetic subdivision of the tunicate
508 Ciona intestinalis in a North Sea archipelago. Mar Biol 165:126.

509 Jones SJ, Southward AJ, Wethey DS. 2012. Climate change and historical biogeography
510 of the barnacle Semibalanus balanoides. Global Ecology and Biogeography 21:716-724.

511 Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory
512 requirements. Nat Methods 12:357-360.

513 Kimura M, Crow J. 1964. The number of alleles that can be maintained in a finite
514 population. Genetics 49:725–738.

515 King JL. 1967. Continuously distributed factors affecting fitness. Genetics 55:483-492.

516 Kofler R, Orozco-terWengel P, De Maio N, Pandey RV, Nolte V, Futschik A, Kosiol C,
517 Schlotterer C. 2011. PoPoolation: a toolbox for population genetic analysis of next
518 generation sequencing data from pooled individuals. PLoS ONE 6:e15925.

519 Kofler R, Pandey RV, Schlotterer C. 2011. PoPoolation2: identifying differentiation
520 between populations using sequencing of pooled DNA samples (Pool-Seq).
521 Bioinformatics 27:3435-3436.

522 Lee Y, Lee Y, Lee J, Bang S, Hyun S, Kang J, Hong ST, Bae E, Kaang BK, Kim J. 2005.
523 Pyrexia is a new thermal transient receptor potential channel endowing tolerance to
524 high temperatures in Drosophila melanogaster. Nat Genet 37:305-310.

525 Levene H. 1953. Genetic Equilibrium When More Than One Ecological Niche Is
526 Available. American Naturalist 87:331-333.

527 Lewontin RC, Hubby JL. 1966. A molecular approach to the study of genic
528 heterozygosity in natural populations. II. Amount of variation and degree of
529 heterozygosity in natural populations of Drosophila pseudoobscura. Genetics 54:595-
530 609.

531 Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-
532 MEM. arXiv:1303.3997.

533 Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G,
534 Durbin R, Genome Project Data Processing S. 2009. The Sequence Alignment/Map
535 format and SAMtools. Bioinformatics 25:2078-2079.

536 Machado HE, Bergland AO, Taylor R, Tilk S, Behrman E, Dyer K, Fabian DK, Flatt T,
537 González J, Karasov TL, et al. 2019. Broad geographic sampling reveals predictable and
538 pervasive seasonal adaptation in Drosophila. bioRxiv (unpublished data)
539 https://www.biorxiv.org/content/biorxiv/early/2019/10/11/337543.full.pdf - last
540 accessed December 12, 2019.

541 Mackay TF, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y,
542 Magwire MM, Cridland JM, et al. 2012. The Drosophila melanogaster Genetic Reference
543 Panel. Nature 482:173-178.

544 Maggs CA, Castilho R, Foltz D, Henzler C, Jolly MT, Kelly J, Olsen J, Perez KE, Stam W,
545 Väinölä R, et al. 2008. Evaluating Signatures of Glacial Refugia for North Atlantic
546 Benthic Marine Taxa. Ecology 89:S108-S122.

547 Malinsky M, Matschiner M, Svardal H. 2020. Dsuite - fast D-statistics and related
548 admixture evidence from VCF files. bioRxiv (unpublished data)
549 https://www.biorxiv.org/content/10.1101/634477v2 - last accessed May 1, 2020.

550 Messer PW, Petrov DA. 2013. Population genomics of rapid adaptation by soft selective
551 sweeps. Trends Ecol Evol 28:659-669.

552 Metz EC, Palumbi SR. 1996. Positive selection and sequence rearrangements generate
553 extensive polymorphism in the gamete recognition protein bindin. Mol Biol Evol 13:397-
554 406.

555 Milkman RD. 1967. Heterosis as a Major Cause of Heterozygosity in Nature. Genetics
556 55:493-&.

557 Nunez JCB, Elyanow RG, Ferranti DA, Rand DM. 2018. Population Genomics and
558 Biogeography of the Northern Acorn Barnacle (Semibalanus balanoides) Using Pooled

559 Sequencing Approaches. In: Oleksiak MF, Rajora OP, editors. Population Genomics:
560 Marine Organisms: Springer, Cham. p. 139-168.

561 Nunez JCB, Flight PA, Neil KB, Rong S, Eriksson LA, Ferranti DA, Rosenblad MA,
562 Blomberg A, Rand DM. 2020. Footprints of natural selection at the mannose-6-
563 phosphate isomerase locus in barnacles. Proc Natl Acad Sci U S A:201918232.

564 Patton H, Hubbard A, Andreassen K, Auriac A, Whitehouse PL, Stroeven AP, Shackleton
565 C, Winsborrow M, Heyman J, Hall AM. 2017. Deglaciation of the Eurasian ice sheet
566 complex. Quaternary Science Reviews 169:148-172.

567 Perez-Losada M, Harp M, Hoeg JT, Achituv Y, Jones D, Watanabe H, Crandall KA.
568 2008. The tempo and mode of barnacle evolution. Mol Phylogenet Evol 46:328-346.

569 Pickrell JK, Pritchard JK. 2012. Inference of population splits and mixtures from
570 genome-wide allele frequency data. PLoS Genet 8:e1002967.

571 Pineda J, Starczak V, Stueckle TA. 2006. Timing of successful settlement:
572 demonstration of a recruitment window in the barnacle Semibalanus balanoides.
573 Marine Ecology Progress Series 320:233-237.

574 Pryszcz LP, Gabaldon T. 2016. Redundans: an assembly pipeline for highly heterozygous
575 genomes. Nucleic Acids Research 44:e113.

576 Rand DM, Spaeth PS, Sackton TB, Schmidt PS. 2002. Ecological Genetics of Mpi and
577 Gpi Polymorphisms in the Acorn Barnacle and the Spatial Scale of Neutral and Non-
578 neutral Variation. Integrative and Comparative Biology 42:825-836.

579 Reich D, Thangaraj K, Patterson N, Price AL, Singh L. 2009. Reconstructing Indian
580 population history. Nature 461:489-494.

581 Ruddiman WF, Mcintyre A. 1981. The North-Atlantic Ocean during the Last
582 Deglaciation. Palaeogeography Palaeoclimatology Palaeoecology 35:145-214.

583 Sanford E, Menge BA. 2001. Spatial and temporal variation in barnacle growth in a
584 coastal upwelling system. Marine Ecology Progress Series 209:143-157.

585 Schiffels S, Durbin R. 2014. Inferring human population size and separation history
586 from multiple genome sequences. Nat Genet 46:919-925.

587 Schlotterer C, Tobler R, Kofler R, Nolte V. 2014. Sequencing pools of individuals-mining
588 genome-wide polymorphism data without big funding. Nature Reviews Genetics 15:749-
589 763.

590 Schmidt PS, Bertness MD, Rand DM. 2000. Environmental heterogeneity and balancing
591 selection in the acorn barnacle Semibalanus balanoides. Proc Biol Sci 267:379-384.

592 Schmidt PS, Rand DM. 2001. Adaptive maintenance of genetic polymorphism in an
593 intertidal barnacle: habitat- and life-stage-specific survivorship of Mpi genotypes.
594 Evolution 55:1336-1344.

595 Schmidt PS, Rand DM. 1999. Intertidal microhabitat and selection at Mpi: Interlocus
596 contrasts in the northern acorn barnacle, Semibalanus balanoides. Evolution 53:135-
597 146.

598 Schmidt PS, Serrão EA, Pearson GA, Riginos C, Rawson PD, Hilbish TJ, Brawley SH,
599 Trussell GC, Carrington E, Wethey DS, et al. 2008. Ecological Genetics in the North
600 Atlantic: Environmental Gradients and Adaptation at Specific Loci. Ecology 89:S91-
601 S107.

602 Stanke M, Waack S. 2003. Gene prediction with a hidden Markov model and a new
603 intron submodel. Bioinformatics 19:ii215-ii225.

604 Sundell K, Wrange AL, Jonsson PR, Blomberg A. 2019. Osmoregulation in Barnacles:
605 An Evolutionary Perspective of Potential Mechanisms and Future Research Directions.
606 Front Physiol 10:877.

607 Sved JA, Reed TE, Bodmer WF. 1967. The Number of Balanced Polymorphisms That
608 Can Be Maintained in a Natural Population. Genetics 55:469-481.

609 Tracey WD, Wilson RI, Laurent G, Benzer S. 2003. painless, a Drosophila Gene
610 Essential for Nociception. Cell 113:261-273.

611 Uyenoyama MK. 2005. Evolution under tight linkage to mating type. New Phytol
612 165:63-70.

613 Väinölä R. 2003. Repeated trans-Arctic invasions in littoral bivalves: molecular
614 zoogeography of the Macoma balthica complex. Marine Biology 143:935-946.

615 Veliz D, Bourget E, Bernatchez L. 2004. Regional variation in the spatial scale of
616 selection at MPI* and GPI* in the acorn barnacle Semibalanus balanoides (Crustacea). J
617 Evol Biol 17:953-966.

618 Vermeij GJ. 1991. Anatomy of an invasion: the trans-Arctic interchange. Paleobiology
619 17:281-307.

620 Wares JP, Cunningham CW. 2001. Phylogeography and Historical Ecology of the North
621 Atlantic Intertidal. Evolution 55:2455-2469.

622 Watt WB, Dean AM. 2000. Molecular-functional studies of adaptive genetic variation in
623 prokaryotes and eukaryotes. Annu Rev Genet 34:593-622.

624 Williams G. 1966. Adaptation and Natural Selection: A Critique of Some Current
625 Evolutionary Thought. Princeton, NJ.: Princeton University Press.

626 Wittmann MJ, Bergland AO, Feldman MW, Schmidt PS, Petrov DA. 2017. Seasonally
627 fluctuating selection can maintain polymorphism at many loci via segregation lift. Proc
628 Natl Acad Sci U S A 114:E9932-E9941.

629 Wray GA. 2007. The evolutionary significance of cis-regulatory mutations. Nature
630 Reviews Genetics 8:206-216.

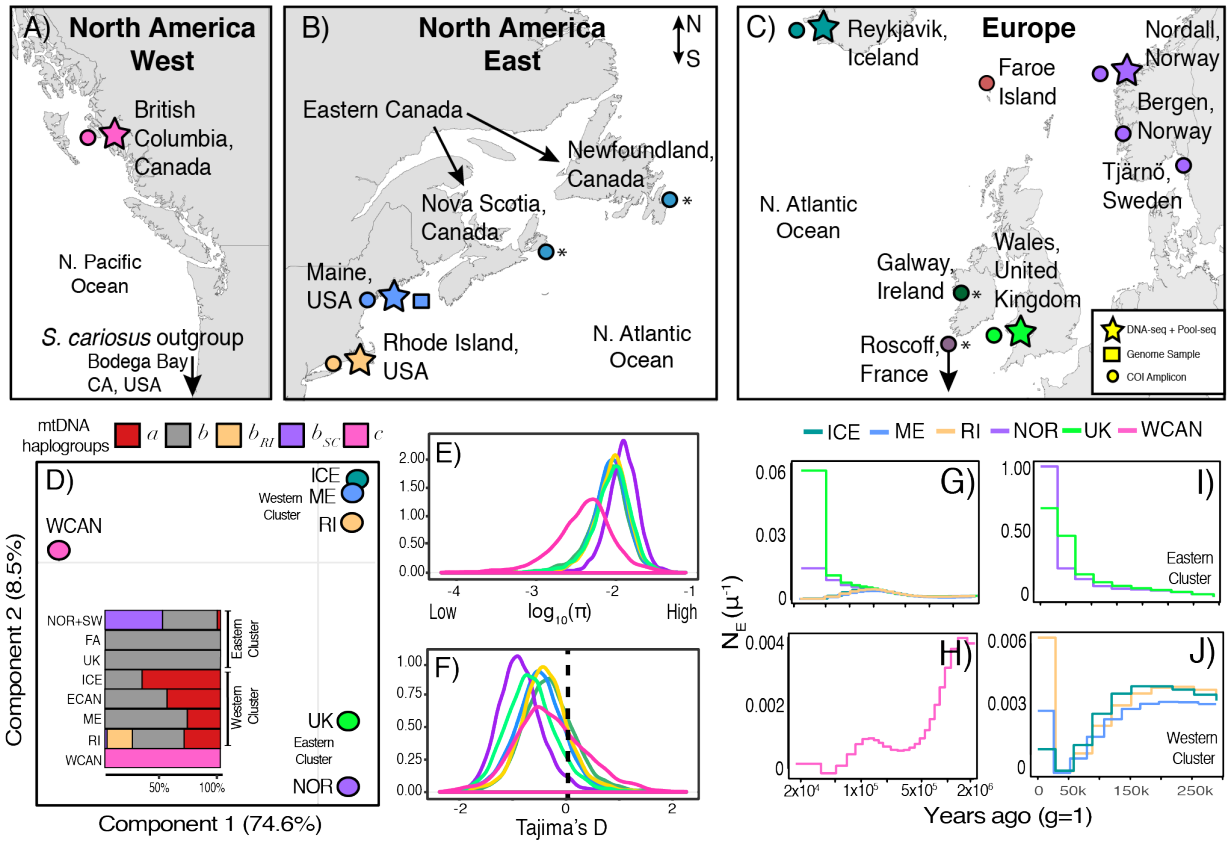
631 Wu Q, Han TS, Chen X, Chen JF, Zou YP, Li ZW, Xu YC, Guo YL. 2017. Long-term
632 balancing selection contributes to adaptation in Arabidopsis and its relatives. Genome
633 Biol 18:217.

634 Xu SY, Cang CL, Liu XF, Peng YQ, Ye YZ, Zhao ZQ, Guo AK. 2006. Thermal nociception
635 in adult Drosophila: behavioral characterization and the role of the painless gene. Genes
636 Brain Behav 5:602-613.

637 Ye C, Hill CM, Wu S, Ruan J, Ma ZS. 2016. DBG2OLC: Efficient Assembly of Large
638 Genomes Using Long Erroneous Reads of the Third Generation Sequencing
639 Technologies. Sci Rep 6:31900.
640

641 **Figure 1. Genetic variation and phylogeography**

642



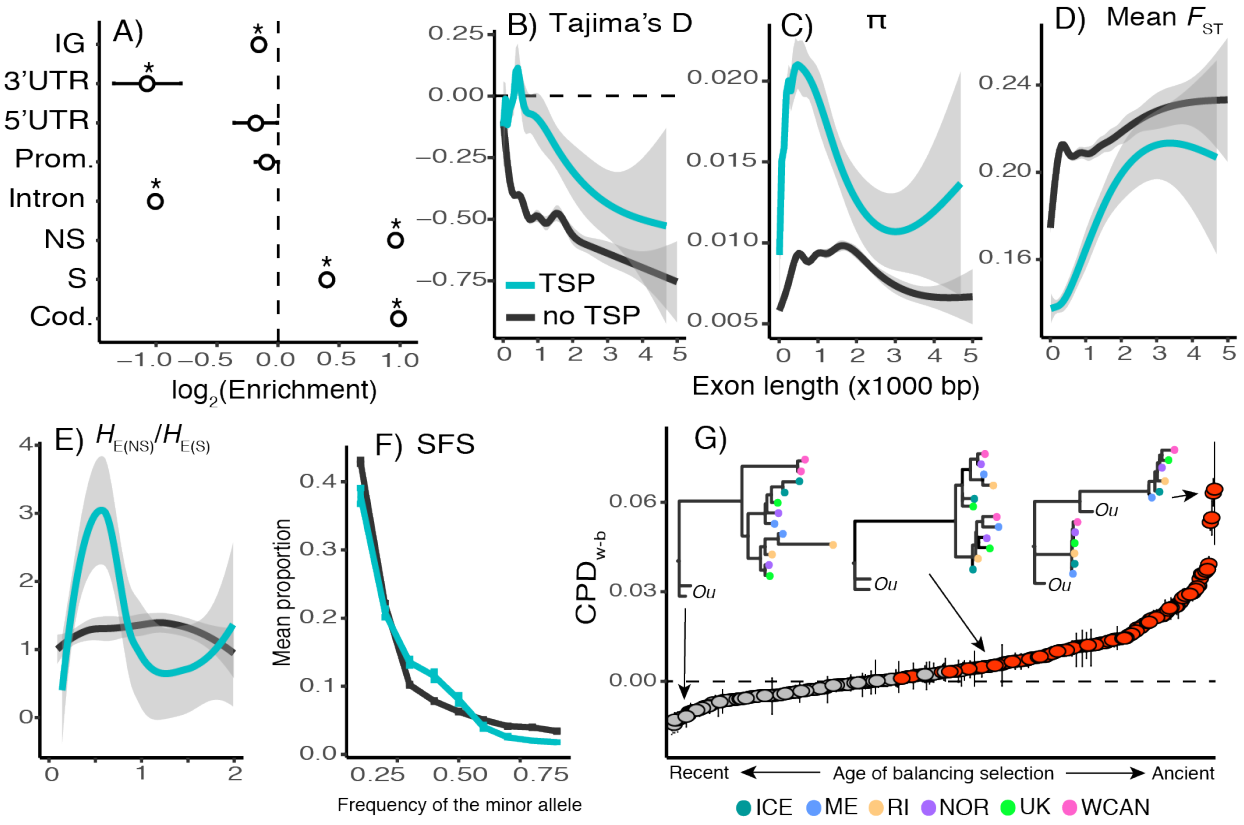
643

644 **A.** Map of the North Pacific coast of North America with collection sites indicated.
645 **B.** Collections in the Atlantic Eastern coast of North America. **C.** Collections in the
646 Atlantic European coast. For A, B, and C, stars indicate sites where a single individual and
647 a pool of multiple individuals were collected, the hexagon indicates the site from which
648 the reference genome was constructed, and the circles indicate sites were *COX I* data was
649 collected. The asterisks indicate cases where *COX I* data was downloaded. **D**. PCA with
650 Pool-seq data from all populations. The colors represent populations. Pacific Canada
651 (WCAN; pink), Maine (ME; blue), Rhode Island (RI; yellow), Iceland (ICE; dark green),
652 Norway (NOR; purple), United Kingdom (UK; light green). **D-inset**. Distribution of
653 mitochondrial haplotypes across all populations. The names *a*, *b* (including b_{RI} and b_{SC}),
654 and *c* represent common mtDNA haplotypes observed in populations. **E**. Nucleotide
655 diversity ($\log_{10} \pi$) for all nuclear genes across all populations. **F**. Tajima's *D* for all nuclear
656 genes across all populations. The dashed vertical line marks 0, the expected value under
657 a neutral model. The y-axis in E and F show the density of observations. **G**. Demographic

reconstruction for North Atlantic individuals showing demographic changes from 2 mya to 200 kya. **I**. demographic changes in British and Norwegian individuals. **H**. North Pacific individual showing demographic changes from 2 mya to 200 kya. **J**. Plot of recent (today – 250 kya) demographic changes in the North American and Icelandic individuals.

662 **Figure 2. Evidence for balancing selection across the genome**

663

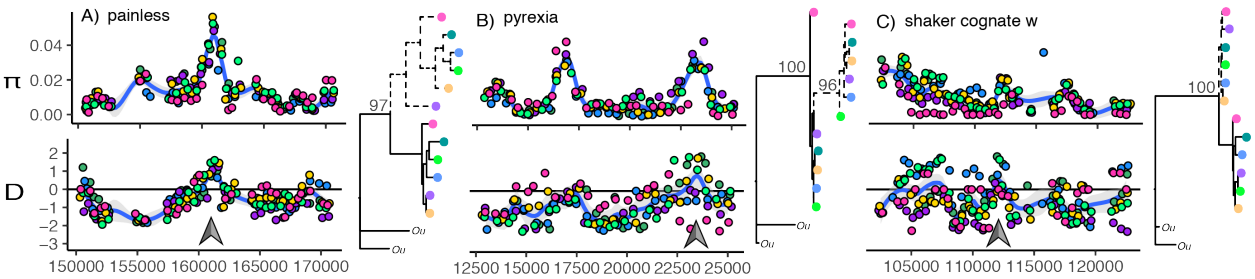


664

665 **A**. Enrichment analysis of TSPs across the genome of *S. balanoides* based on all
666 populations studied. The asterisks symbols represent statistical significance.
667 Abbreviations: promoters (Prom.), nonsynonymous loci (NS), synonymous loci (S),
668 coding loci (Cod.). **B**. Plot of Tajima's *D* (as a function of length) of exons bearing TSPs
669 versus all other exons not bearing TSPs. **C**. Same as B but for nucleotide diversity (π). **D**.
670 Same as B but for mean F_{ST}. **E**. Same as B but for the ratio of nonsynonymous
671 heterozygosity to synonymous heterozygosity. **F**. Site frequency spectrum for whole genes
672 with TSPs vs other genes. Vertical bars are 95% confidence intervals. **G**. Candidate genes
673 under balancing selection ranked according to their $CPD_{w\text{-}b}$ values (interquartile ranges
674 shown as error bars). Red values indicate statistical significance. Horizontal dashed line
675 indicates $CPD_{w\text{-}b} = 0$. Three example allele tree topologies are shown. The sister taxon, *S.*
676 *cariosus*, is shown as "*Ou*" (for outgroup). The x-axis for B, C, D, and E is exon length (x
677 1000 bp).

678

Figure 3. Balancing selection on ecologically important genes



We present patterns of genetic variation (π and D estimated from pool-seq data, and allele tree topologies estimated from single individuals) for 3 example genes: A) painless (*Pain*), B) pyrexia (*Pyx*), C) shaker cognate w (*Shaw*). Grey arrows show regions that contain TSPs. In Tajima's D panels, the horizontal line marks the D = 0 point. For all trees, the sister taxon, *S. cariosus*, is shown as "*Ou*". The colors represent populations. WCAN (pink), ME (blue), RI (yellow), ICE (dark green), NOR (purple), UK (light green). The x-axis shows base pair position within scaffolds.