History and extent of introgressive hybridization in Puget Sound rockfishes (*Sebastes auriculatus, S. caurinus, and S. maliger*)

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Chapter 1: Asymmetric introgression among three rockfish species (*Sebastes* spp.) in the Salish Sea, Northeast Pacific Ocean

Abstract

Natural hybridization is common in closely related species especially where they invade novel habitat. The patterns of introgressive hybridization are often asymmetrical and are attributed to various influences from selection to gene flow and dispersal. Hybridization has previously been detected in *S. auriculatus, S. caurinus,* and *S. maliger* in Puget Sound (the southern Salish Sea) but the details of the history and direction of introgression are incomplete. These Pacific rockfish species are sympatric over most of their geographic range but hybridization has only been detected in Puget Sound. In order to measure interspecific gene flow, we used sequence data from one mitochondrial locus, three nuclear intron loci, and one coding gene to compare interspecific gene flow between collections from the Salish Sea and the Pacific coast. Although ancestral polymorphisms could not be excluded in the analysis of phylogenetic trees, coalescence analysis provided clear evidence for broad-scale, asymmetrical introgression from *S. maliger* into *S. auriculatus* and *S. caurinus* and a much lower incidence of introgression between *S. auriculatus* and *S. caurinus*. The absence of F1 hybrids was consistent with historical hybridization events or ongoing, low-level hybridization in the Salish Sea. Although hybrids were found in high frequency, introgressed rockfish in the Salish Sea appear to maintain the morphological characters and coloration of pure parental species morphology. This rockfish hybrid system, with asymmetrical introgression and the maintenance of parental species, may prove useful to study both mechanisms that maintain species boundaries and processes that facilitate speciation.

Introduction

Introgressive hybridization is an important process in evolution (Arnold 1997; Barton 2001) that promotes diversification and adaptability by providing a rich source of genetic variability in species (Harrison 1990; Coyne and Orr 2004; Arnold and Martin 2010). Hybrids can play an important role in adaptive radiation as they become more genetically diverse and distinct from parental species, thus allowing them to colonize and compete in new habitats (Arnold 1997). Natural hybridization is common at the periphery of species ranges, where closely related species invade new habitat, find low population densities, and encounter few conspecifics as potential mates (Rieseberg et al. 2003, Grant et al. 2004; Seehausen 2004; Mallet 2005; Nolte et al. 2005). In these ecological peripheries or geographical range edges, hybrids theoretically have less competition with parental species, and introgressive hybridization can be maintained (Arnold 1997). Asymmetrical introgression is often found in novel habitats (Barton and Hewitt 1989, Currat et al. 2008) and these patterns are attributed to various influences from selection (Barton 2001) to gene flow and dispersal (Currat et al. 2008).

Research on introgressive hybridization depends crucially on powerful detection of hybrids and the accurate identification of later generation hybrids. Over the past several decades molecular genetic markers have become the standard for detecting hybrids; moreover, modern techniques have made it possible to detect backcrossed individuals and genetic introgression in hybrid populations (Avise 2001). Hybridization among closely related species has been described in most major taxonomic groups (Burke and Arnold 2001; Mallet 2005). Historically, hybrids were identified using morphological characters with the assumption that hybrids were intermediate to the parental types and that there is no plasticity within the morphological trait. Backcrossed individuals are often morphologically indistinguishable from parental species and low levels of introgression may not be detected in the wild (Naisbit et al. 2003). As more molecular genetic markers become available, evidence of cryptic hybridization and reticulate speciation is rapidly increasing (Arnold 1997, Arnold and Meyer 2006).

Despite the power of molecular markers, introgressive hybridization is often difficult to disentangle from other evolutionary signals in molecular data (Funk and

Omland 2003; McKay and Zink 2010). Many closely related species share a portion of their genome either from retention of ancestral polymorphism or recent hybridization followed by introgression (Mallet 2005). Hybrids are often detected during phylogenetic analysis when morphological species are not monophyletic (Funk and Omland 2003) or when gene trees at independent loci are incongruent (Linder and Rieseberg 2004). The interpretation of such patterns is complicated, however, because incomplete lineage sorting will create the same signal in gene trees as hybridization (Wakely 1996; Holder et al. 2001).

One method often used to distinguish between incomplete lineage sorting and introgressive hybridization in closely related species is to compare gene trees from mitochondrial and several independent nuclear genetic loci (Funk and Omland 2003). The maternally inherited haploid mitochondrial locus is expected to evolve to species monophyly faster than diploid biparentally inherited nuclear loci (Palumbi 2001). Monophyly at nuclear loci genealogies and shared polymorphisms at the mitochondria DNA can therefore be interpreted as evidence for mitochondrial DNA introgression (Redenback and Taylor 2002). However, this assumes that mitochondrial DNA is more susceptible to introgressive hybridization than nuclear loci (Rieseberg and Soltis 1991; Avise 1994; Currat et al. 2008).

Another way to detect hybridization from phylogenetic trees is through geographic comparison (Small et al. 2004). Shared polymorphisms in areas of sympatry together with reciprocal monophyly in areas of allopatry provide clear evidence of hybridization (Hare and Avise 1998; Donnelly et al. 2004). Additional evidence for long divergence times between species can strengthen the evidence for hybridization (Hare and Avise 1998; Small et al. 2004; Ray et al. 2008); yet stochastic genetic processes in the genealogies of species necessitates systematic hypothesis testing to evaluate causes of paraphyly in gene tress (Rosenberg and Nordborg 2002; Maddison and Knowles 2006).

Testing the hypothesis of hybridization or interspecific migration can also be performed using coalescence approaches in an isolation with migration (IM) model because the IM method accounts for these stochastic genetic variances by evaluating all locus genealogies consistent with the data (Nielsen and Wakeley 2001; Hey and Nielsen 2004). The IM method evaluates the following six parameters simultaneously: size of

ancestral population and each daughter population, migration rates among daughter populations for each direction, and the splitting times (Hey and Nielsen 2004). Interspecific gene flow among species can be quantified using the IM method and migration rates can then be compared between geographic regions to test for localized hybridization.

With the increasing power of molecular approaches, hybridization has been documented in a growing number of marine species (Roques et al. 2001; Yaakub et al. 2006; Garrett et al. 2007, Tringali et al. 2011, Morgan et al. 2012) including three closely related (Hyde and Vetter 2007) Pacific rockfish species, *Sebastes auriculatus*, *S. caurinus*, and *S. maliger* (Seeb 1998, Buonaccorsi et al. 2002). Like other species of *Sebastes*, these taxa are internal fertilizers and ovoviviparous, long lived and late maturing (Stout et al. 2001; Love et al. 2002). Adult *S. auriculatus*, *S. caurinus*, and *S. maliger* show strong site fidelity and remain sedentary in rocky reef areas at shallow depth (<50 m), although *S. maliger* is sometimes found deeper than *S. auriculatus* and *S. caurinus* (Love et al 2002; Palsson et al. 2009) while *S. auriculatus* can often be found in shallow intertidal and estuary habitat (Stout et al. 2001; Love et al. 2002). The geographic distribution for these species mostly overlaps on the Pacific Ocean coast from South California to the Gulf of Alaska with S. auriculatus more in Southern California and *S. maliger* more in Alaska (Love et al. 2002). One exception is *S. auriculatus* which has a break in distribution from Washington to North Vancouver Island, B.C. except for a population in the Puget Sound Basin, the most southern portion of the Salish Sea (Love et al. 2002, Buonaccorsi et al. 2002).

Sebastes auriculatus, *S. caurinus*, and *S. maliger* in Puget Sound, in the south Salish Sea, are isolated from the coast (Stout et al. 2001) and therefore experience distinct differences in habitat and water quality compared with the coast. Puget Sound is a semi-enclosed glacial fjord that formed towards the end of the Pleistocene epoch, approximately 12,000 years ago. Compared to the coast, Puget Sound has limited rocky reef habitat (Pacunski and Palsson 1998), lower salinity, more variable temperatures and anoxic conditions (Ebbert et al. 2000). Recruitment of juvenile rockfish from outside the Puget Sound basin appears to be limited due to low surface water exchange from outside the main basin (Engie and Klinger 2007). Indeed, *S. auriculatus*, *S. caurinus*, and *S.*

maliger gene flow show high differentiation from coastal populations (Seeb 1998; Stout et al. 2001; Buonaccorsi 2002, 2005) and Puget Sound is also home to distinct populations of several other marine species (Stout et al. 2001).

Sebastes auriculatus, *S. caurinus*, and *S. maliger* are sympatric in Puget Sound and they are the most abundant rockfish species found in the basin. Hybridization and intermediate phenotypes between these species have only been detected in Puget Sound, although these species are also found in sympatry along the Pacific coast (Seeb 1998). While hybridization among these species of *Sebastes* has been described previously, the information on the extent of introgression across their range as well detailed data on direction of introgression is incomplete. Buonaccorsi et al. (2005) found directional introgression from *S. maliger* into *S. auriculatus* in Puget Sound, while another study (Seeb 1998) found evidence for introgression from *S. auriculatus* and *S. caurinus* into *S. maliger*, while still another study found no evidence of introgression in Puget Sound *S. caurinus* (Buonaccorsi et al 2002). Here, we provide a more detailed evaluation of the direction and extent of introgression among *S. auriculatus*, *S. caurinus*, and *S. maliger* by employing multilocus sequence data with phylogenetic and coalescent analyses to measure interspecific gene flow between *S. auriculatus*, *S. caurinus*, and *S. maliger* on the Pacific coast and the Salish Sea.

Methods

Tissue collection and sample preparation

Two tissue sample collections were obtained for these analyses. The Pacific coast collection contained tissue samples from morphologically identified *S. auriculatus* (n =13), S. *caurinus* (n =12), and *S. maliger* (n =17), provided by the Southwest Fisheries Science Center (SWFSC). The coastal samples were collected across the full coastal range from Alaska to California (1994 – 2002), (Figure 1.1, Table 1.1). The Salish Sea collection contained tissue samples from morphologically identified *S. auriculatus* (n =24), S. *caurinus* (n =33), and *S. maliger* (n =40) provided by the Washington Department of Fish and Wildlife (WDFW). The Salish Sea collection was mostly from the Puget Sound Basin but also included a few samples from the Straight of Juan de Fuca

and the San Juan Archipelago. (1997 – 2003, Figure 1.2, Table 1.1). Tissues were provided as fin clips or muscle stored in 95 % ethanol. Data collected from each whole specimen included the capture locality (latitude, longitude, and depth), species, length, sex, and weight. Although whole specimens were not retained, all fish identifications were made by experienced rockfish biologists and were collected as true species representatives (Wayne Palsson, NOAA Alaska Fisheries Science Center, pers. comm., 2010; Carol Kimbrell, NOAA SWFSC, pers. comm., 2008). Three whole fish specimens from closely related species, *Sebastes dallii*, *S. atrovirens*, and *S. elongatus*, were also used for these analyses. Whole specimens were described by morphological characteristics, photographed, and tissue samples were taken and stored in 95% non-denatured ethanol. The specimens were then preserved in formalin, stored in ethanol, and catalogued at the University of Washington Burke Museum Fish Collection (UW 114033, UW 114048, UW 48830) http://www.burkemuseum.org/ichthyology.

Genomic DNA was extracted from a 2 mm^2 piece of fin or muscle tissue using a Qiagen DNeasy 96 Tissue Kit on the Qiagen BioRobot 8000. Five DNA regions were targeted using PCR amplification and primers for the mitochondrial DNA locus cytochrome b mitochondrial (*Cytb*), 5' external transcribed spacer (*Ets*), *S7* ribosomal intron 2 (*S7*), malate dehydrogenase (*Mdh*), and malic enzyme (*Mep*). Primers for Cytb, *Ets*, and *S7* were obtained from the published articles (Table 1.2). Primers for *Mdh* and *Mep* were designed using conserved regions across gene sequences from fish and other vertebrates available in NCBI Genbank (Park, L.K. unpublished). Additional primers for *S7*, *Mdh*, and *Mep* were designed here to improve sequence data in rockfish. Individual PCR amplifications were performed on each genomic DNA template using a BioRad 96-well DNA Engine Tetrad. The primer sequences and cycling conditions for the PCR amplifications were specific to each locus (Table 1.2). Generally, PCR reactions were performed in a 40 µl reaction with 0.5 unit Go*Taq* DNA polymerase (Promega), 1X Go*Taq* Buffer, 200µM dNTP (Promega), each primer at 100 – 400 nM, and approximately 10 – 20 ng of genomic DNA. PCR cycles were carried out for each locus with an initial denature step at 95° C for 2 minutes and 32 cycles of 94 °C for 40 seconds, locus specific annealing conditions (Table 1.2), and 72 °C for 60 seconds. PCR products were visualized using agarose gel electrophoresis and ethidium bromide / UV transducer

in order to verify quality of amplification. PCR products were purified using the Montage MultiScreen 96-well plate protocol (Millipore) and sequenced in both directions using PCR primers and Big Dye Terminator Cycle Sequencing Ready Reaction version 3.1 (Life Sciences). Sequencing reactions were purified using CleanSeq Dye Terminator Removal Kit (Beckman Coulter Genomics) and size-separated on an ABI3100 Genetic Analyzer using POP4 polymer and an 80 cm capillary array.

Sequencing and DNA polymorphism:

The sequences were visualized, edited, and aligned using the software program Codon Code Aligner software version 3.7 which uses a quality based method to determine sequence consensus. Interpretations of heterozygous peaks were evaluated by eye from high quality sequence data (Q > 0.95). Locus alignments were evaluated for segregating sites in both the coastal and Salish Sea populations separately. All individuals used for analysis had complete sequence and locus data. All nucleotide gaps were removed from the sequence data and diploid nuclear data for each species group and locus were phased into haplotypes using the program PHASE implemented in the software program DNAsp v5 (Librado and Rozas 2009) using the following MCMC parameters: Burn of 10,000 steps, 10 step thinning intervals, and 100,000 iterations. The same software was used to analyze each locus and populations for total haplotype number, number of segregating sites, number of unique haplotypes, haplotype diversity and its variance (Nei 1987, equations 8.4 and 8.12 but replacing 2n by n), nucleotide diversity (π , Nei 1987, eq. 10.5); nucleotide divergence (K, Nei 1987, eq. 10.20), population mutation rate per nucleotide site (θ , Nei 1987 equation 10.3), and population mutation rate per gene, Watterson's θ (Watterson 1975).

Tests for recombination, neutrality and population structure:

The isolation with migration models used in coalescent analysis was derived under several assumptions of molecular evolution (Hey and Nielsen 2004, 2007) including no recombination, no selection, and no population structure. Locus recombination and selection could bias the results from coalescent analyses (Strasburg and Rieseberg 2010) and the genetic polymorphism data can also reflect demographic

changes in populations. To determine non-recombining (NR) blocks from the nuclear DNA sequence, we tested for intralocus recombination using the "4 gamete test" method (Hudson and Kaplan 1985) implemented in DNAsp. The program output identified the recombination sites, which we used to report the total number of NR blocks for each locus. To test for selective neutrality at each locus, we used the Tajima's D (Tajima 1989) statistic implemented in DNAsp, which tests the assumption that under mutation-drift equilibrium π and θ should be the same parameter ($4N_e\mu$). The D statistic is used to test for signs of recent population expansion or purifying selection (large negative values), which is caused by an excess of low frequency polymorphisms. Population structure was tested using F_{st} between species and populations from haplotype frequencies, using the DNAsp implemented method following Hudson et al. 1992b, equation 3. The software program Arlequin version 3.5 (Excoffier et al. 1992) was used for the analysis of molecular variance (AMOVA) to quantify variation between populations in Puget Sound and the coast and between species.

Phylogenetics

For phylogenetic analysis, a haplotype sequence from *Sebastes elongatus* was used as the outgroup species and two haplotype sequences from *S. dallii* and *S. atrovirens* were used as ingroup control species. The ingroup species are all members of the sub-genus *Pteropodus* as described by Hyde and Vetter 2007 and are closely related to *S. auriculatus, S. caurinus*, and *S. maliger*. Initially, all individual haplotypes were evaluated using the phylogenetic neighbor joining (NJ) algorithm (Kimura 2-parameter model) in the software program Mega v4.0. This initial data evaluation (not shown) was used to determine overall phylogenetic patterns for individual fish from all morphological species across loci. Evaluation of the individual fish haplotype data also allowed conformation of the segregating sites for species from the sequence alignments. For each locus, the individual sequence data were collapsed into unique haplotypes and invariable sites were removed. The software program ModelTest v3.7 was used to determine the best DNA mutation model fit for the locus haplotype data using the software's default maximum likelihood settings. Phylogenies of the haplotypes were reconstructed using PAUP* 4.0 (Swofford 2003) with 1000 bootstrap data resamplings and maximum

likelihood heuristic search with random stepwise sequence addition, TBR branch swapping, and 1000 replicates. The resulting gene trees were evaluated for distinct clades and genetic clusters comprising haplotypes from morphological species given their geographic location. We identified nonconforming haplotypes that were shared among species in order to evaluate evidence of genetic introgression. A "nonconforming haplotype" is a genetic clade, cluster, or haplotype not corresponding to the morphological identification.

Coalescent Analysis

The isolation with migration model (Nielsen and Wakeley 2001) is modified in the program IMa2 (Hey and Nielsen 2007), which employs a Bayesian implemented Markov chain Monte Carlo (MCMC) method of distinguishing migration from isolation. IMa2 simultaneously estimates the marginal posterior probability densities (PPD) for the mutation rate scaled parameters: population size (q), migration rate (m), and divergence time (t) in generations. These probabilities are the estimated probability of the data given the genealogy and the probability of the genealogies given the data, $p(D|\Theta,G) = p(G|\Theta)\ p(D|G)$.

The data partitions used for the IMa2 analysis were one or two of the largest NR blocks from each nuclear locus sequence in addition to the entire *Cytb* locus sequence (see results). To get a rough estimate of population size (q) as a starting point for priors, we used the geometric mean of Watterson's θ across loci to estimate the population mutation rate (per gene per generation) and took 5 x θ for q (IMa2.2 program documentation). The starting priors were initially set as follows: q = 10; m = 1; t = 4. Multiple short, independent MCMC runs were performed using different parameter priors to determine optimal prior settings that produced the most complete posterior probabilities density plots. The final MCMC input priors were uniform for each species and were set to q = 1, m = 4, and t =1.

In order to test for differences in hybridization levels between the three species in the Salish Sea compared to the coast, we analyzed the two data sets independently. The input parameters were identical between the two data sets: Each locus was set for the HKY mutation model and migration was only allowed between sampled populations.

The MCMC chain was run for 100,000 burn-in steps with 1,000,000 run steps. Based on neutral expectation from maternal inheritance of a haploid mitochondrial genome compared to bi-parental inheritance of diploid nuclear loci, an inheritance scalar for the cytochrome b mitochondrial locus was set to 0.25 and the nuclear loci each were set to 1. Results for migration rate are reported as effective number of migrants ($2NM$) and are independent of mutation rate (μ) because the variable, μ, cancels out of the equation, $2NM = \theta M /2 = 4N\mu (m/\mu)/2$ (Hey 2010b). In contrast, absolute estimates for population size and divergence time are dependent on accurate estimates of mutation rate for each locus, though such point estimates were not necessary here. The resulting data were evaluated to ensure that the MCMC parameter probability surface was well explored, the marginal distributions were adequately sampled, and that duplicate MCMC run results were similar.

Results

DNA sequence data from five genes consisted of 293 – 785 nucleotides from 139 individuals across three species, each with two populations (Table 1.3). The sequence data for each locus resolved 8 – 32 unique haplotypes including ingroup controls and outgroup species (GenBank numbers: to be accessioned). Haplotype diversity was greater in coastal populations versus the Salish Sea in all species at the locus *Cytb*, as well as in *S. maliger* at *S7*, and in *S. auriculatus* and *S. maliger* at *Mdh* (Table 1.4). However, Watterson θ (population mutation rate per gene) estimates for all three species were higher in the Salish Sea than the coast (Table 1.4). The test for locus neutrality, Tajima's D, was significant ($p < 0.05$) for *Cytb* sequences from Salish Sea *S. maliger* (Table 1.3), but not for the other populations. This significant D value was due to a 3rd nucleotide variant and is not likely a signal from selection (Tajima 1989). No recombination was detected in the *Mdh* locus, but locus *S7*, *Ets*, and *Mep* contained varying numbers of non-recombining blocks across populations (Table 1.3). The largest NR block from *Ets* and the two largest NR blocks from *S7* and *Mep* were used to create data partitions for the IMa2 analyses. These five NR blocks along with the complete sequence data from the *Mdh* and *Cytb* loci comprised the seven data partitions used in the IMa2 (Table 1.3).

AMOVA results showed that most genetic variation could be explained by differences among species, with the exception of *Mep* and differentiation between populations within species was significant for all loci (Table 1.5). F_{st} values between species in the Salish Sea were significant at all loci except for Salish Sea *S. auriculatus* and *S. maliger* at *Mdh*; all species were significantly differentiated at all loci in the coastal populations *Mdh* (Table 1.6). Genetic differentiation between Salish Sea and the coastal population within each species were significant with the following exceptions: *S. auriculatus* at *S7* and *Mdh*, *S. caurinus* at *Cytb*, and *S. maliger* at *Cytb* and *Ets*.

Phylogenetics

The phylogenetic analyses showed paraphyly for all morphological species at all loci; however, the haplotype data from coastal populations for each species revealed some monophyletic species clades at all loci. Monophyletic clades with moderate bootstrap support (>65%) containing all coastal individuals were found for *S. auriculatus* and *S. caurinus* at *Cytb* (clade A and C, Figure 1.3) and *S. auriculatus* at *Ets* (clade D, Figure 1.4) and *S7* (clade F, Figure 1.5). Distinct genetic clades, though with low bootstrap support, were also found for coastal *S. auriculatus* at *Mep* (clade G, Figure 1.6) and coastal *S. maliger* at *Cytb* (clade B, Figure 1.3). These distinct genetic clades were mostly defined based on haplotypes from fish with the same morphological species identity; however, some haplotypes did not correspond with the morphological identification, especially those from fish collected in the Salish Sea. These haplotypes that did not correspond to their morphological species identification were considered "nonconforming" if they did not group with the distinct genetic clade defined by coastal populations described above. Mostly, these were shared haplotypes among species from distinct morphological species clades though a few unique haplotypes were nonconforming (open circles, Figures 1.3 and 1.6). At *Ets*, *Mep*, and *Mdh*, the shared morphological species haplotypes were also shared with an ingroup control or the outgroup species (Figures 1.4, 1.6, and 1.7). All fish could be verified as correctly categorized to species because the majority of their genetic data conform to morphological identification (Table 1.S1).

The *Cytb* tree had 30 unique haplotypes with strong bootstrap support for two distinct clades (A and C, Figure 1.3). The *S. caurinus* clade (C) had 92% bootstrap support and grouped most *S. caurinus* morphological species. The nine nonconforming haplotypes were Salish Sea *S. caurinus* and were shared with a common coastal *S. maliger* haplotype (haplotype18, clade B, Figure 1.3, Table 1.S1). The *S. auriculatus* clade (A) had 86% bootstrap support and included all but two haplotypes from the morphological *S. auriculatus*. Those nonconforming haplotypes (haplotype 20 and 23) were from Salish Sea *S. auriculatus* and clustered with common *S. maliger* haplotypes (clade B, Figure 1.3, Table 1.S1), though haplotype 23 was unique allele and not a shared haplotype. Interestingly, clade A2 grouped all Salish Sea *S. auriculatus* with 75% bootstrap support (Figure 1.3), with the exception of the two non-conforming haplotypes mentioned above. Clade B grouped the majority of *S. maliger* morphological species (Figure 1.3) except for a few nonconforming haplotypes from Salish Sea *S. maliger* that were shared with *S. auriculatus* clade (A) and *S. caurinus* clade (C) (haplotypes 22 and 12, Figure 1.3, Table 1.S1).

Trees from nuclear loci were generally less resolved among coastal morphological species, complicating the distinction of hybridization and ancestral polymorphism. The *Ets* tree had 15 unique haplotypes with 68% support for clade D that grouped all morphological *S. auriculatus* (Figure 4). Morphological *S. maliger* and *S. caurinus* shared one haplotype with each other (haplotype 3, Figure 4) and one haplotype both with each other and the ingroup control *S. atrovirens* (haplotype 8, Figure 4). Although morphological *S. maliger* and *S. caurinus* were not completely resolved into monophyletic clades, haplotype 3 was mostly *S. caurinus* and haplotype 8 was mostly *S. maliger*.

The *S7* tree had 20 unique haplotypes with 97% support for clade F that grouped all morphological *S. auriculatus* haplotypes except two and the ingroup control *S. dallii* (Figure 1.5). The two nonconforming haplotypes were Salish Sea *S. auriculatus* that were shared with common coastal *S. maliger* haplotypes (4 and 6, Figure 1.5). Clade E grouped most morphological *S. caurinus* haplotypes except for six nonconforming haplotypes that grouped with common coastal *S. maliger* haplotypes (4 and 5, Figure 1.5). Although haplotypes from morphological *S. maliger* did not form a distinct clade at

S7, they were not grouped with the other morphological species in clade F or E, with the exception of a single haplotype from a coastal *S. maliger* found in clade E (Figure 1.5). The *Mep* tree had 32 unique haplotypes (Figure 1.6) with clade G that grouped most haplotypes from morphological *S. auriculatus* and the ingroup control, *S. dallii*. Three shared haplotypes were from Salish Sea *S. auriculatus* and did not cluster with the rest of the morphological species in clade G (5, 9, 28, Figure 1.6), though haplotype 28 was a unique haplotype. The *Mdh* tree had 8 unique haplotypes and appeared to be polyphyletic for all morphological species; however, there is a single shared haplotypes that represented almost all the morphological *S. caurinus* (2, Figure 1.7). The *S. caurinus* shared this haplotypes with two coastal *S. maliger* morphological species, one Salish Sea *S. auriculatus*, and the outgroup *S. elongatus*. The nine haplotypes from Salish Sea *S. caurinus* which did not share haplotype 2 were shared among two common haplotypes (1 and 3) found in the other morphological species plus both ingroup controls (Figure 1.7).

In summary, the phylogenetic data across all loci showed that "nonconforming" and shared haplotypes were mostly from individuals collected in the Salish Sea, with the exception of two individuals from the coast. The overall multilocus haplotype patterns from individual fish suggest hybridization but retention of ancestral polymorphism could not be ruled out.

Coalescent Analysis

The coalescent analysis results for coastal and Salish Sea collections had similar results for size and divergence but showed distinct differences for migration rates. Mutation rate scaled population size parameters (q) varied between species but not between Salish Sea and coastal populations within each species (Figure 1.8). Similarly, estimates of divergence times between *S. maliger* and *S. caurinus* (t0), and between *S. auriculatus* and the other two species were similar in the Salish Sea and the coast (Figure 1.9). In contrast, patterns of interspecific gene flow differed considerably between the two areas, with more gene flow among species in the Salish Sea than on the coast (Figure 1.10). The highest effective number of migrants per generation (2NM) in the Salish Sea was from *S. maliger* into the other two species, while there was less gene flow between *S.*

caurinus and *S. auriculatus*. Most migration rates estimates on the coast were close to zero except for *S. caurinus* into *S. maliger* (Figure 1.9).

Discussion

Our results provided strong evidence for introgression among Salish Sea *S. auriculatus, S. caurinus* and *S. maliger*, with multiple lines of evidence from genetic diversity, phylogenetic trees and coalescent analysis. The direction of introgression was asymmetrical mostly from *S. maliger* into *S. caurinus* and *S. auriculatus* and less between *S. caurinus* and *S. auriculatus*. We found little evidence for hybridization in coastal populations with only some introgression of *S. caurinus* into *S. maliger*. Results on the direction of hybridization were in contrast to previous reports in which *S. maliger* was thought to be more introgressed (Seeb 1998) or reports that *S. caurinus* in the Salish Sea was not largely impacted by hybridization (Buonaccorsi et al. 2002). The paraphyly of species in the gene trees pointed towards introgressive hybridization, though the existence of ancestral polymorphism could not be unequivocally excluded. Nevertheless, the spatial pattern of nonconforming haplotypes concentrated in fish from the Salish Sea strongly supported introgressive hybridization within a confined geographic area. In addition, higher within species nucleotide diversity in Salish Sea populations despite lower haplotype diversity was most likely explained by interspecific introgression. However, the strongest support for introgressive hybridization came from the coalescent analyses, which provided well supported evidence for high migration rates into *S. caurinus* and *S. auriculatus* from *S. maliger* in Salish Sea populations.

Introgression vs. retained ancestral polymorphism

The coalescent analysis paired with phylogenetic analysis at multiple loci can help disentangle the signals of ancestral polymorphism from introgression. Shared polymorphisms need to be considered carefully as retained ancestral polymorphism from incomplete linage sorting can mimic gene flow (Avise 2001; Funk and Omland 2003). The ability to detect introgressive hybridization among species is dependent on effective population size and divergence time. As divergence time increases the chance of incomplete lineage sorting decreases, especially if coupled with small population size

(Joly et al. 2009). Furthermore, as predicted by coalescent theory, mitochondrial loci are likely to reach monophyly three times faster than nuclear loci (Palumbi et al. 2001). The divergence time estimates between *S. auriculatus* to *S. caurinus/S. maliger* using a molecular clock is nearly 3 Mya and the divergence time for *S. caurinus* and *S. maliger* is approximately 1.7 Mya (Hyde and Vetter 2007). Our mitochondrial locus data from *Cytb* show species monophyly for costal population in contrast to the nuclear loci that show species paraphyly for the sister taxa, *S. caurinus* and *S.* maliger. However, two nuclear loci *Ets* and *S7* each show monophyletic clades for *S. auriculatus*, which is more distantly related to the other two species. Therefore, the species divergences are likely deep enough to interpret shared haplotypes as introgression for some loci, but incomplete lineage sorting is also likely apparent in our phylogenetic trees. The incongruence among gene trees, the shared haplotypes among species, and nonconforming species haplotypes were likely due to introgression, although these patterns could also arise from retention of ancestral polymorphism and incomplete linage sorting. The mitochondrial locus C*ytb* resolved all three species, whereas some of the shared haplotypes observed in the nuclear gene phylogenies may likely represent retained ancestral polymorphisms between species. For example at *Mdh*, the outgroup *S. elongatus* was shared with most of the *S. caurinus* haplotypes while the ingroup control haplotypes from *S. atrovirens* and *S. dallii* were shared with haplotypes common in *S. maliger*. In the phylogenetic analyses, the ingroup controls and outgroup species helped establish where shared haplotypes could be signals from retained ancestral polymorphism as well as from hybridization. Nonetheless, the phylogenetic data tracked across individuals showed that some nonconforming haplotypes were from two independent loci in the same individual, and thus these results are evidence of introgression and not retained ancestral polymorphisms.

The strongest evidence for introgression over ancestral polymorphism stems from the comparison of phylogenetic and coalescent analyses for the two geographic samples. In the Salish Sea populations, the phylogenetic results showed more shared haplotypes from different morphological species compared with the coastal population. Also in the coalescent analyses, the populations from the Salish Sea show higher interspecific migration rates compared with the coast. The coalescent analyses had the best power to resolve signals of introgression versus ancestral polymorphism. The IM model uses a

Markov chain Monte Carlo method to estimate the effects of both migration and isolation on genetic variation observed among species; consequently, parsing the genetic variation among species to either shared ancestral polymorphism or gene flow (Nielsen and Wakeley 2001). Evidence for population isolation over migration is supported by posterior distributions that include zero migration rate values or the lowest migration rate value tested (Hey 2005). If ancestral polymorphism was the only explanation for the paraphyly in the phylogenetic trees, then we would observe high probabilities for zero migration rates for all species comparisons in the coalescent analyses (Figure 1.10). Our interspecific migration rate PPD data did not include zero in three of six species comparisons from the Salish Sea and one of six on the coast ($2NM<0.001$, $p<0.05$, Figure 1.10). Therefore, both migration and incomplete linage sorting elucidate the species paraphyly from our phylogenetic analysis. Because the IM method uses population size, divergence time, and migration rate estimates simultaneously for all possible genealogies, it can be useful for studying the divergent population genetics allowing for gene flow in multi-locus genealogies (Hey and Nielsen 2007).

Violations of assumptions in the coalescent

The overall assumption in the coalescent model is that the sampled populations are the only populations contributing to gene flow (Beerli 2004). To consider all sources of gene flow, the model requires all species to be sampled, yet the addition of populations exponentially increases the number of parameters to be estimated. On the coast, *S. auriculatus*, *S. caurinus*, and *S. maliger* are found in sympatry with other closely related species, yet in the Salish Sea these three species are the most closely related. If gene flow from unsampled species biased the migration rates upwards then we would expect to see higher migration rates on the coast, which we did not.

It is unlikely that the data conformed to the theoretical Wright-Fisher model; thus allowance for some violations of the assumptions in the theoretical model must be considered to make inferences from empirical data. Genetic variation from recombination, gene flow, linkage, selection, and population size changes may impact results more or less severely (Strasburg and Rieseberg 2010). Recombination can be a significant source of bias for all parameters except migration (Hey and Nielsen 2004).

We detected recombination in three of the four nuclear loci (Table 1.3) and we used only NR blocks in the IMa2 analysis. To prevent loss of data, we used two of the largest NR blocks from two loci (*S7* and *Mep*) in the analysis (Hey and Nielsen 2004). Using two NR blocks from the same locus could theoretically introduce bias from genetic linkage effects, but simulations showed that in practice linkage introduced little bias (Strasburg and Rieseberg 2010). Theoretically, population structure could bias divergence time estimates upward and artificially increase migration rates (Wakeley 2000), but similarly simulations show little bias from population structure (Strasburg & Rieseberg 2010). Another potential source of bias was the larger sample size in the Salish Sea compared to the coast, which may lead to the detection of more hybrids in the larger sample (Felsenstein 2006). However, coalescence analyses with smaller subsets of the Salish Sea sample revealed similar estimates of migration rates (data not shown) and so the analysis appears to be robust to variation in sample sizes. Therefore, while some assumptions were clearly not met, they are unlikely to introduce bias that could seriously affect the interpretation of results.

Patterns of introgressive hybridization

The history of hybridization apparently unique to Salish Sea rockfish can be inferred from both geological history and the patterns of introgression in each species. Initially, Puget Sound hybrids were likely produced during the range expansion into the Salish Sea as the glaciers receded at the end of the Pleistocene. Novel environments during range expansion allow more overlap for closely related species compared with their historical environments (Arnold 1997) and with fewer conspecifics to mate with, the new colonizer might initiate the hybridization if species are interfertile (Hubbs 1955). The genetic patterns here are consistent with historical hybridization because no F1 hybrids were detected and hybridization was detected at only one or two loci (Table 1.S1). This confirms that initial hybrids and their backcrosses were fertile; and second, that introgressive hybridization is from many generations of backcrosses. In addition, these patterns do not appear to be signals from ancient hybridization events. The introgressive hybridization patterns seen in the phylogenetic trees were from shared haplotypes in both nuclear and mitochondrial loci. Over long evolutionary timescales

random mutation events would create a pattern of derived haplotypes within these species clades. These unique haplotypes are most likely from random mutations that have accumulated after the hybridization event (Funk and Omland 2003). We observed two unique haplotypes, which were nonconforming haplotypes at *Cytb* and *Mep*, both were from morphological *S. auriculatus* (Figures 1.3 and 6; Table 1.S1). The contemporary patterns seen in Salish Sea *Sebastes* were likely either from frequent hybridization events during early range expansion or from a persistent, low level hybridization since expansion. Coalescent approaches are powerful to detect hybridization events, but the timing of migration events cannot be estimated reliably with the IM method (Strasberg and Rieseberg 2011).

The direction of introgression among species can provide information on biological, environmental, or demographic mechanisms influencing interspecific hybridization. The biological attributes important for differential survival of hybrids and assortative mating may contribute to the asymmetrical patterns of introgression in Salish Sea *Sebastes*. Little is known about the mating behaviors of rockfish though there is the element of mate choice due to internal fertilization. The asymmetrical pattern of introgression from *S. maliger* into the other two species could be explained by hybrid mate selection, in which F1 hybrids preferentially mate with *S. caurinus* or *S. auriculatus* and rarely with *S. maliger*. On the other hand, asymmetrical introgression may also be due to reduced fitness in some hybrid crosses (Burke and Arnold 2001).

Habitat use and bathymetric distributions of *S. auriculatus, S. caurinus*, and *S. maliger* in the Salish Sea versus the coast also may play a role in the patterns of introgression, particularly in the Puget Sound Basin. Puget Sound has sparse and patchy rockfish habitat with tight niche separation (Palsson et al. 2009). Competition for the few reef habitat spots could force multiple species to share reefs (Palsson et al. 2009) and thus increase opportunities for hybridization. Depth segregation has been an important way for closely related rockfish species to avoid competition (Hyde and Vetter 2007; Hyde et al. 2008). Compression of depth distributions due to water quality or habitat limitations in Puget Sound compared with the coast could be one mechanism that drives opportunity for hybridization. In Puget Sound natural, periodic anoxic conditions may force deeper species closer to the surface (Palsson et al. 2009) creating more species overlap in

nearshore areas. Over the geographic species range, *S. maliger* is found at the deepest distribution while *S. auriculatus* is found in more shallow depths (Love et al. 2002; Palsson et al. 2009). As the lack of oxygen periodically forces *S. maliger* into more shallow depths, there may be more opportunities for hybridization due to the increased contact with *S. caurinus* and *S. auriculatus*. If F1 hybrids then stay at shallow depths, directional introgression from *S. maliger* into *S. caurinus* and *S. auriculatus* could ensue.

Gene flow and population size play important roles in maintaining diversity in populations and are also important for understanding the dynamics of introgressive hybridization. Physical and oceanographic forces appear to prevent dispersal of hybrids out of the Salish Sea thus retaining introgressed alleles, especially in more isolated basins such as Puget Sound. All three species have been reported to be genetically different in the Puget Sound Basin compared to the coast (Seeb 1998; Stout et al. 2001; Buonaccorsi et al. 2002, 2005), though influence from hybridization cannot be ruled out. *Sebastes auriculatus* in Puget Sound are much more physically isolated from coastal populations than the other two species, because they are rarely found along the Pacific Ocean coast from Oregon to B.C. (Stout et al. 2001; Palsson et al. 2009). Correspondingly, our phylogenetic analysis of mitochondrial DNA showed significant population differentiation for *S. auriculatus* in Puget Sound compared with coastal *S. auriculatus* (Figure 1.3). Limited intraspecific gene flow with the coast may allow introgressed alleles to accumulate in Salish Sea populations of *S. auriculatus* and *S. caurinus*; moreover, *S. maliger* may actually be more connected to the coastal populations. Connection to the coast coupled with population size differences may be important in Salish Sea hybridization. Coalescent analyses estimated smaller effective population size in S. *auriculatus* compared to *S. caurinus* or *S. maliger*, which corresponds to relative census size estimates (Stout et al. 2001, Palsson et al. 2009). Smaller populations are more vulnerable to dilution of their gene pool by hybridization from more abundant species over time (Currat et al. 2008). On the other hand, in the case of large abundance differences, hybrids could more likely mate with the most available species thus redistributing hybrid genotypes into the more abundant species (Allendorf et al. 2001). In the Salish Sea, introgression is most pronounced into *S. auriculatus*, the least abundant species, however high levels of introgression were also found in *S. caurinus*.

The historical colonization order of *Sebastes* into new habitat vacated by receding glaciers at the end of the Pleistocene could be important for understanding the contemporary patterns of introgression (Currat et al. 2008). Yet, introgressive hybridization patterns today in the Salish Sea would also be influenced by demography over the past 12,000 years; for example, changes in abundance, gene flow from the coast, competition for resources, and habitat availability, as discussed above. The amount and direction of introgression during range expansion are influenced by neutral gene flow with parental species and may even predict the order of species arrival (Currat et al. 2008). Based on this prediction, the patterns of introgressive hybridization from our data in the Salish Sea *Sebastes* suggests that *S. maliger* was established before the invasion of *S. caurinus* or *S. auriculatus.*

Introgressed *Sebastes* in the Salish Sea appear to conform to the morphological and coloration found in pure parental species, which seems to be maintained in *S. caurinus, S. maliger*, and *S. auriculatus*. Rockfish have diverse interspecific color and pattern heterogeneity (Orr et al. 1998; Love et al. 2002), which has likely co-evolved with complex mating systems (Narum et al. 2004). Nevertheless, intraspecific color and pattern heterogeneity pose taxonomic challenges (Orr and Blackburn 2004) and may obscure cryptic species (Gharret et al. 2006; Hyde et al. 2008). *Sebastes* are similar to cichlid fishes, which are the result of an explosive speciation event (Johns and Avise 1998) and the species segregate in similar ways such as depth segregation and coloration differences (Alesandrini and Bernardi 1999). In African cichlids, asymmetrical hybridization is due to mate choice and reproductive isolation of sympatric congeneric species (Martin and Genner 2009; Egger et al. 2010). Assortative mating and elaborate mating behaviors are a hallmark of speciation in sympatric and parapatric cichlid fishes (Egger et al. 2010); furthermore, cichlids have dramatic courtship and distinct and elaborate color morphs that play an important role in reproductive isolation and adaptive radiation (Seehausen 2006). Thus, regions with asymmetrical interspecific hybridization can permit us to study mechanisms that maintain species boundaries or processes that facilitate speciation (Field et al. 2011).

Future work on Salish Sea rockfish hybrids needs to include more genes, both to detect further hybrid backcrossed individuals and to explore specific genetic regions

influenced by hybridization in these species. Interspecific gene flow might be temporarily or spatially restricted or it may occur in some parts of the genome but not in others (Baack and Rieseberg 2007; Mallet 2005; Yatabe et al. 2007). Genetic loci with restricted gene flow in hybridizing species can provide insights on reproductive barriers in sympatric species (Buonaccorsi et al. 2011). The hybrid zone in the Salish Sea is an ideal location to investigate localized interspecific interactions, yet the species dynamics need to be explored in other areas of sympatry. Further research should include expanding the geographic scope to include additional regions with post Pleistocene glacial influence, such as Queen Charlotte Sound just north of the Salish Sea. Also further information about the effects of hybridization on morphology need to be accessed using known F1 and F2 hybrids. Understanding what and how morphological traits are influenced by hybridization will be critical to understand the biological response to hybridization in the wild.

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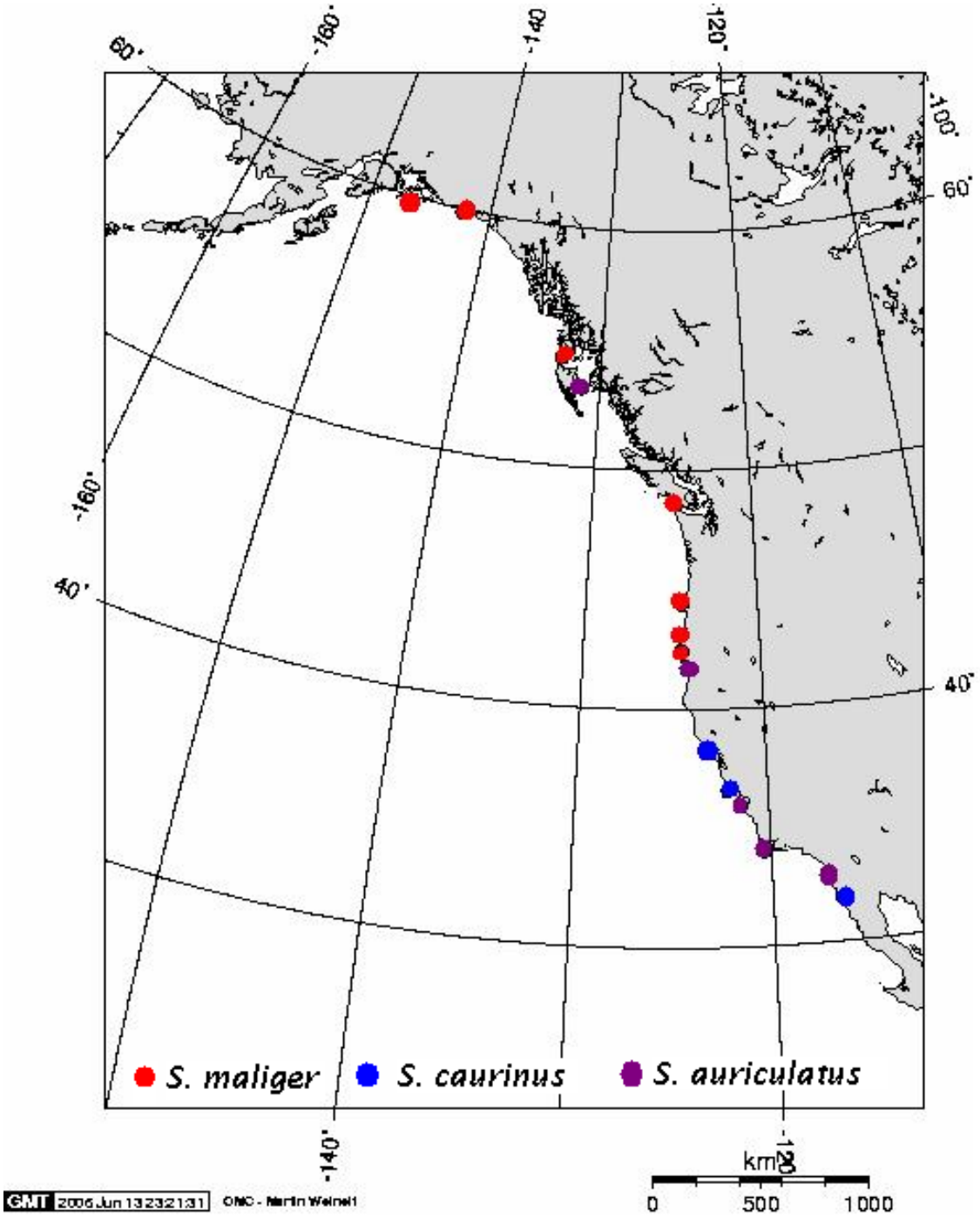

Figure 1.1. Localities for costal collections. Red circles are *S. maliger* (n=17), blue are *S. auriculatus* (n=13), and purple are *S. caurinus* (n=12). Each sample location represent 1 – 6 individuals.

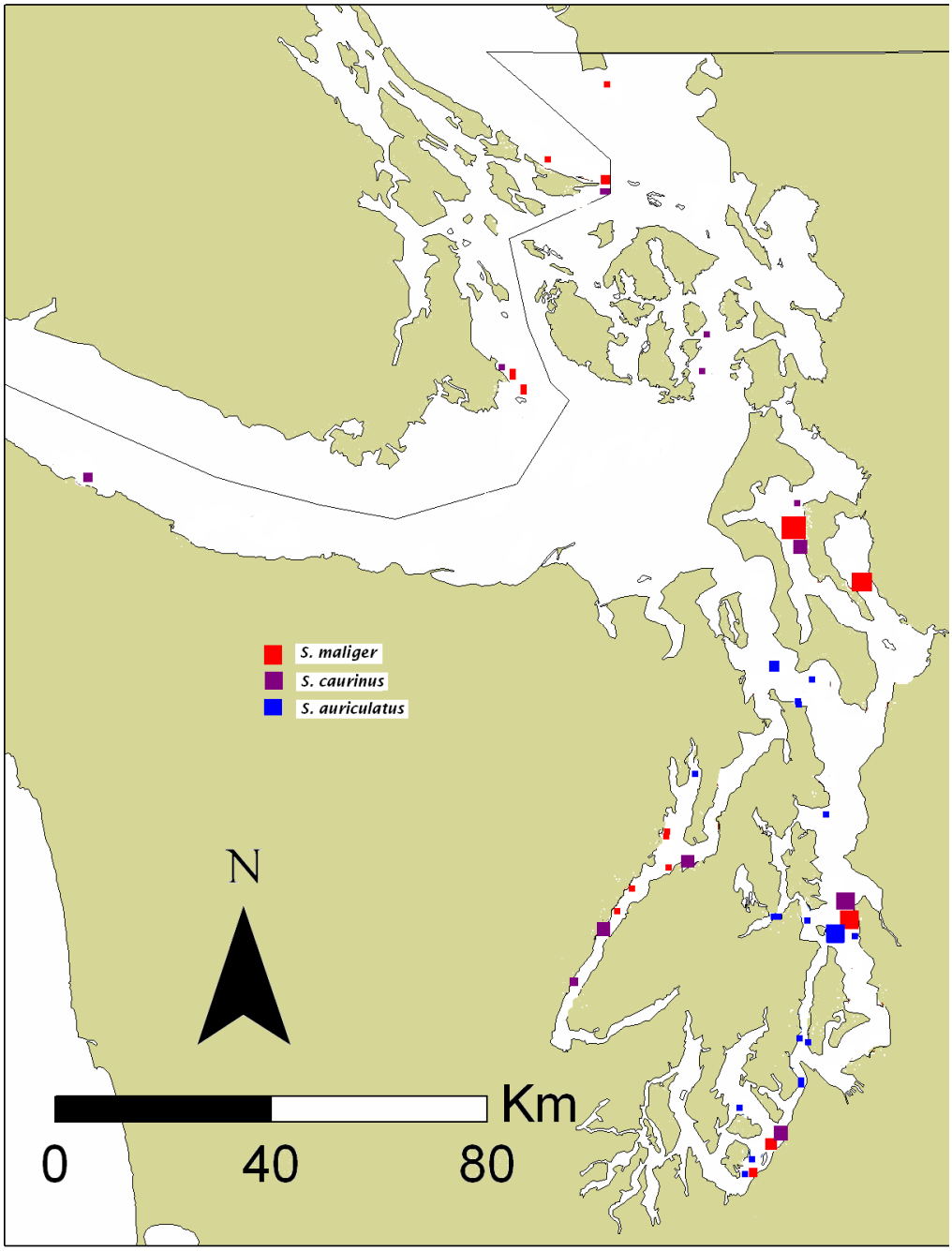

Figure 1.2. Localities for the Salish Sea collections. Red squares are *S. maliger* (n=40), blue are *S. auriculatus* (n=24) and purple are *S. caurinus* (n=33). The size of the square is proportional to the size of collection (1 – 8 individuals). More than one species collected from the same location is represented by adjacent squares.

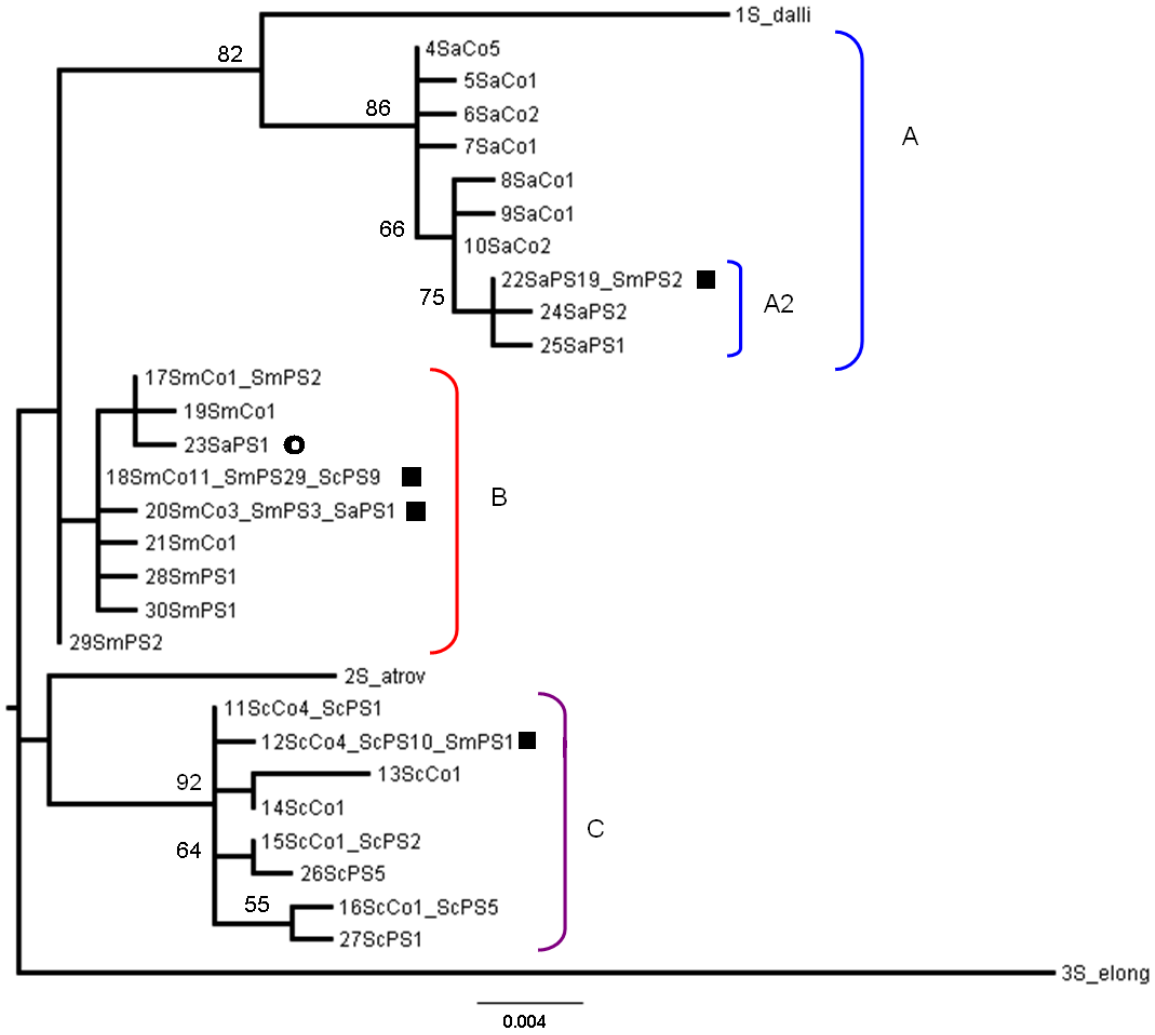

Figure 1.3. *Cytb* (717 nucleotides and KHY+G mutation model). Maximum likelihood phylogenetic tree with >50% support indicated beside branch. Node label coded with the following information: unique haplotype identifier, species, population, haplotype count. Species are coded by two letters: species (Sa–*S. auriculatus*, Sc–*S. caurinus*, Sm–*S. maliger*). The next two letters are the population code (Co – Coastal, PS – Puget Sound (Salish Sea)). The nonconforming haplotypes that were shared are marked with a solid square and clades are labeled by the letters A, A2, B, and C. Open circle represents a nonconforming haplotypes that was not shared. The tree is rooted with outgroup *Sebastes elongatus*.

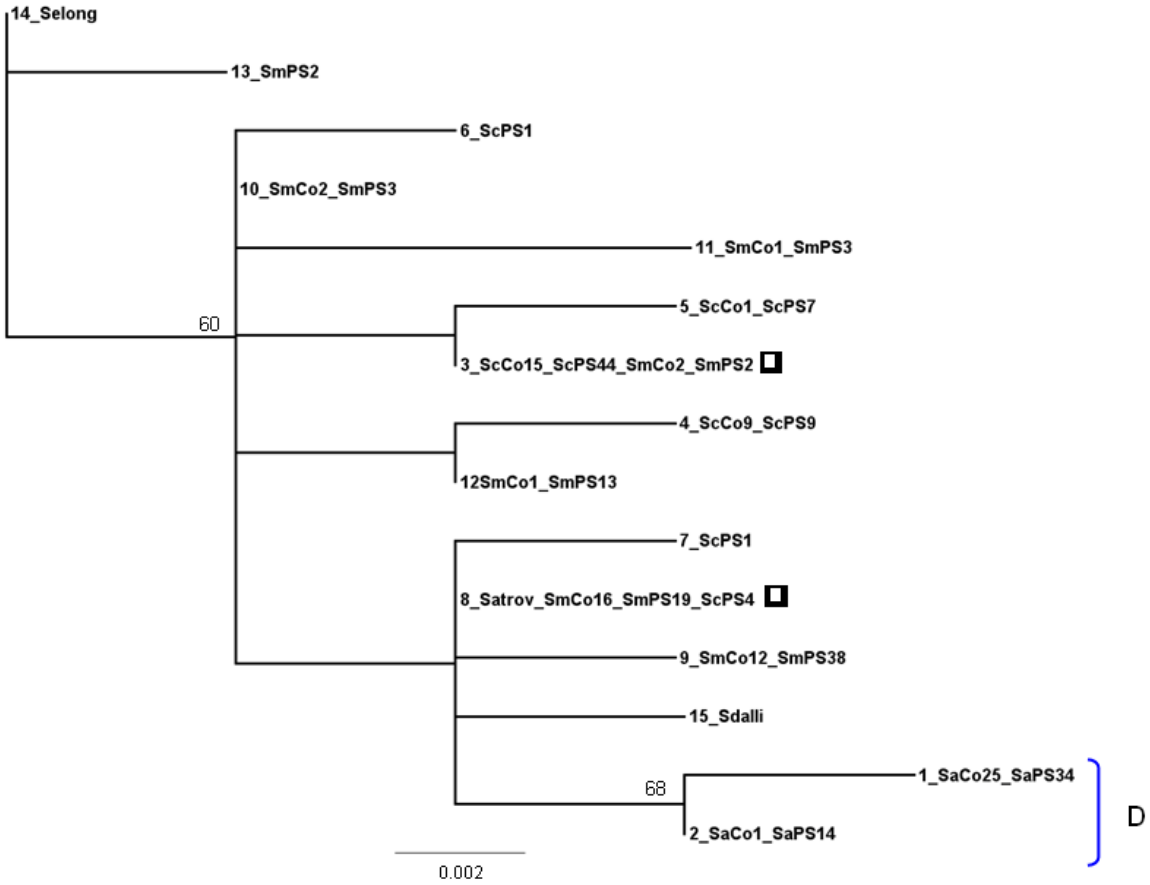

Figure 1.4. *Ets* (293 nucleotides and K80+I mutation model). Maximum likelihood phylogenetic tree with >50% support indicated beside branch. Node label coded with the following information: unique haplotype identifier, species, population, haplotype count. Species are coded by two letters: species (Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*). The next two letters are the population code (Co – Coastal, PS – Puget Sound (Salish Sea). The shared haplotypes are marked with an open square and the distinct clade is labeled with D. The tree is rooted with outgroup *Sebastes elongatus*.

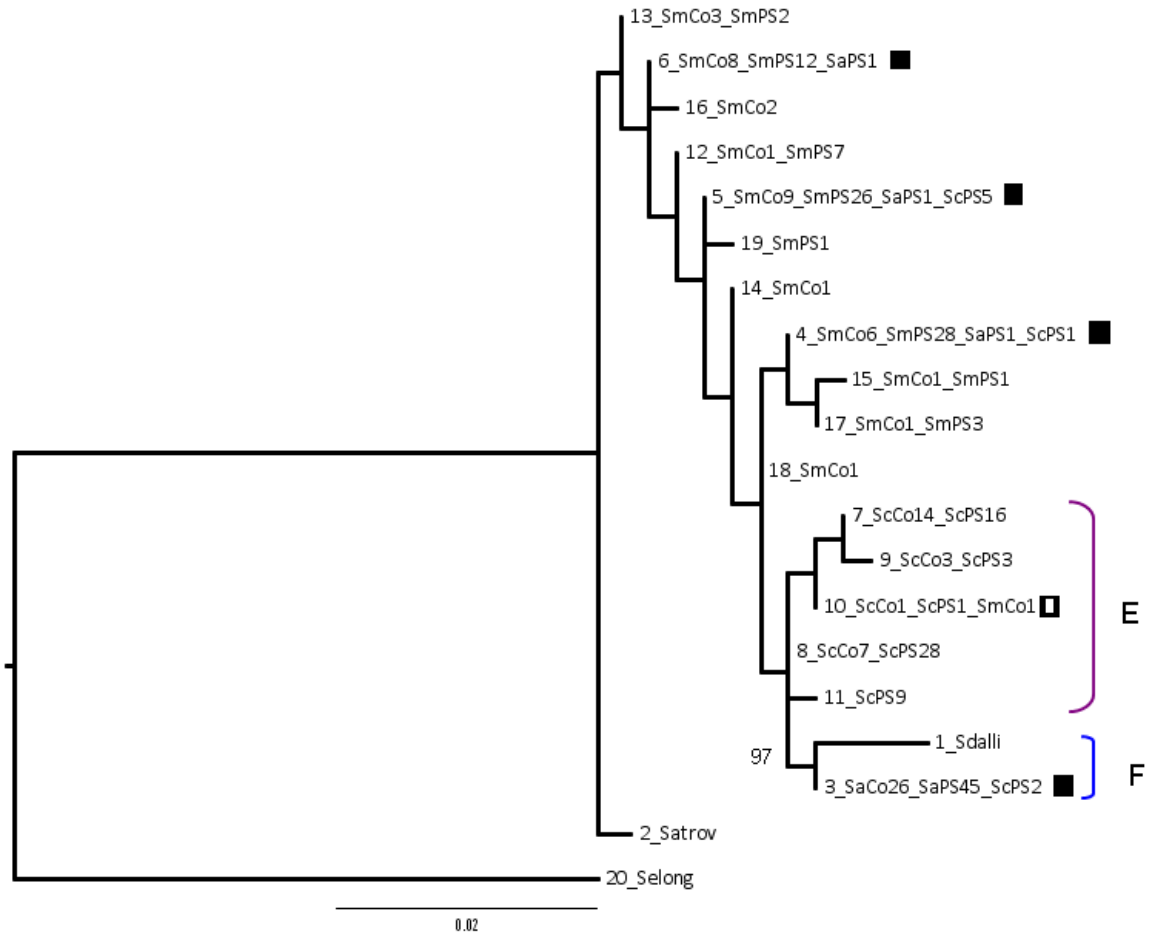

Figure 1.5. *S7* (560 nucleotides and F81+I+G mutation model). Maximum likelihood phylogenetic tree with >50% support indicated beside branch. Node label coded with the following information: unique haplotype identifier, species, population, haplotype count. Species are coded by two letters: species (Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*). The next two letters are the population code (Co – Coastal, PS – Puget Sound (Salish Sea). The nonconforming haplotypes that were shared are marked with a solid square and distinct clades are labeled E and F. The open square marks a shared haplotype. The tree is rooted with outgroup *Sebastes elongatus*.

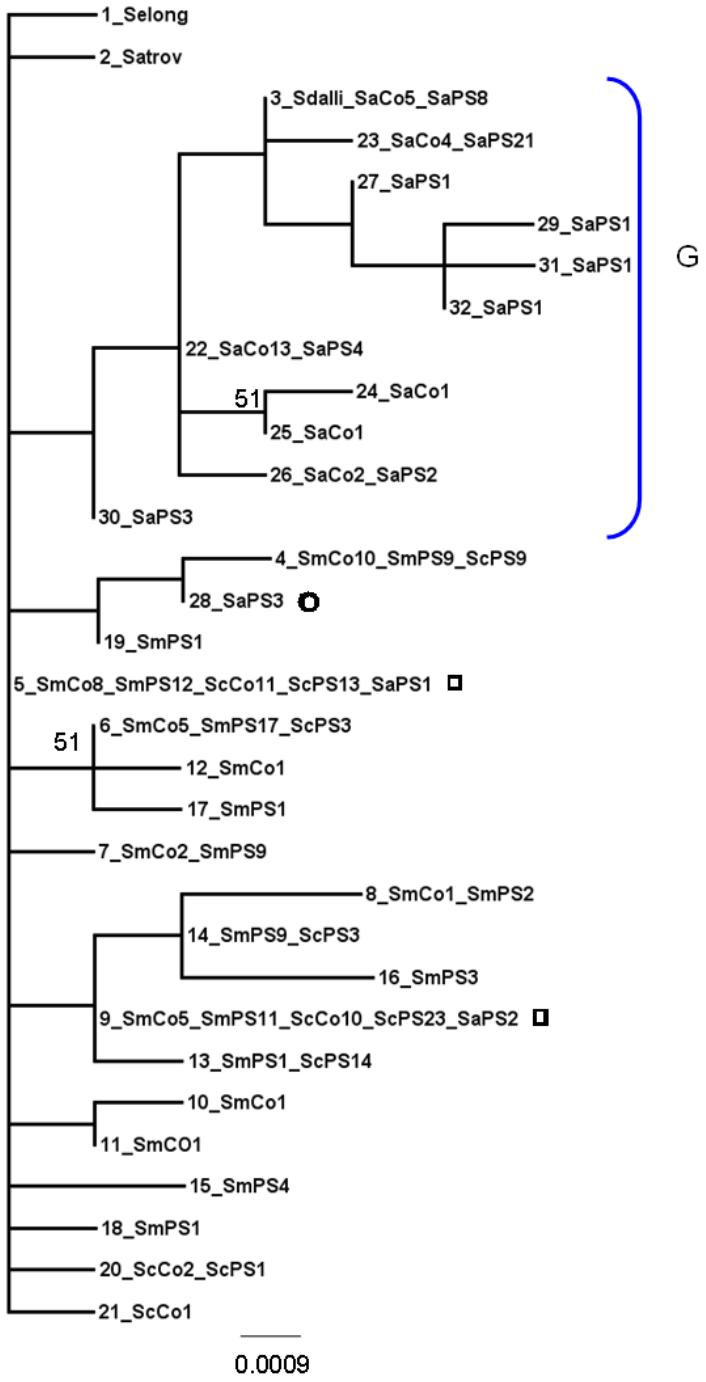

Figure 1.6. *Mep* (785 nucleotides and HKY+I+G mutation model). Maximum likelihood phylogenetic tree with >50% support indicated beside branch. Node label coded with the following information: unique haplotype identifier, species, population, haplotype count. Species are coded by two letters: species (Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*). The next two letters are the population code (Co – Coastal, PS – Puget Sound (Salish Sea). The nonconforming species haplotypes are marked with a solid square and the distinct clade is labeled G. The open circle represents a nonconforming haplotypes that was not shared. The open square marks a shared haplotype. The tree is rooted with outgroup *Sebastes elongatus*.

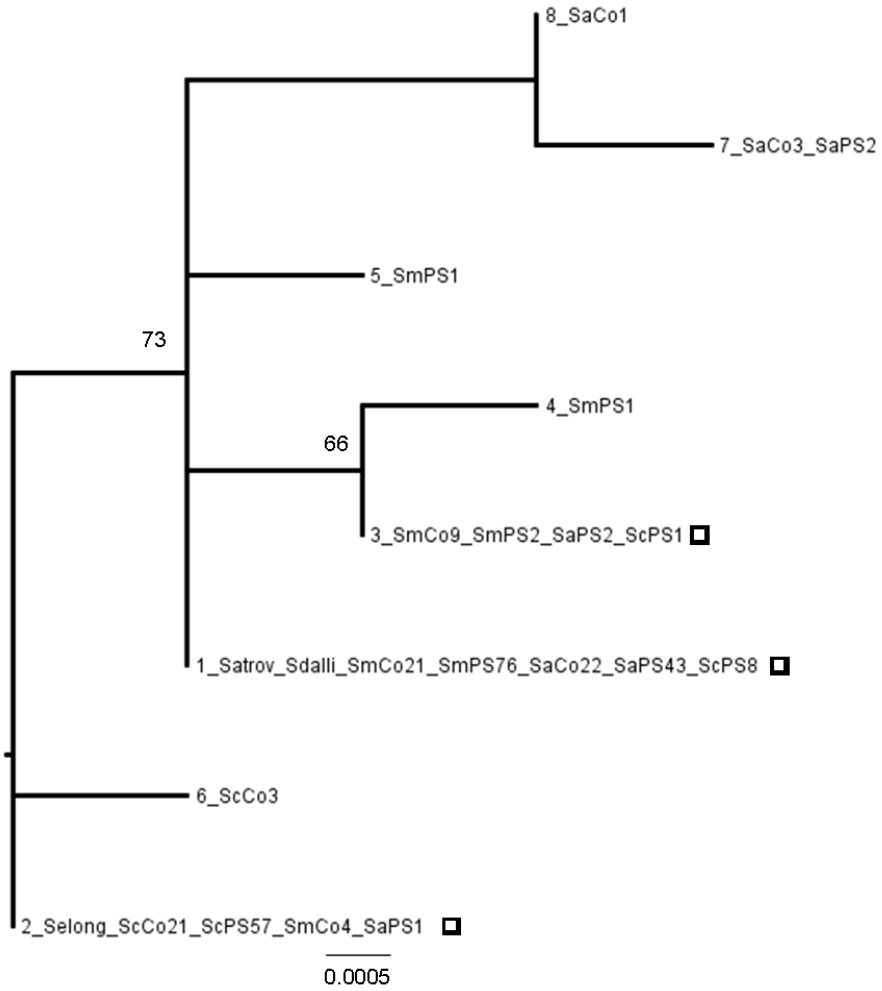

Figure 1.6. *Mdh* (730 nucleotides and HKY mutation model). Maximum likelihood phylogenetic tree with >50% support indicated beside branch. Node label coded with the following information: unique haplotype identifier, species, population, haplotype count. Species are coded by two letters: species (Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*). The next two letters are the population code (Co – Coastal, PS – Puget Sound (Salish Sea). Shared haplotypes are marked with an open square. The tree is rooted with outgroup *Sebastes elongatus*.

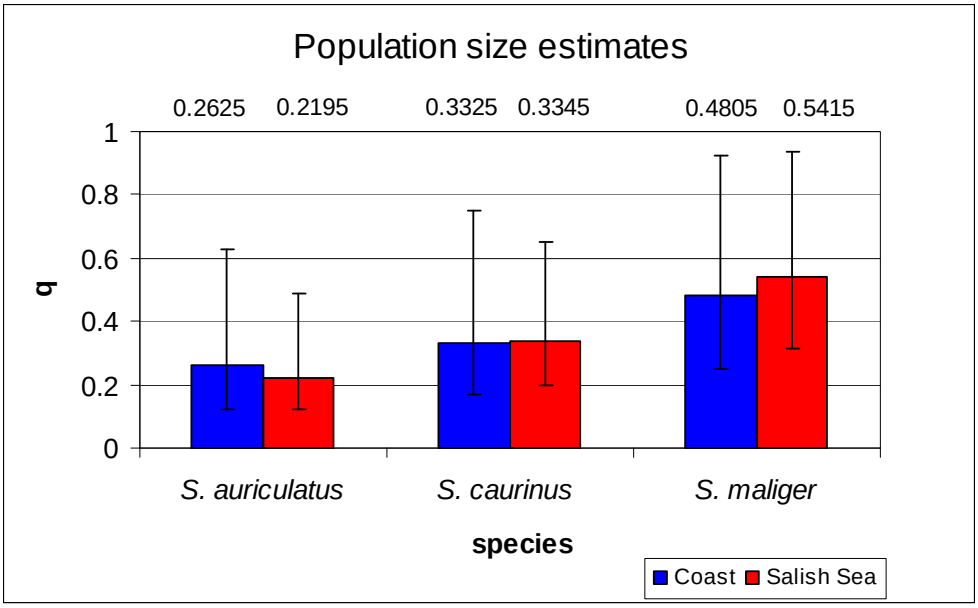

Figure 1.8. Population size parameter estimates (q) for *S. auriculatus*, *S. caurinus*, and *S. maliger* and between coastal and Puget Sound populations. The PPD peak values are posted above the column and 95% CI are indicated with bars.

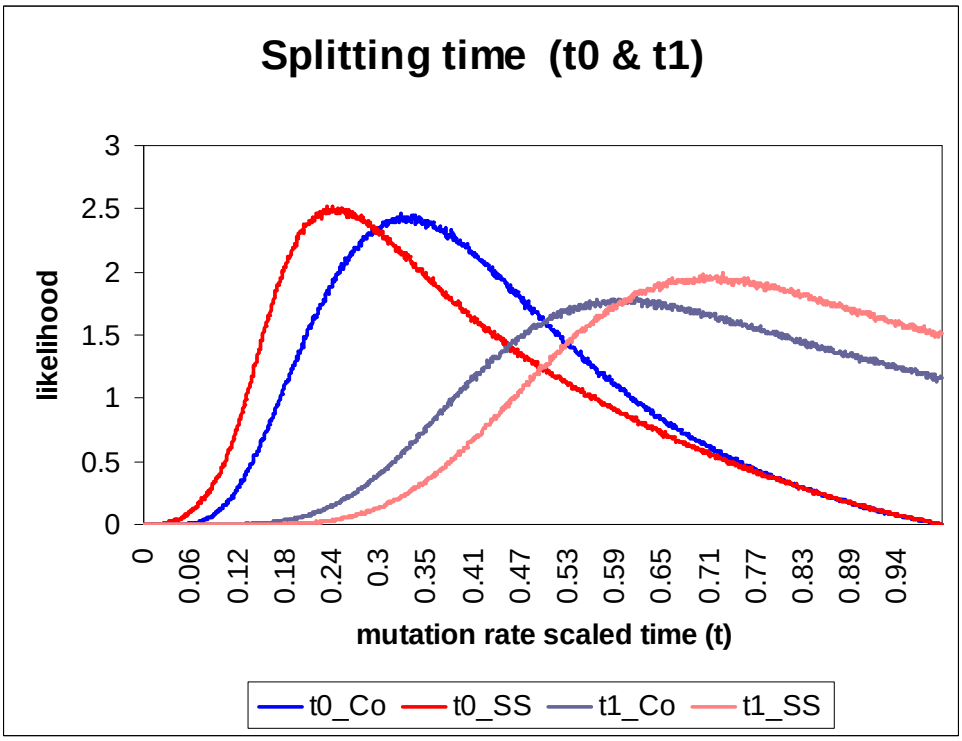

Figure 1.9. PPD for mutation rate scaled splitting time in generations (t) between coastal (Co) and Salish Sea (SS) populations. The t0 value is the spitting time for *S. caurinus* and *S. maliger* and t1 splitting time for *S. auriculatus* split from *S. caurinus* and *S. maliger*.

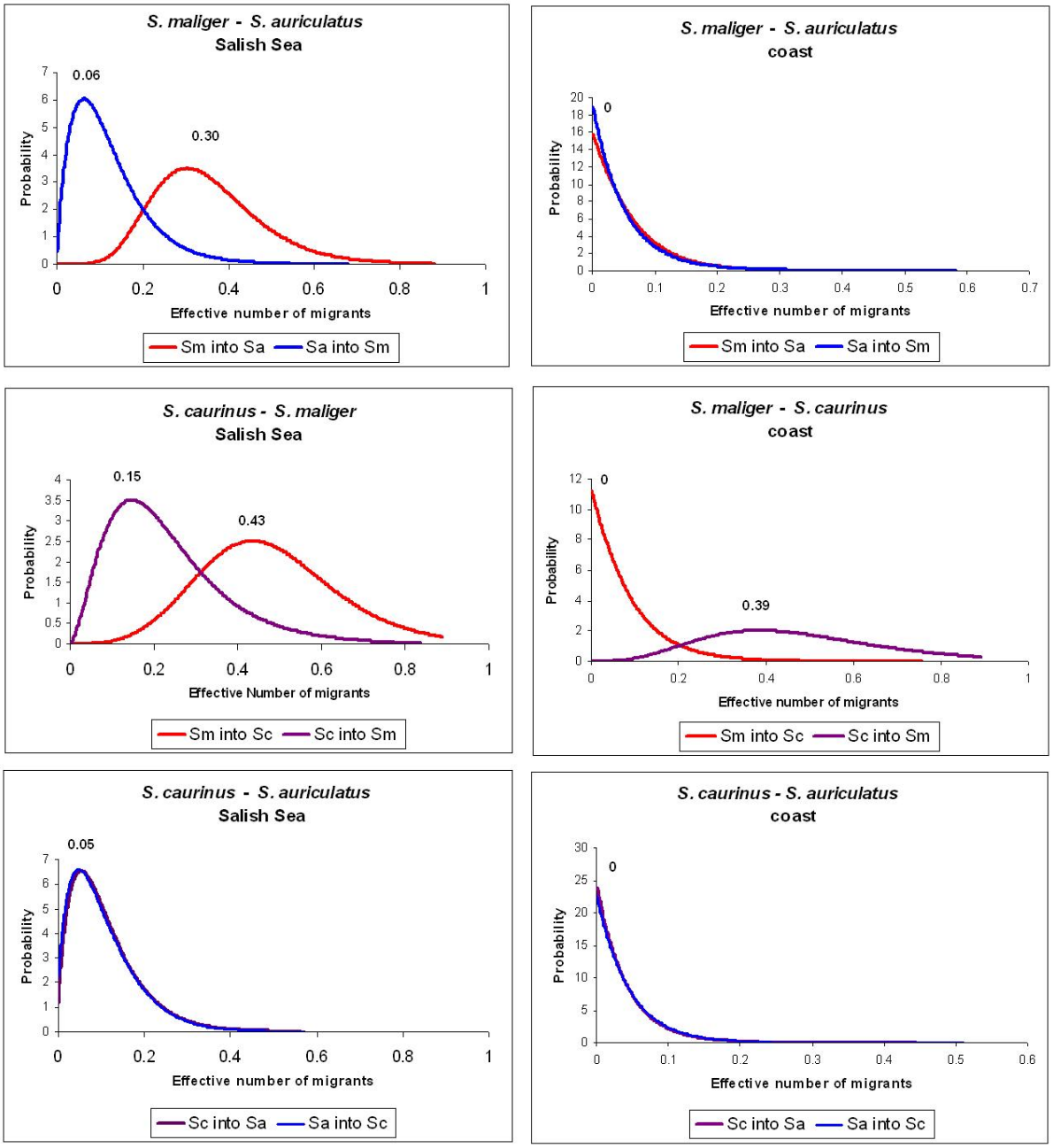

Figure 1.10. PPD for effective number of migrants (2NM) between species for the coastal and Salish Sea populations. Species are coded by two letters: Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*. The PPD highest peak values are posted above the distribution.

Table 1.1. Tissue samples from all specimens

Species & Region	**Number**	**Collection Date**
S. auriculatus		
Central California	5	1999
Southern California	4	1999
Baja, Mexico	4	2000
Coast Total	*13*	
Salish Sea	*24*	2002
S. caurinus		
Queen Charlotte Island	2	1994
Northern California	5	1999
Central California	3	1993
Southern California	1	1998
Baja, Mexico	1	2001
Coast Total	*12*	
Salish Sea	*33*	1997, 2001, 2002
S. maliger		
Northern California	3	1999
Southern Oregon	6	2002
Northern Oregon	1	1998
West Vancouver Island	3	1998
Queen Charlotte Island	1	1994
Southeast Alaska	2	1995
Prince William Sound	1	1999
Coast Total	*17*	
Salish Sea	*40*	2002, 2003, 2005

Table 1.2. Locus data.

Locus	**Primers**	**PCR Annealing Conditions**	**Reference**	**Name**
Cytb	GluF 5' TGA CTT GAA RAA CCA YCG TTG 3' *Cytb*R 5'ATA TCA TTC TGG CTT AAT GTG 3'	58 °C for 40 sec.	Rocha-Olivares et al. (1999)	Cytochrome b mitochondrial
5'*Ets*	F 5' CGG CCA TGG GCA GTT CAG G 3' R 5' ATA TGC TTA AAT TCA GCG GG 3'	52 °C for 40 sec.	Le et al. (1993)	5' external transcribed spacer, intron
S7	F 5' AGCGCCAAAATAGTGAAGCC 3' R 5' GCCTTCAGGTCAGAGTTCAT 3' C272 R 5' CAT CTA CTG ACA CTT GTA TAC TA 3' (internal with *S7* – F)	60 °C for 60 sec. 58 °C for 60 sec.	(Chow and Hazama, 1998) This paper	*S7* Ribosomal, intron 2
Mdh	F1 5' CCT CTC TCA CTG CTG CTG AA 3'R1 5' TTC TTC TCG ATG CCG TTC TT 3' RF R2 5' TCC CCA GAA GAA GAG GTG TG 3' (internal with *Mdh* – F1)	61°C for 30 sec.	Park, L.K. unpublished This paper	malate dehydrogenase, coding
Mep	F 5' GCT GTA ATG GAA TGG GCA TCC 3' R 5' AGC CTC TCC AGC TCC CTG G 3' RF R2 5' GGT TAA CTT TAT GGC ATT ATG AAG AA 3' (internal with *Mep* – F) RF F2 5' TTG GAA ACC ACA ATG CCT TC 3' RF R3E 5' CAC GGT AAA CAA TGA AGT AT CTG 3'	60 °C for 60 sec. 60 °C for 40 sec. 58 °C for 60 sec	Park, L.K. unpublished This paper This paper	malic enzyme, intron

Table 1.3. Polymorphism data: Populations (pop): Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*, Co – coast, and SS – Salish Sea

Locus	nuc	IMa2 nuc	pop	n	S	Hap	Hd	VarHd	θ	Π	K	TajimaD	NRB
Cytb	717	717	SaCo	13	6	7	0.879	0.0057	0.0028	0.002	0.00435	-1.0217	n.a.
			SaSS	24	17	5	0.391	0.0157	0.0064	0.0035		-1.6503	n.a.
			ScCo	12	7	6	0.836	0.0079	0.0033	0.0027	0.00524	-0.7855	n.a.
			ScSS	33	13	7	0.81	0.0013	0.0045	0.0063		1.3329	n.a.
			SmCo	17	3	4	0.517	0.0175	0.0013	0.0009	0.00192	-0.8148	n.a.
			SmSS	40	22	8	0.484	0.0095	0.0076	0.0028		-2.1409*	n.a.
S7	542	181	SaCo	26	0	1	0	0	n.a.	0	0.00046	n.a.	n.a.
		145	SaSS	48	6	4	0.125	0.0043	0.0025	0.0009		-1.6372	1
			ScCo	24	3	4	0.605	0.0062	0.0015	0.002	0.00305	0.8474	0
			ScSS	66	7	8	0.742	0.0015	0.0027	0.0033		0.5456	2
			SmCo	34	8	12	0.877	0.0012	0.0036	0.0042	0.00357	0.4319	2
			SmSS	80	6	9	0.761	0.0009	0.0022	0.0027		0.5048	2
Ets	293	238	SaCo	26	1	2	0.077	0.0049	0.0009	0.0003	0.00105	-1.1556	0
			SaSS	48	1	2	0.422	0.0031	0.0008	0.0014		1.1852	0
			ScCo	24	2	3	0.518	0.0053	0.0018	0.0019	0.00244	0.0473	0
			ScSS	66	5	6	0.53	0.0044	0.0036	0.0027		-0.5438	1
			SmCo	34	5	6	0.665	0.003	0.0042	0.0035	0.0041	-0.4433	1
			SmSS	80	6	7	0.696	0.0014	0.0041	0.0046		0.2583	1
Mdh	730	730	SaCo	26	3	3	0.29	0.012	0.0011	0.0011	0.00079	-0.0453	0
			SaSS	48	5	4	0.202	0.0059	0.0016	0.0005		-1.6408	0
			ScCo	24	1	2	0.237	0.011	0.0004	0.0003	0.00038	-0.2132	0
			ScSS	66	2	3	0.246	0.0042	0.0006	0.0004		-0.5915	0
			SmCo	34	2	3	0.538	0.0057	0.0007	0.0008	0.00058	0.4271	0
			SmSS	80	3	4	0.099	0.0021	0.0008	0.0002		-1.4907	0
Mep	785	121	SaCo	26	3	6	0.723	0.0053	0.001	0.0013	0.00209	0.7466	0
		82	SaSS	48	7	12	0.768	0.003	0.002	0.0022		0.2544	3
			ScCo	24	3	4	0.636	0.0038	0.001	0.001	0.00174	-0.194	1
			ScSS	66	7	7	0.788	0.0006	0.0019	0.0021		0.2912	1
			SmCo	34	9	9	0.839	0.0011	0.0028	0.0028	0.00301	-0.0594	1
			SmSS	80	11	13	0.882	0.0002	0.0028	0.003		0.1204	3

nuc = number of nucleotides; Ima2 nuc= number of nucleotides in analysis block; n = haplotypes; S = number of segregating sites; Hap = number of unique haplotypes; Hd and VarHd = Haplotype diversity and variance (Nei 1987, equations 8.4 and 8.12 but replacing 2n by n); $\boldsymbol{\pi}$ = nucleotide diversity (Nei 1987, eq. 10.5); K = nucleotide divergence (Nei 1987, eq. 10.20), θ = pop mutation rate /site/generation (Nei 1987, equation 10.3); TajimaD = gene neutrality test (Table 2 in Tajima 1989) * significant $p < 0.05$; NRB = number of non-recombining blocks (Hudson and Kaplan 1985).

Table 1.4. Population mutation rate (per gene per generation)

Species	**Population**	**Watterson's θ**
S. auriculatus	coast	0.9595
	Salish Sea	1.7814
S. caurinus	coast	0.9644
	Salish Sea	1.5303
S. maliger	coast	1.3617
	Salish Sea	2.1400

θ (Watterson 1975)

Table 1.5. AMOVA results (haplotype data).

Source of Variation (%)	**Cytb**	***S7***	***Ets***	***Mdh***	***Mep***
Among species	72.32	55.28	71.28	57.81	27.12
Among populations	4.69	3.20	1.08	6.33	4.43
Within populations	22.98	41.52	27.64	37.76	66.54
Fixation indices					
F_{ct} (species/total)	0.723	0.553	0.713	0.578	0.271
F_{sc} (population/species)	0.170**	0.072**	0.038*	0.105**	0.087**
F_{st} (population/total)	0.770**	0.585**	0.713**	0.622**	0.335**

*P<0.05, **P<0.005

Table 1.6. F_{ST} values between species and populations. Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*, Co – coast, and SS – Salish Sea

Pop1	**Pop2**	**Locus**	F_{st}	**Locus**	F_{st}	**Locus**	F_{st}	**Locus**	F_{st}	**Locus**	F_{st}	**Average F_{st}**
Between populations within species												
SaCo	SaSS	Cytb	0.3970	*S7*	0.02837#	*Ets*	0.1893	*Mdh*	0.02511#	*Mep*	0.16417	0.2502
ScCo	ScSS	Cytb	0.14156#	*S7*	0.1386	*Ets*	0.0565	*Mdh*	0.1000	*Mep*	0.12947	0.1061
SmCo	SmSS	Cytb	0.03204#	*S7*	0.0416	*Ets*	0.01706#	*Mdh*	0.1317	*Mep*	0.04711	0.0735
Between Species on Coast												
SaCo	ScCo	Cytb	0.9060	*S7*	0.7856	*Ets*	0.9281	*Mdh*	0.6828	*Mep*	0.64312	0.7891
SaCo	SmCo	Cytb	0.9070	*S7*	0.7136	*Ets*	0.7619	*Mdh*	0.1503	*Mep*	0.52336	0.6112
ScCo	SmCo	Cytb	0.8463	*S7*	0.5199	*Ets*	0.6945	*Mdh*	0.6686	*Mep*	0.20769	0.5874
Between Species in Puget Sound												
SaSS	ScSS	Cytb	0.7819	*S7*	0.4823	*Ets*	0.8396	*Mdh*	0.6903	*Mep*	0.43961	0.6468
SaSS	SmSS	Cytb	0.8026	*S7*	0.7350	*Ets*	0.6767	*Mdh*	0.00555#	*Mep*	0.3533	0.6419
ScSS	SmSS	Cytb	0.4955	*S7*	0.4488	*Ets*	0.5800	*Mdh*	0.7912	*Mep*	0.07651	0.4784

Comparisons are not significant ($p>0.05$); All other comparisons are significant ($p<0.05$); F_{st} calculation from Hudson et al. 1992b, equation 3.

Table 1.S1. Individual samples with a nonconforming haplotype (in bold) or individuals whose haplotypes are 'shared' haplotypes with another morphological species. The columns for each locus see Cytb = Fig.3; Ets=Fig.4; S7=Fig.5; Mep=Fig.6; Mdh=Fig.7. The population column abbreviates species and population as follows: Sa – *S. auriculatus*, Sc – *S. caurinus*, Sm – *S. maliger*, Co – coast, and SS –Salish Sea. The number in each column is the haplotype number for that locus and that individual. The colors for the haplotype numbers correspond to the most common morphological species it was shared with. The 'u' designates where the haplotype is unique otherwise they were all 'shared'. The nuclear loci show both haplotypes with a '/' separator if the second haplotype conforms to morphological species then '/ – .'

Sample ID	Population of Origin	CYTB	ETS	S7	MEP	MDH
Sa008	**SaSS**	**23 u**				
Sa011	SaSS				23 u/ –	
Sa012	**SaSS**			**5/ –**		
Sa023	SaSS				5/9	2/2
Sa025	**SaSS**			**5/6**		
Sa006	SaSS				23 u/ –	
Sa004	**SaSS**	**20**			9/ –	
Sa017	SaSS				23 u/ –	
Sc051	**ScSS**			**4/ –**		
Sc053	**ScSS**			**5/ –**		
Sc054	ScSS					1/ –
Sc055	**ScSS**		8/8	**3/ –**		
Sc056	**ScSS**	**18**		**3/ –**		
Sc063	ScSS		8/8			
Sc065	**ScSS**	**18**				
Sc069	**ScSS**	**18**				
Sc071	**ScSS**	**18**		**5/ –**		
Sc073	**ScSS**			**5/ –**		1/ –
Sc074	ScSS					1/ –
Sc045	**ScSS**	**18**		**5/ –**		1/ –
Sc046	**ScSS**			**5/ –**		
Sc048	**ScSS**	**18**				1/ –
Sc087	ScSS					3/ –
Sc002	**ScSS**	**18**				
Sc033	**ScSS**	**18**				
Sc035	**ScSS**	**18**				1/ –
Sc039	ScSS					1/ –
Sc040	ScSS					1/ –
Sm181	**SmSS**	**12**				
Sm161	**SmSS**	**22**				
Sm164	**SmSS**	**22**				
Sm173	SmSS		3/3			
Sm019	SmCo		3/3			2/2
Sm022	SmCo			10/ –		2/2

Chapter 2: Extent and distribution of introgression of three rockfish species (*Sebastes auriculatus, S. caurinus,* and *S. maliger*) in the Salish Sea, Northeast Pacific Ocean

Abstract

Hybridization is a concern for conservation of species impacted by habitat alteration and fragmentation; but may also important for species evolution by creating a rich source of genetic variability. Hybridization between three closely related species of rockfish, *Sebastes auriculatus, S. caurinus,* and *S. maliger* has previously been reported in Puget Sound (southern Salish Sea), yet they are also found in sympatry along the Pacific coast where they apparently do not hybridize. Here, we describe the geographic patterns of hybrid abundance in Puget Sound and evaluate the level of genetic introgression in Puget Sound hybrids. Sequence data from four nuclear intron markers and one mitochondrial marker were collected in 139 fish from coastal and Salish Sea populations. Individual admixture was evaluated using Bayesian genetic clustering analyses and hybrid proportions were estimated in each geographic region to determine distribution of introgression. We found broad-scale introgressive hybridization from *S. maliger* into *S. caurinus* and *S. auriculatus,* with a third of all fish in the Salish Sea of hybrid origin. All rockfish hybrids were later generation backcrosses indicating long-term introgressive hybridization in the Salish Sea, specifically in Puget Sound. The proportion of hybrids was significantly lower on the coast compared with all Puget Sound regions and increased with increasing isolation from the coast. Also, we found strong directional introgression from *S. maliger* into *S. caurinus* and *S. auriculatus*. The bathymetry, oceanography and ecology of Puget Sound is distinct from the coast and may be particularly suited for the development of a hybrid zone, however the exact mechanisms influencing hybridization in Puget Sound require further research.

Introduction

Hybridization and introgression are often concerns for conservation of species impacted by fragmented or altered habitats (Rhymer and Simberloff 1996; Allendorf et al. 2001); however, introgressive hybridization is also important for species evolution by providing a rich source of genetic variability (Harrison 1990; Coyne and Orr 2004; Arnold and Martin 2010). The evolutionary potential of hybrids and their parental species depend on specific mechanisms that influence the development and maintenance of hybrid zones in nature (Barton and Hewitt 1985). The conservation implications of hybridization in natural populations are therefore context dependent, depending both on anthropogenic factors and the evolutionary processes maintaining the hybrid zone (Allendorf et al. 2001).

Hybridization is often spatially limited to hybrid zones, which may be maintained by a combination of exogenous and endogenous selection (Moore 1977; Barton and Hewitt 1985). Exogenous selection is dependent on environmental factors (extrinsic) on hybrid survival, whereas endogenous selection depends on physiological and genetic factors (intrinsic) such as genetic incompatibility (Barton 2001) and disruption of coadapted gene-complexes (Moore 1977). One explanation for the maintenance of hybrid zones is the "tension zone" hypothesis, in which endogenous selection acts against hybrids and prevents dispersal of parental genotypes across the zone (Barton and Hewitt 1985; Barton 2001). An alternative hypothesis is "bounded hybrid superiority", which posits that novelty created by hybridization and exogenous selection favor hybrids within a specific ecotone (Moore 1977). A combination of these theories is represented by the "mosaic hybrid zone," which is maintained by exogenous selection in a heterogeneous environment and/or endogenous influences (Harrison 1986). Although there is an ongoing debate in the literature on which mechanism is most important for maintenance of hybrid zones, all intrinsic and extrinsic processes along with dispersal are important to consider when investigating hybridization and genetic introgression in a specific ecosystem.

Hybridization is common in terrestrial and freshwater vertebrates (Hubbs 1955; Burke and Arnold 2001; Mallet 2005) and recently, hybridization has also been reported in marine fish (Seeb 1998; Roques et al. 2001; Yaakub et al. 2006; Garrett et al. 2007; Tringali et al. 2011; Morgan et al. 2012). The scarcity of reports of marine hybridization may be due in part to low genetic differentiation and the lack of diagnostic characters in closely related marine taxa (Knowlton 1993; Roques, et al. 2001); nonetheless, the increased availability of molecular markers (Avise 1994) has made investigations of hybridization in marine fish more accessible (Avise 2001; Roques et al. 2001). Several studies found hybridization between three species of rockfish, *Sebastes auriculatus, S. caurinus,* and *S. maliger* in Puget Sound, although they also occur in sympatry along the Pacific coast where they apparently do not hybridize (Seeb 1998; Buonaccorsi et al. 2005). *Sebastes caurinus, S. maliger,* and *S. auriculatus* are closely related to each other (Hyde and Vetter 2007), and are the most common species of rockfish in Puget Sound (Palsson et al. 2009), southern Salish Sea. Like most other rockfish species, they are long lived, late maturing, maintain a pelagic life history as larvae and juveniles, have internal fertilization, and are ovoviviparous (Love et al. 2002). Adult *S. caurinus, S. maliger,* and *S. auriculatus* show strong site fidelity and remain sedentary in rocky reef areas. Although all three overlap in their geographic distribution, they tend to separate on different habitats according to depth, with *S. maliger* generally occupying deeper waters and *S. auriculatus* occupying shallow intertidal and estuarine habitats (Stout et al. 2001; Love et al. 2002; Palsson et al. 2009).

The bathymetry, oceanography, and ecology of Puget Sound may be particularly suited for the development of hybrid zones. Puget Sound is an estuarine environment with distinct ecosystems that support diverse communities from plants to marine mammals (Gelfenbaum et al. 2006). Puget Sound was formed towards the end of the last glacial maximum, approximately 12,000 years ago (Thorson 1980), and consists of narrow, deep channels and shallow sills (Burns 1985). One major shallow sill separates the main basin of Puget Sound) from the Salish Sea at Admiralty Inlet. Within Puget Sound shallow sills define major geographic regions: South Puget Sound, Hood Canal, Central Puget Sound and Whidbey Basin (Stout et al. 2001, Palsson et al. 2009). Compared to the coast and the San Juan Islands, Puget Sound has less reef habitat

(Pacunski and Palsson 1998), lower salinity, more variable temperatures, and anoxic conditions; furthermore, this variability in water quality is most pronounced in Puget Sound sub-basins (Ebbert et al. 2000). Because of sills and oceanography, populations of marine species in Puget Sound tend to be isolated from coastal populations. Indeed, Puget Sound populations of *S. auriculatus, S. caurinus,* and *S. maliger* show genetic differentiation from the coastal populations in each species (Seeb 1998; Buonaccorsi et al. 2002; Buonaccorsi et al. 2005).

The spatial patterns of introgression as well as the distribution of characters or genetic traits can provide an important foundation for research on factors important for the evolution and maintenance of a marine hybrid zone (Barton and Hewitt 1985; Arnold 1997). The research objectives of this study are to describe the geographic patterns of hybrids in Puget Sound and evaluate the level of genetic introgression in Puget Sound hybrids.

Methods

Tissue Collection

Tissues were provided by the Washington Department of Fish and Wildlife (WDFW) and the Southwest Fisheries Science Center (SWFSC). Samples provided by the SWFSC were tissues of *S. auriculatus* (n =13), S. *caurinus* (n =12), and *S. maliger* (n =17) collected across the full coastal range from Alaska to California (1994 – 2002), (Figure 2.1, Table 2.1). The WDFW provided tissues samples from *S. auriculatus* (n =24), S. *caurinus* (n =33), and *S. maliger* (n =40) from collections from the southern Salish Sea: South Puget Sound (SPS), Central Puget Sound (CPS), Hood Canal (HC), Whidbey Basin (WB), and areas north of Puget Sound (NPS) including the Strait of Georgia, San Juan Islands and the Strait of Juan de Fuca. (Figures 2.2 and 2.3, Table 2.2). Tissues were provided as fin clips or muscle stored in 95 % ethanol. Data collected from each specimen included the capture locality (latitude, longitude, and depth), species, length, sex, and weight. Although whole specimens were not retained, the identifications were made by experienced rockfish biologists and were collected as true species representatives (Wayne Palsson, NOAA Alaska Fisheries Science Center, pers. comm. 2010; Carol Kimbrell, NOAA SWFSC, pers. comm. 2008).

Three whole hybrid specimens were included in the analysis (Table 2.3). Two were suspected to be hybrids (UW 113205 and UW 113206) because of atypical species morphology (Wayne Palsson pers. comm. 2009). One whole specimen (UW 47319) was identified morphologically as *S. caurinus*, but mitochondrial DNA identified the specimen as *S. auriculatus* (Schwenke et al. unpublished). Whole specimens were described by morphological characteristics, photographed, and tissue samples were taken and stored in 95% non-denatured ethanol. The specimens were then preserved in formalin, stored in ethanol, and catalogued at the University of Washington /Burke Museum Fish Collection (http://www.burkemuseum.org/ichthyology).

Laboratory Sample Preparation

Genomic DNA was extracted from a 2 mm^2 piece of fin or muscle tissue using a Qiagen DNeasy 96 Tissue Kit on the Qiagen BioRobot 8000. Five DNA regions were targeted using PCR amplification and primers for the mitochondrial DNA locus cytochrome b (*Cytb*), 5' external transcribed spacer (*Ets*), *S7* ribosomal intron 2 (*S7*), malate dehydrogenase (*Mdh*), and malic enzyme (*Mep*). Primers for *Cytb*, *Ets*, and *S7* were obtained from the published literature (Table 2.4). Primers for *Mdh* and *Mep* were designed using conserved regions across gene sequences from fish and other vertebrates available in NCBI Genbank (Park, L.K. unpublished). Additional primers for *S7*, *Mdh*, and *Mep* were designed to improve sequence data in rockfish. Individual PCR amplifications were performed on each genomic DNA template using a BioRad 96-well DNA Engine Tetrad. The primer sequences and cycling conditions for the PCR amplifications were specific to each locus (Table 2.3). Generally, PCR reactions were performed in a 40-µl reaction with 0.5 unit Go*Taq* DNA polymerase (Promega), 1X Go*Taq* Buffer, 200µM dNTP (Promega), each primer at 100 – 400 nM, and approximately 10 – 20 ng of genomic DNA. PCR cycles were carried out for each locus with an initial denature step at 95° C for 2 minutes and 32 cycles of 94 C for 40 seconds, locus specific annealing conditions (Table 2.3) and 72 °C for 60 seconds. PCR products were visualized using agarose gel electrophoresis and ethidium bromide / UV transducer in order to verify quality of amplification. PCR products were purified using the Montage MultiScreen 96-well plate protocol (Millipore) and sequenced in both directions

using PCR primers and Big Dye Terminator Cycle Sequencing Ready Reaction version 3.1 (Life Sciences). Sequencing reactions were purified using CleanSeq Dye Terminator Removal Kit (Beckman Coulter Genomics) and size-separated on an ABI3100 Genetic Analyzer using POP4 polymer and an 80 cm capillary array.

Sequencing and Structure analysis

The sequences were visualized, edited, and aligned using the software program Codon Code Aligner software version 3.7. Interpretations of heterozygous peaks were evaluated by eye and called using data with high PHRED scores (>0.95). Locus alignments were evaluated for segregating sites in both the coastal and Puget Sound populations separately. Only individuals with data for all loci were used for analysis. All gaps were removed from the data and diploid nuclear data for each species group and locus were phased into haplotypes using the program PHASE implemented in the software program DNAsp v5 (Librado and Rozas 2009) using the following MCMC parameters: Burn of 10,000 steps, 10 step thinning intervals, and 100,000 iterations.

Sequence data were formatted for analysis for the program, *Structure*, using the program, xmfa2struct (http://www.xavierdidelot.xtreemhost.com/clonalframe.htm). This conversion program takes the haplotype sequence data and partitions each polymorphic site as a separate locus with the length of the sequence between polymorphic sites as the linkage distances. The software program *Structure* version 2.3.3 (Prichard et al. 2000, Falush et al. 2003) was used to identify hybrids in coastal and Salish Sea populations of *S. auriculatus*, *S. caurinus*, and *S. maliger*. The linkage model was used to evaluate unphased, diploid data in 142 samples at 92 sites across 5 sequence fragments. *Structure* implements a model based clustering method for inferring population structure by minimizing Hardy-Weinberg and linkage disequilibrium within groups to form homogenous genetic clusters. Recent versions of *Structure* can consider the linkage disequilibrium inherent in recently admixed populations (Falush et al. 2003). *Structure* infers population structure from the data using a Markov chain Monte Carlo (MCMC) approach. The *Structure* analysis initiated a single MCMC chain with 10,000 burn-in steps, 100,000 run steps, 10 thinning interval steps, and 3 inferred clusters (k=3). To

determine the number of populations (k) that best fit the data, we evaluated the log likelihood score output for simulations using k=1 to k=7.

The species for each genetic group was confirmed by morphological assignment in coastal samples in which hybridization is assumed to be rare. An individual fish was assumed to be a hybrid if its largest Q value was less than 0.90. The data from the three hybrid whole specimens were also included in the *Structure* analysis and grouped post-hoc with Salish Sea *S. caurinus* in order to evaluate their individual level of introgression and to validate our hybrid detection methods.

Independence and correlation

Each region or basin in Puget Sound is naturally separated by a shallow sill (Stout et al. 2001) that was used to draw geographical regional boundaries (Figure 2.3). The geographic borders correspond to approximate locations of these shallow sills which are located at the mouth of each basin. Whidbey Basin has the main inlets at CPS and a much narrower one at NPS. Each individual fish was counted as hybrid if *Structure* revealed any evidence of admixture, and the proportion of hybrids was calculated by geographic region for all species combined and for each species separately. A Fisher's Exact Test was used to test for independence among regions with all species combined and to test for independence between coast and the Salish Sea for each species. We categorized each geographic region by its isolation from the coast using the number of shallow sills and the Strait of Juan de Fuca as barriers for dispersal (or dispersal distance) to the coast (0=coast; 1=NPS; 2=CPS;, 3=WB, HC, and SPS). The three sub-basins in Puget Sound were considered the same category. A spearman's rho was used to test for correlation between location category and the proportions of hybrids for each species. The data used for the hybrid proportion analyses included only the tissue collection samples; the three hybrid voucher specimens were excluded.

Results

The Bayesian clustering in *Structure* showed that the best fit number of populations given the data was k=3, which had the highest log likelihood value for all simulations (k=1 – 7, Table 2.S2) and which was consistent with the collection of three

species. The admixture results showed 31 out of 139 individuals with mixed ancestry (Q<0.90), and confirmed the mixed ancestry of the three hybrid whole specimens (Figure 2.4, Table 2.S1). In the Salish Sea, the relative proportions of hybrids were significantly larger in *S. caurinus* and *S. auriculatus* than in *S. maliger* (Figure 2.4, Tables 2.5 and 2.6). No hybrids were detected in coastal *S. caurinus* and *S. auriculatus*, and coastal *S. maliger* had a low incidence of hybrids. The proportion of hybrids from Salish Sea *S. maliger* and the coast were similar (Figure 2.4, Table 2.6). The only two hybrids found on the coast were collected as *S. maliger* off West Vancouver Island and near the Queen Charlotte Islands (Figure 2.4, Table 2.S1).

The *Structure* analysis showed interesting patterns for individual admixture and direction of introgression. First, all hybrids appeared to be later generation backcrosses and the absence of F1 hybrids was confirmed with raw sequence data (not shown) Second, there was higher introgression of *S. maliger* into *S. auriculatus* and *S. caurinus* than the other way around, or between *S auriculatus* and *S caurinus* (Figure 2.4). Third, two *S. caurinus* from the tissue collection and one hybrid whole specimen had ancestry from all three species (Figure 2.4). Interestingly, the only evidence of *S. auriculatus* hybrid ancestry in *S. caurinus* was found in a single individual with three species ancestry (Figure 2.4). Fourth, one whole specimen morphologically identified as *S. caurinus* collected in CPS appeared to be mostly pure *S. auriculatus* (UW 447319 in Table 2.S1). This curious result was verified with repeat morphological examinations of the voucher specimen and replicate tissue sampling and sequence identification. Both of the suspected hybrid *S. caurinus* whole specimens collected in SPS appeared to have high levels of mixed ancestry (Figure 2.4, A, B), and one tissue sample of *S. caurinus* (morphological specimen unavailable) from SPS appears to also have high mixed ancestry (Figure 2.4, CoSPS045 in Table 2.S1).

A strong relationship was found between collection location and hybrid frequency. For all species combined, the proportion of hybrids in each geographic region within Puget Sound were significantly higher compared to the coast (Fisher's exact test, $p<0.05$, Figure 2.5, Table 2.7). The hybrid proportion comparisons between the coast and areas north of Puget Sound were not significantly different, also for regions within Puget Sound (Table 2.7). Although tests for independence could not be rejected for hybrid

proportions among Salish Sea regions, hybrid proportions for all species combined are higher in CPS, WB, HC, and SPS compared to the coast and areas North of Puget Sound (Figure 2.5, Table 2.7). Hybrid proportions within species were not compared among Salish Sea regions because of small sample sizes for some species in some regions. However for *S. caurinus,* there was a strong positive relationship between the proportion of hybrids and the collection location within Puget Sound (Figure 2.6 and 2.7).

Discussion

Although rockfish hybridization in Puget Sound has been described before (Seeb 1998; Buonaccorsi et al. 2005), the sampling and molecular methods employed here provided higher resolution into the patterns of introgression in Puget Sound. Our study provided evidence for asymmetrical introgression and unprecedented data on spatial and temporal patterns of hybridization, suggesting higher incidences of hybridization in inshore, isolated basins and long-established processes resulting primarily in backcrosses rather than F1 offspring. The admixture analysis showed broad-scale introgressive hybridization from *S. maliger* into *S. caurinus* and *S. auriculatus,* with thirty percent of all fish in the Salish Sea of hybrid origin. All rockfish hybrids were later generation backcrosses indicating long-term patterns of introgressive hybridization in the Salish Sea, specifically in Puget Sound. The proportion of hybrids was significantly lower on the coast compared with all Puget Sound regions south of Admiralty Inlet and increased with increasing isolation from the coast (Figure 2.7).

Even though a high frequency of hybrids in Puget Sound *S. caurinus* and *S. auriculatus* was detected, the true frequency may be even higher given the limited number of markers employed and their ability to resolve all three species (Chapter 1). If introgressive hybridization has been ongoing for generations in Puget Sound, then more markers would increase the detection power for later generation hybrids (Boecklen and Howard 1997). We would further expect that increased detection would reinforce the geographic patterns seen within Puget Sound. Although we found extensive introgression in Puget Sound, this hybrid zone does not appear to be a hybrid swarm because retention of parental morphological types is evident. Rather than two or three gene pools fusing in Puget Sound *Sebastes*, most hybrids were morphologically and

genetically similar to one of the three species; although, some fish with intermediate morphology are found in Puget Sound *Sebastes*, as seen here and in other studies (Seeb 1998).

There are various factors that may influence the occurrence and the spatial patterns in the Puget Sound *Sebastes* hybrid zone including selection and dispersal. Yet, the relative influence from each of these factors remains unknown, given that little is understood about the life history and ecology of many Sebastes species. Nonetheless, hybrid frequency was strongly correlated across an environmental gradient defined by Puget Sound sub-basins. More hybridization associated with a defined environmental gradient suggests that exogenous selection is important in Puget Sound. On the other hand, asymmetrical introgression in Puget Sound suggests that hybrid mate choice and fitness differences between hybrids are also important influences (Chapter 1). Biological factors and endogenous selection such as mating behaviors or hybrid fitness are also likely influencing the patterns of introgression in Puget Sound. All *Sebastes* species are internal fertilizers with potentially strong sexual selection. The details of mating behavior are not well understood (Love et al. 2002) so detailed factors in mate preference and interspecific hybridization are difficult to ascertain. However, the asymmetrical pattern of introgression from *S maliger* into the other two species could be explained by F1 hybrids backcrossing with *S. caurinus* or *S. auriculatus* and rarely with *S. maliger*. On the other hand, asymmetrical introgression may also be due to differences in fitness between hybrids (Burke and Arnold 2001). *Sebastes caurinus* and *S. maliger* are more closely related to each other than to *S. auriculatus* (Hyde and Vetter 2007), and hybrids between closely related taxa may be more viable than those between distantly related species. Indeed, we found more introgression between *S. maliger* and *S. caurinus*, but relatedness between these species does not entirely explain the asymmetry of introgression into *S. caurinus*.

Habitat

Opportunities for hybridization in Puget Sound compared with those on the coast are likely influenced by limited habitat in Puget Sound. Puget Sound (south of Admiralty Inlet) has 20 times less reef habitat compared to the San Juan Islands north of Puget

Sound (Pacunski and Palsson 1998). An important life history component of adult rockfish is their close association with rocky reef habitats and the limited amounts of available rocky reef habitat in Puget Sound could force multispecies assemblages onto limited reefs that may provide more opportunity for hybridization in Puget Sound. Such habitat constraints may be reinforced by other environmental needs: for example, *S. auriculatus* is thought to be less tolerant to currents along rocky reef structure than *S. caurinus* and *S. maliger*, leading to a more fragmented distribution (Palsson et al. 2009). *Sebastes auriculatus* in Puget Sound is mostly distributed within the main basin and is rare in the rest of the Salish Sea (Palsson et al. 2009). There are also artificial reefs in Puget Sound that are reported to be more densely used by rockfish than natural reef habitat (Buckley and Hueckel 1985, Hueckel and Buckley 1989).

In addition to competition for fewer rocky reef sites, species of *Sebastes* may also be affected by gradients in water quality among basins, allowing more opportunities to hybridize in Puget Sound. Compared to the coast, Puget Sound naturally has lower salinity, variable temperatures, and natural anoxic conditions (Stout et al. 2001; Palsson et al. 2009). The Puget Sound sub-basins experience increased anoxic conditions because they are exposed to heavy, seasonal freshwater input compared to the main basin (Albertson et al. 2002). Such oxygen depletion generally starts in deep waters and forces species into shallower habitat. Many rockfish species are segregated by their depth preference (Hyde and Vetter 2007, Hyde et al. 2008): for example, *S. maliger* is usually found at the deepest distribution while *S. auriculatus* occurs at more shallow depths (Love et al. 2002, Palsson et al. 2009). Periodic low oxygen levels, which occur frequently in Hood Canal (Palsson 2005), force *S. maliger* into more shallow depths, which may provide more opportunities for hybridization due to the increased contact with *S. caurinus* and *S. auriculatus*. Asymmetric introgression could result if hybrids stay in shallow water while pure *S. maliger* retreat back to depth when oxygen levels return to normal. Anoxic conditions may thus provide opportunities for extrinsic selection in Puget Sound sub-basin hybrid zones and may also explain the directional patterns of hybridization.

Dispersal

The higher incidence of hybridization in Puget Sound is likely influenced by restricted geneflow with the outer coast and also by limited dispersal of adult rockfish from Puget Sound. Evidence for restricted geneflow between Puget Sound and coastal populations has been found in *S. auriculatus*, *S. caurinus*, and *S. maliger* (Seeb 1998, Buonaccorsi et al. 2002, Buonaccorsi et al. 2005). Recruitment of juvenile rockfish from outside the main basin appears to be limited, due to low surface-water exchange from outside the main basin as estimated by drift card studies (Engie and Klinger 2007). Genetic evidence for limited dispersal from Puget Sound in rockfish (Seeb 1998; Buonaccorsi et al. 2005) and clams (*Protothaca staminea* and *Macoma balthica*, Parker et al. 2003) (Parker et al. 2003). Within Puget Sound, natural barriers to dispersal are likely found at two major shallow sills at Admiralty Inlet (between NPS and CPS) and the Tacoma Narrows (between CPS and SPS); also, shallow sills separate Whidbey Basin and Hood Canal from the main basin (Stout et al. 2001; Palsson et al. 2009; Drake et al. 2010). Indeed, Pacific herring (*Clupea pallasii*), for example, from SPS are genetically distinct from other Puget Sound populations (Small et al. 2005; Mitchell 2006). In this study, the increased hybrid frequency in *S. auriculatus* and *S. caurinus* is correlated with greater isolation from the coast (Figure 2.7). The sills could be acting as physical barriers to dispersal thus limiting geneflow between Puget Sound and coastal populations. In Puget Sound, the low intraspecific geneflow from neighboring populations may be causing introgressed alleles to be retained in Puget Sound. Broader sampling in Puget Sound, the Strait of Georgia, and the outer coast is needed to further investigate hybrid boundaries. Also, additional genetic markers are needed for added resolution for individual admixture thus allowing a better measure of hybrid genotypes across environmental gradients.

The dynamics that affect extent and spatial distribution of hybridization are complex in Puget Sound because of multiple influences of demographic, ecological, and biological processes. The Puget Sound rockfish hybrid zone appears to somewhat fit the mosaic zone hybrid hypothesis because of the heterogeneity of habitat and the integrated role of exogenous and endogenous selection. In addition, Puget Sound rockfish are exposed contemporary anthropogenic influences from habitat change to fishing pressure.

The Puget Sound shoreline is home to over 1.1 million people with over half developed as urban or agriculture (Drake et al. 2010). In addition, the overall abundance of Puget Sound *S. maliger*, *S. caurinus*, and *S. auriculatus* has declined in the past 40 years (Palsson et al 2009). Conservation of hybridization in natural populations is an important management concern (Allendorf et al. 2001); therefore, understanding the ecological processes that affect Puget Sound rockfish is important for maintenance of rockfish species and their hybrids.

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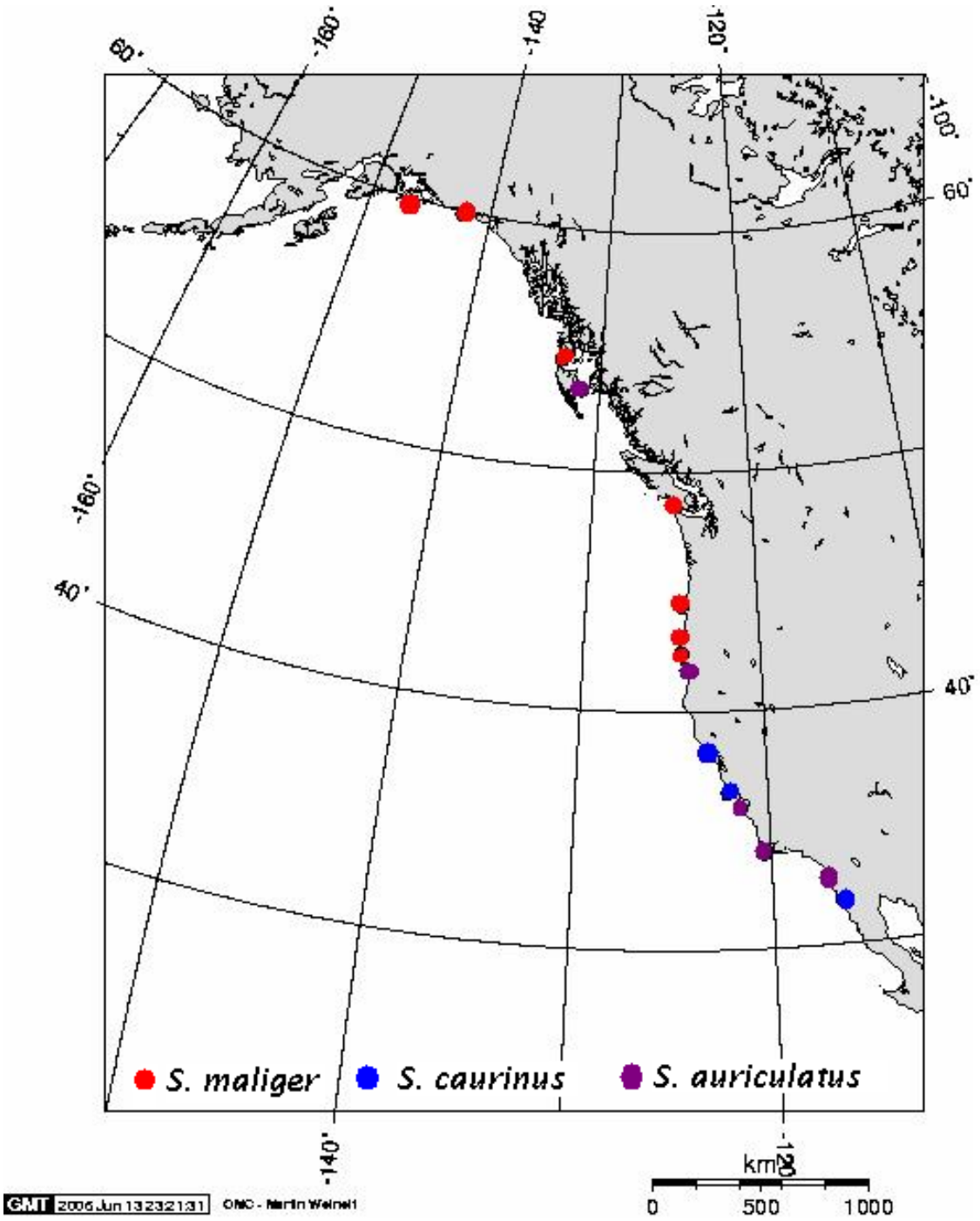

Figure 2.1. Localities for costal collections. Red circles are *S. maliger* (n=17), blue are *S. auriculatus* (n=13), and purple are *S. caurinus* (n=12). Each sample location represent 1 – 6 individuals.

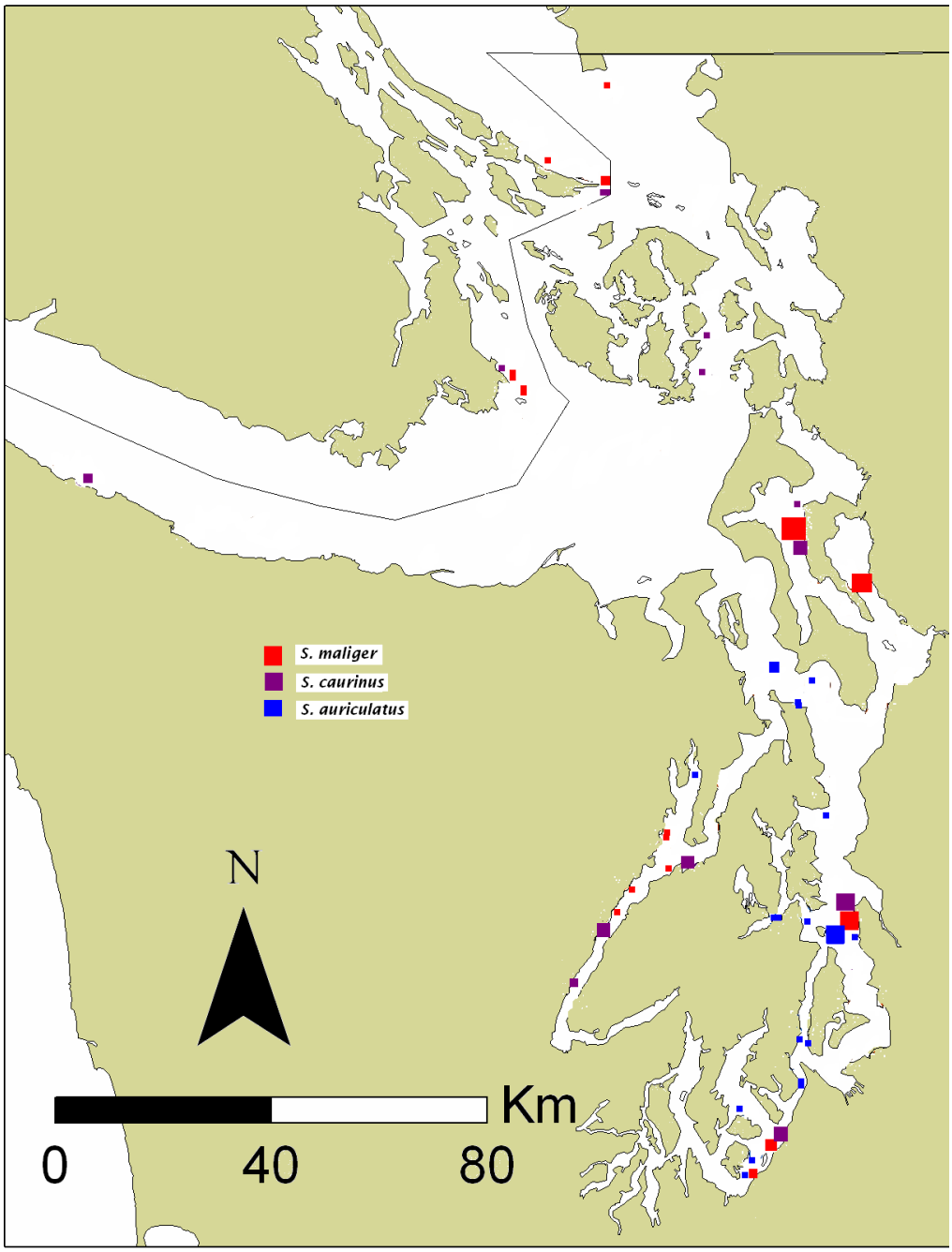

Figure 2.2. Localities for Salish Sea collections. Red squares are *S. maliger* (n=40), blue are *S. auriculatus* (n=24) and purple are *S. caurinus* (n=33). The size of the square is proportional to the size of collection (1 – 8 individuals). More than one species collected from the same location is represented by adjacent squares.

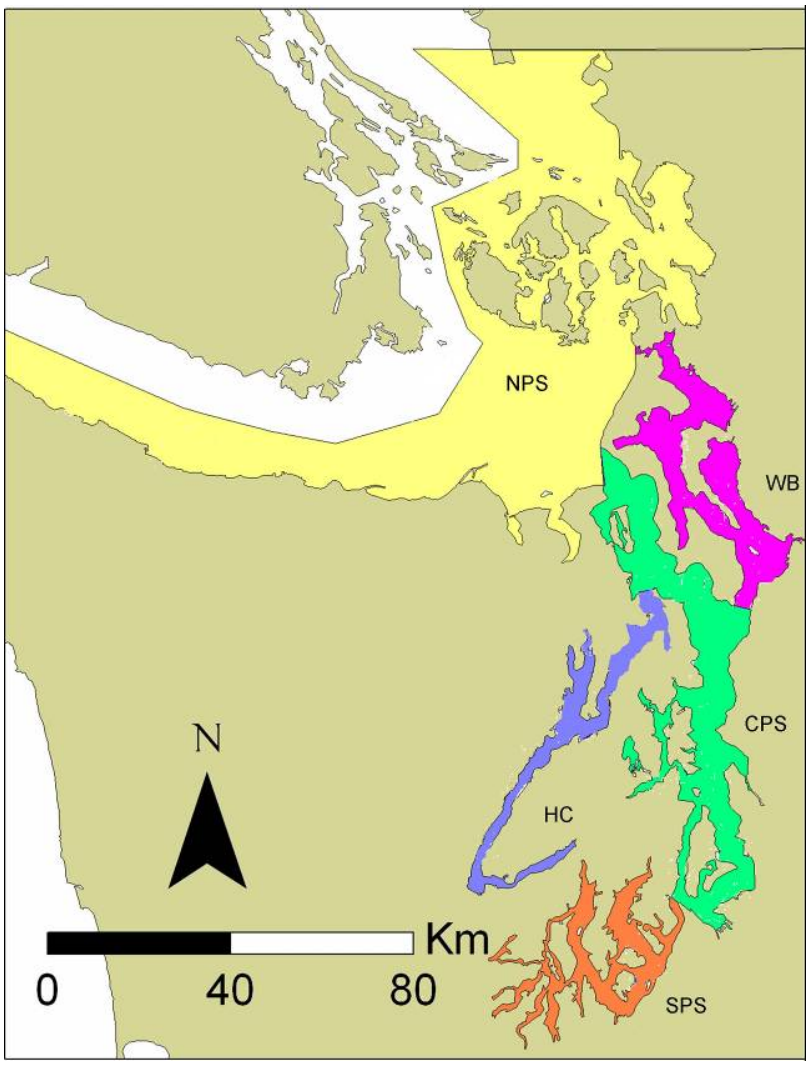

Figure 2.3. Major sampling regions in the Salish Sea collection: South Puget Sound (SPS), Central Puget Sound (CPS), Hood Canal (HC), Whidbey Basin (WB), and north of Puget Sound (NPS) including the Strait of Georgia, San Juan Islands, and the Strait of Juan de Fuca. Each basin is naturally separated by a shallow sill. The geographic region borders correspond to approximate locations of these shallow sills which are located at the mouth of each basin. Whidbey Basin has two inlets one at NPS and one at CPS.

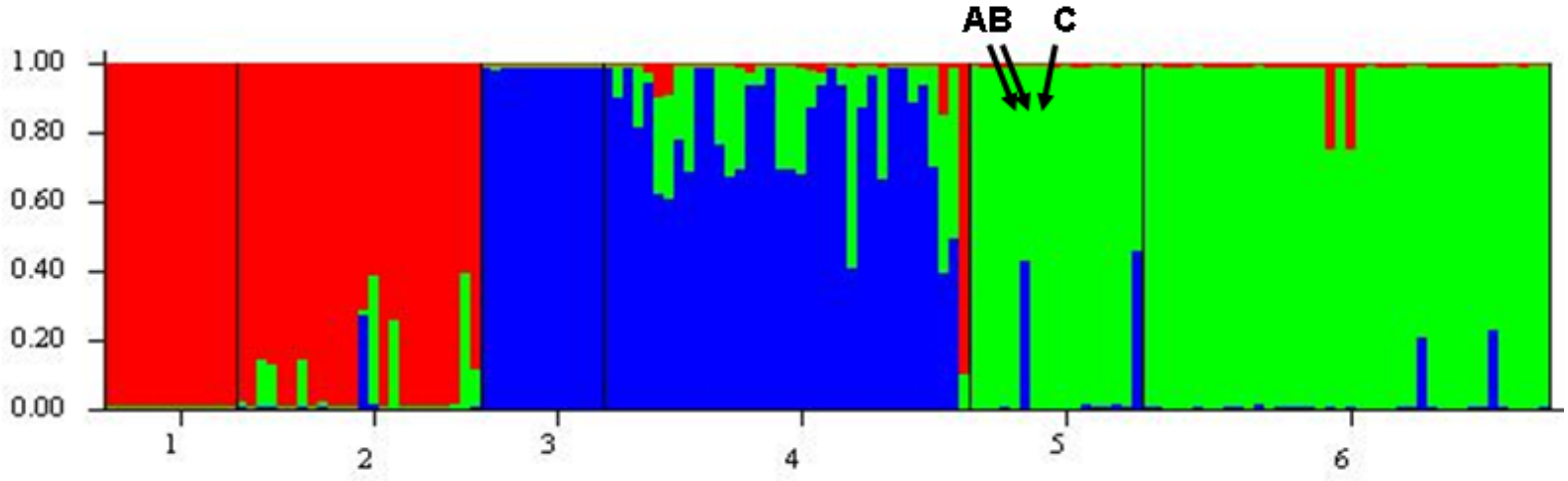

Figure 2.4. The probability of assignment for each individual to one of three genetic groups (k=3). Each vertical bar represents a single individual and the colors shows the proportion of ancestry to each genetic group. The three genetic clusters are represented by red as *S. auriculatus*, blue as *S. caurinus* and green as *S. maliger*. The results for each individual are arranged vertically by morphological species and population: 1= *S. auriculatus* (coast), 2 = *S. auriculatus* (Salish Sea), 3=*S. caurinus* (coast), 4 = *S. caurinus* (Salish Sea), 5 = *S. maliger* (Salish Sea), 6 = *S. maliger* (Salish Sea); A and B are *S. caurinus* hybrid whole specimens from SPS, and C is the *S. caurinus* hybrid voucher from CPS.

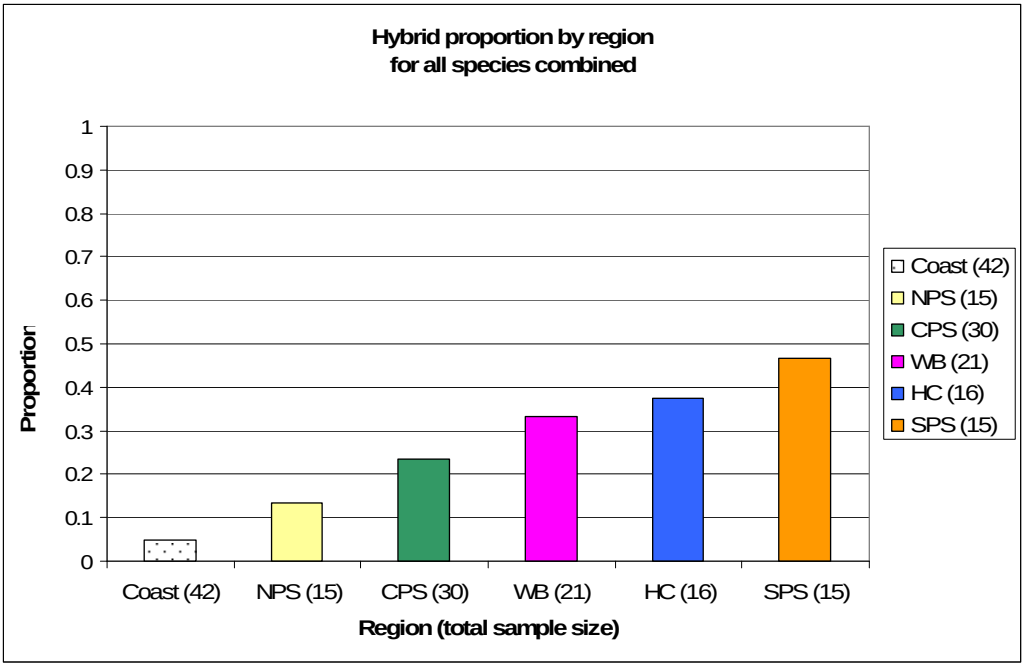

Figure 2.5. Hybrid proportions in each Salish Sea geographic region. All species and hybrids were pooled into each region. The colors correspond to each geographic region shown in Figure 3. Sample sizes are given in parenthesis along with region codes.

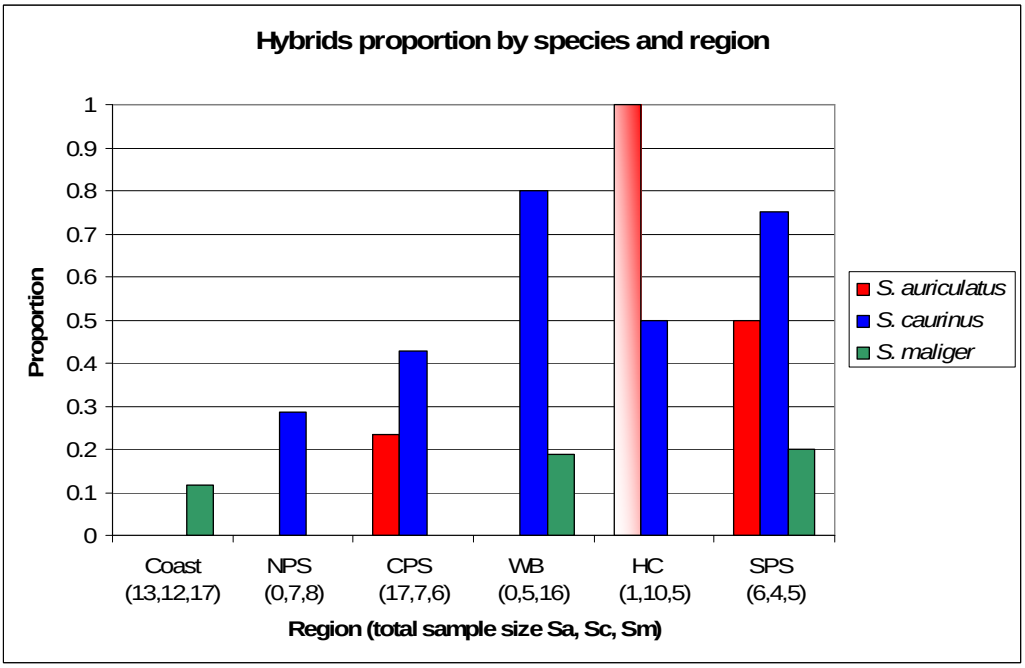

Figure 2.6. Hybrid proportion by species and geographic region. Sample sizes are given in parenthesis (Sa = *S. auriculatus*, Sc = *S. caurinus*, Sm = *S. maliger*). Sa was not sampled in NPS or WB and hybrid proportions from Sa in HC was from a single sample (color diminished) thus interpret with caution.

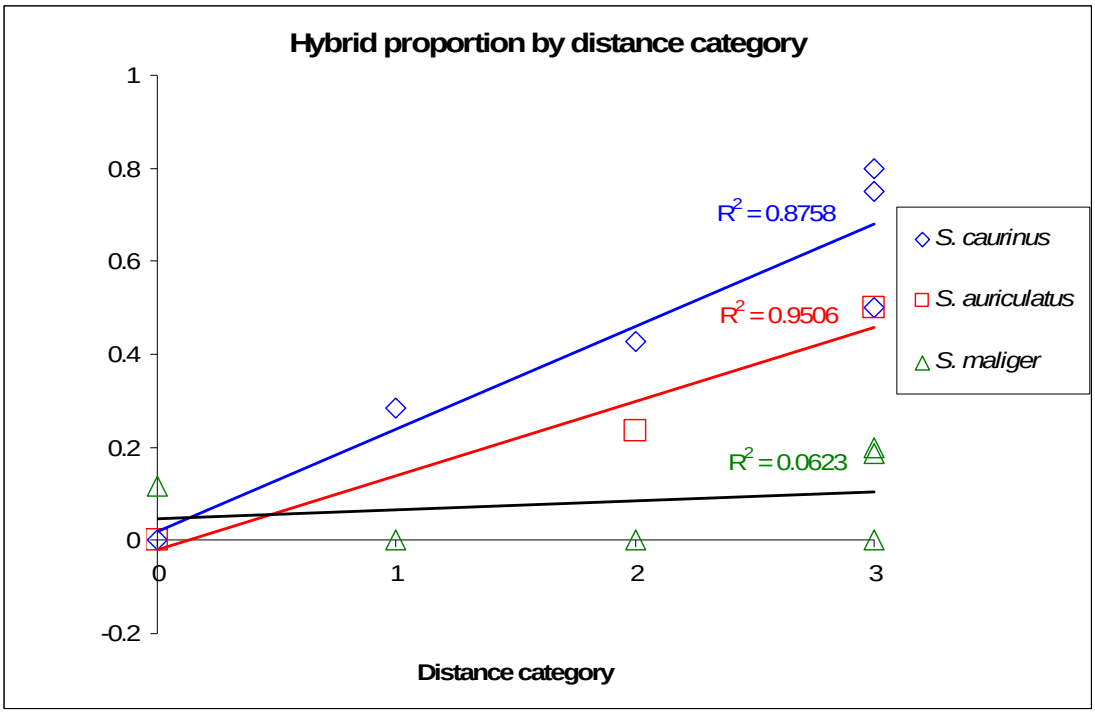

Figure 2.7. Spearman's rank correlation coefficient for proportion of hybrids in each species by distance category. The location category was determined by dispersal distance from the coast and also grouped by location type (0=coast; 1=NPS; 2=CPS; 3=WB, HC and SPS). Puget Sound sub-basins were all category 3.

Table 2.1. Tissue samples from the coastal region

Species and Region	Number	Collection Date
S. auriculatus		
Central California	5	1999
Southern California	4	1999
Baja, Mexico	4	2000
Total	*13*	
S. caurinus		
Queen Charlotte Island	2	1994
Northern California	5	1999
Central California	3	1993
Southern California	1	1998
Baja, Mexico	1	2001
Total	*12*	
S. maliger		
Northern California	3	1999
Southern Oregon	6	2002
Northern Oregon	1	1998
West Vancouver Island	3	1998
Queen Charlotte Island	1	1994
Southeast Alaska	2	1995
Prince William Sound	1	1999
Total	*17*	

Table 2.2. Tissue samples collected from the Salish Sea.

Species and Region	Number	Collection Year
S. auriculatus		
North of Puget Sound*	0	
Central Puget Sound	17	2002
Whidbey Basin	0	
Hood Canal	1	2002
South Puget Sound	6	2002
total	24	
S. caurinus		
North of Puget Sound*	7	2002
Central Puget Sound	7	2002
Whidbey Basin	5	1997, 2002
Hood Canal	10	2002
South Puget Sound	4	2001
total	33	
S. maliger		
North of Puget Sound*	8	2002
Central Puget Sound	6	2002
Whidbey Basin	16	2002
Hood Canal	5	2002
South Puget Sound	5	2003, 2005
total	40	

*Localities from Salish Sea north of Admiralty Inlet were combined and included the Strait of Georgia, San Juan Islands, and the Strait of Juan de Fuca.

Table 2.3. Hybrid whole specimens catalogued at the University of Washington / Burke Museum Fish Collection (http://www.burkemuseum.org/ichthyology).

Catalogue Number	Morphology	Region	Collection Year
UW 47319	*S. caurinus*	Central Puget Sound	2004
UW 113205	*S. caurinus* (atypical)	South Puget Sound	2005
UW 113206	*S. caurinus* (atypical)	South Puget Sound	2005

Table 2.4. Locus data.

Locus	**Primers**	**PCR Annealing Conditions**	**Reference**	**Name**
Cytb	GluF 5' TGA CTT GAA RAA CCA YCG TTG 3' *Cytb*R 5'ATA TCA TTC TGG CTT AAT GTG 3'	58 °C for 40 sec.	Rocha-Olivares et al. (1999)	Cytochrome b mitochondrial
5'*Ets*	F 5' CGG CCA TGG GCA GTT CAG G 3' R 5' ATA TGC TTA AAT TCA GCG GG 3'	52 °C for 40 sec.	Le et al. (1993)	5' external transcribed spacer, intron
S7	F 5' AGCGCCAAAATAGTGAAGCC 3' R 5' GCCTTCAGGTCAGAGTTCAT 3' C272 R 5' CAT CTA CTG ACA CTT GTA TAC TA 3' (internal with *S7* – F)	60 °C for 60 sec. 58 °C for 60 sec.	(Chow and Hazama, 1998) This paper	*S7* Ribosomal, intron 2
Mdh	F1 5' CCT CTC TCA CTG CTG CTG AA 3'R1 5' TTC TTC TCG ATG CCG TTC TT 3' RF R2 5' TCC CCA GAA GAA GAG GTG TG 3' (internal with *Mdh* – F1)	61°C for 30 sec.	Park, L.K. unpublished This paper	malate dehydrogenase, coding
Mep	F 5' GCT GTA ATG GAA TGG GCA TCC 3' R 5' AGC CTC TCC AGC TCC CTG G 3' RF R2 5' GGT TAA CTT TAT GGC ATT ATG AAG AA 3' (internal with *Mep* – F) RF F2 5' TTG GAA ACC ACA ATG CCT TC 3' RF R3E 5' CAC GGT AAA CAA TGA AGT AT CTG 3'	60 °C for 60 sec. 60 °C for 40 sec. 58 °C for 60 sec	Park, L.K. unpublished This paper This paper	malic enzyme, intron

Table 2.5. Hybrid proportions by region and species. North of Puget Sound includes the Strait of Georgia, San Juan Islands, and the Strait of Juan de Fuca.

Species and Region	**Total no. samples**	**No. of Hybrids**	**Hybrid Proportion**
S. auriculatus			
Coast	**13**	**0**	**0.00**
North of Puget Sound	0	0	
Central Puget Sound	17	4	0.24
Whidbey Basin	0	0	
Hood Canal	1	1	1.00
South Puget Sound	6	3	0.50
Salish Sea Total	24	8	0.33
S. caurinus			
Coast	12	0	0.00
North of Puget Sound	7	2	0.29
Central Puget Sound	7	3	0.43
Whidbey Basin	5	4	0.80
Hood Canal	10	5	0.50
South Puget Sound	4	3	0.75
Salish Sea Total	33	17	0.52
S. maliger			
Coast	17	2	0.12
North of Puget Sound	8	0	0.00
Central Puget Sound	6	0	0.00
Whidbey Basin	16	3	0.19
Hood Canal	5	0	0.00
South Puget Sound	5	1	0.20
Salish Sea Total	40	4	0.10

Table 2.6. Probability of equal hybrid proportion between Salish Sea and the coast from the Fisher's Exact Test. Numbers in bold signify significant two tailed test at $p<0.05$.

Species population	*S. auriculatus* Coast	*S. auriculatus* Salish Sea	*S. caurinus* Coast	*S. caurinus* Salish Sea	*S. maliger* Coast	*S. maliger* Salish Sea
S. auriculatus Coast		**0.0324**	1	**0.0007**	0.4920	0.5614
S. auriculatus Salish Sea			**0.0334**	0.1909	0.1524	**0.0435**
S. caurinus Coast				**0.00133**	0.4975	0.4975
S. caurinus Salish Sea					**0.0068**	**0.0002**
S. maliger Coast						1
S. maliger Salish Sea						

Table 2.7. Probability of equal hybrid proportions across all regions from the Fisher's Exact Test. All species are combined within region. Numbers in bold signify significant two tailed test at $p<0.05$. North of Puget Sound includes the Strait of Georgia, San Juan Islands, and the Strait of Juan de Fuca.

Region	Coastal	North of Puget Sound	Hood Canal	Central Puget Sound	Whidbey Basin	South Puget Sound
Coastal		1	**0.0003**	**0.0289**	**0.0046**	**0.0001**
North of Puget Sound			0.1134	0.4539	0.1038	0.1086
Hood Canal				0.3276	1	0.4795
Central Puget Sound					0.5291	0.0912
Whidbey Basin						0.3104
South Puget Sound						

Table 2.S1. The structure probability of ancestry output (Q) for individual tissue samples and three voucher specimens. In the 'population' column the species are coded by two letters: species (Sa – *S. auriculatus,* Sc– *S. caurinus,* Sm – *S. maliger*). The next two letters are the population code (Co – Coastal, SS – Salish Sea). The sample order here is the same left to right in Figure 4.

Sample ID	(Q) Sa	(Q) Sm	(Q) Sc	Population	Region	Hybrid (Q<0.90)
BrCoCC016	0.986	0.008	0.006	SaCo	coast	
BrCoCC017	0.987	0.007	0.006	SaCo	coast	
BrCoCC018	0.986	0.008	0.006	SaCo	coast	
BrCoCC019	0.986	0.008	0.006	SaCo	coast	
BrCoCC020	0.986	0.008	0.006	SaCo	coast	
BrCoMx002	0.987	0.007	0.006	SaCo	coast	
BrCoMx003	0.986	0.008	0.006	SaCo	coast	
BrCoMx004	0.986	0.008	0.006	SaCo	coast	
BrCoMx005	0.986	0.008	0.006	SaCo	coast	
BrCoSC011	0.986	0.008	0.006	SaCo	coast	
BrCoSC013	0.987	0.006	0.006	SaCo	coast	
BrCoSC014	0.986	0.008	0.006	SaCo	coast	
BrCoSC015	0.987	0.006	0.006	SaCo	coast	
BrCPS007	0.976	0.013	0.011	SaSS	CPS	
BrCPS010	0.986	0.008	0.006	SaSS	CPS	
BrCPS011	0.854	0.133	0.013	SaSS	CPS	hybrid
BrCPS012	0.865	0.121	0.014	SaSS	CPS	hybrid
BrCPS013	0.986	0.008	0.006	SaSS	CPS	
BrCPS015	0.986	0.008	0.006	SaSS	CPS	
BrCPS017 †	0.852	0.135	0.013	SaSS	SPS	hybrid
BrCPS018 †	0.986	0.008	0.006	SaSS	SPS	
BrCPS019 †	0.976	0.013	0.011	SaSS	SPS	
BrNPS001 †	0.986	0.008	0.006	SaSS	CPS	
BrNPS002 †	0.988	0.006	0.006	SaSS	CPS	
BrNPS003 †	0.986	0.008	0.006	SaSS	CPS	
BrNPS023 †	0.711	0.012	0.277	SaSS	CPS	hybrid
BrSPS004	0.613	0.37	0.018	SaSS	SPS	hybrid
BrSPS005	0.986	0.008	0.006	SaSS	SPS	
SaCPS008	0.737	0.256	0.007	SaSS	CPS	hybrid
SaCPS009	0.986	0.008	0.006	SaSS	CPS	
SaCPS014	0.986	0.008	0.006	SaSS	CPS	
SaCPS020	0.986	0.008	0.006	SaSS	CPS	
SaCPS021	0.986	0.008	0.006	SaSS	CPS	
SaCPS022	0.986	0.008	0.006	SaSS	CPS	
SaCPS024	0.985	0.008	0.007	SaSS	CPS	
SaHC025	0.603	0.391	0.006	SaSS	HC	hybrid
SaSPS006	0.881	0.105	0.014	SaSS	SPS	hybrid

Table 2.S1, continued.

Sample ID	(Q) Sa	(Q) Sm	(Q) Sc	Population	Region	Hybrid Q<0.90)
CoCoCC011	0.006	0.007	0.986	ScCo	Coast	
CoCoCC018	0.006	0.009	0.985	ScCo	Coast	
CoCoCC019	0.006	0.007	0.987	ScCo	Coast	
CoCoMx026	0.006	0.008	0.986	ScCo	Coast	
CoCoNC006	0.006	0.007	0.987	ScCo	Coast	
CoCoNC007	0.006	0.007	0.987	ScCo	Coast	
CoCoNC008	0.006	0.007	0.986	ScCo	Coast	
CoCoNC009	0.006	0.007	0.987	ScCo	Coast	
CoCoNC010	0.006	0.007	0.987	ScCo	Coast	
CoCoQC002	0.006	0.007	0.987	ScCo	Coast	
CoCoQC005	0.006	0.007	0.986	ScCo	Coast	
CoCoSC023	0.006	0.007	0.987	ScCo	Coast	
CoCPS049	0.006	0.007	0.987	ScSS	CPS	
CoCPS051	0.006	0.088	0.906	ScSS	CPS	
CoCPS052	0.006	0.007	0.987	ScSS	CPS	
CoCPS053	0.006	0.177	0.817	ScSS	CPS	
CoCPS054	0.026	0.03	0.944	ScSS	CPS	
CoCPS055	0.099	0.277	0.624	ScSS	CPS	
CoCPS056	0.09	0.301	0.609	ScSS	CPS	
CoHC063	0.006	0.211	0.783	ScSS	HC	
CoHC065	0.006	0.306	0.688	ScSS	HC	
CoHC066	0.007	0.007	0.986	ScSS	HC	
CoHC067	0.006	0.007	0.987	ScSS	HC	
CoHC069	0.007	0.227	0.766	ScSS	HC	
CoHC071	0.006	0.322	0.672	ScSS	HC	
CoHC073	0.009	0.297	0.695	ScSS	HC	
CoHC074	0.026	0.032	0.942	ScSS	HC	
CoHC096	0.006	0.058	0.936	ScSS	HC	
CoHC105	0.006	0.007	0.987	ScSS	HC	
CoNPS002 †	0.006	0.296	0.698	ScSS	WB	
CoNPS033 †	0.006	0.298	0.696	ScSS	WB	
CoNPS035 †	0.008	0.311	0.68	ScSS	WB	
CoNPS039 †	0.016	0.11	0.874	ScSS	WB	
CoNPS040 †	0.026	0.032	0.942	ScSS	WB	
CoNPS084	0.006	0.007	0.987	ScSS	NPS	
CoNPS085	0.006	0.053	0.941	ScSS	NPS	
CoSPS045	0.008	0.58	0.413	ScSS	SPS	
CoSPS046	0.006	0.116	0.878	ScSS	SPS	
CoSPS047	0.006	0.029	0.965	ScSS	SPS	
CoSPS048	0.009	0.326	0.665	ScSS	SPS	
ScJF079 †	0.006	0.006	0.987	ScSS	NPS	
ScJF080 †	0.006	0.006	0.987	ScSS	NPS	
ScJF087 †	0.007	0.102	0.891	ScSS	NPS	
ScNPS081	0.006	0.058	0.935	ScSS	NPS	
ScNPS082	0.006	0.29	0.704	ScSS	NPS	

Table 2.S1, continued.

Sample ID	(Q) Sa	(Q) Sm	(Q) Sc	Population	Region	Hybrid (Q<0.90)
UW 113205	0.146	0.461	0.394	ScSS voucher	atypical specimen 'A'	
UW 113206	0.01	0.491	0.499	ScSS voucher	atypical specimen ' B'	
UW 47319	0.898	0.095	0.007	ScSS voucher	atypical specimen 'C'	
QuCoNC001	0.007	0.987	0.006	SmCo	Coast	
QuCoNC002	0.008	0.985	0.007	SmCo	Coast	
QuCoNC003	0.008	0.985	0.007	SmCo	Coast	
QuCoNO011	0.008	0.982	0.01	SmCo	Coast	
QuCoPW026	0.006	0.987	0.006	SmCo	Coast	
QuCoQC022	0.006	0.559	0.435	SmCo	Coast	
QuCoSA024	0.007	0.987	0.007	SmCo	Coast	
QuCoSA025	0.006	0.987	0.006	SmCo	Coast	
QuCoSO004	0.008	0.985	0.007	SmCo	Coast	
QuCoSO005	0.007	0.986	0.007	SmCo	Coast	
QuCoSO006	0.008	0.985	0.007	SmCo	Coast	
QuCoSO007	0.008	0.972	0.02	SmCo	Coast	
QuCoSO008	0.007	0.985	0.008	SmCo	Coast	
QuCoSO009	0.007	0.984	0.008	SmCo	Coast	
QuCoWV012	0.008	0.972	0.02	SmCo	Coast	
QuCoWV013	0.007	0.985	0.008	SmCo	Coast	
QuCoWV019	0.007	0.53	0.464	SmCo	Coast	
QuCPS187	0.008	0.979	0.013	SmSS	CPS	
QuCPS188	0.007	0.985	0.008	SmSS	CPS	
QuCPS189	0.008	0.985	0.008	SmSS	CPS	
QuCPS191	0.008	0.985	0.007	SmSS	CPS	
QuCPS192	0.008	0.985	0.007	SmSS	CPS	
QuCPS193	0.007	0.984	0.008	SmSS	CPS	
QuHC243	0.008	0.985	0.008	SmSS	HC	
QuHC244	0.008	0.986	0.007	SmSS	HC	
QuHC251	0.008	0.984	0.008	SmSS	HC	
QuHC252	0.008	0.984	0.008	SmSS	HC	
QuHC253	0.008	0.986	0.007	SmSS	HC	
QuNPS102	0.007	0.974	0.019	SmSS	NPS	
QuNPS103	0.008	0.986	0.007	SmSS	NPS	
QuNPS104	0.008	0.983	0.009	SmSS	NPS	
QuNPS105	0.008	0.983	0.009	SmSS	NPS	
QuNPS158 †	0.008	0.981	0.011	SmSS	WB	
QuNPS159 †	0.008	0.983	0.009	SmSS	WB	
QuNPS160 †	0.008	0.985	0.008	SmSS	WB	
QuNPS161 †	0.249	0.743	0.008	SmSS	WB	
QuNPS163 †	0.008	0.985	0.007	SmSS	WB	
QuNPS164 †	0.249	0.743	0.008	SmSS	WB	
QuNPS167 †	0.008	0.985	0.008	SmSS	WB	
QuNPS168 †	0.007	0.986	0.006	SmSS	WB	

Table 2.S1, continued.

Sample ID	(Q) Sa	(Q) Sm	(Q) Sc	Population	Region	Hybrid (Q<0.90)
QuNPS169 †	0.008	0.986	0.007	SmSS	WB	
QuNPS170 †	0.008	0.985	0.008	SmSS	WB	
QuNPS171 †	0.008	0.984	0.008	SmSS	WB	
QuNPS172 †	0.007	0.985	0.007	SmSS	WB	
QuNPS173 †	0.007	0.784	0.209	SmSS	WB	
QuNPS174 †	0.008	0.983	0.01	SmSS	WB	
QuNPS175 †	0.008	0.986	0.006	SmSS	WB	
QuNPS176 †	0.008	0.986	0.007	SmSS	WB	
QuSPS178	0.008	0.985	0.007	SmSS	SPS	
QuSPS179	0.008	0.981	0.011	SmSS	SPS	
QuSPS180	0.008	0.98	0.013	SmSS	SPS	
QuSPS181	0.008	0.761	0.23	SmSS	SPS	
QuSPS182	0.007	0.981	0.012	SmSS	SPS	
SmJF018 †	0.007	0.987	0.007	SmSS	NPS	
SmNPS002	0.008	0.985	0.007	SmSS	NPS	
SmNPS003	0.007	0.986	0.007	SmSS	NPS	
SmNPS005	0.007	0.985	0.008	SmSS	NPS	

† These sample names were used in the laboratory but here have some variation on region codes. The final codes used in the analyses are in 'region' columns.

Table 2.S2. *Structure* likelihood values.

	k=1	k=2	k=3	k=4	k=5	k=6	k=7
LnP(D)	-7226.9	-5328.9	-4269.1	-4447.5	-5240.2	-4881.8	-6537.2