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MICROSATELLITE ANALYSIS ON PACIFIC LAMPREY ALONG THE WEST COAST OF NORTH AMERICA

Executive Summary

- This project used nine newly-developed microsatellite markers to determine if there is broad-scale population structure in Pacific lamprey populations.
- It expanded on a previous study which applied these markers to nine Pacific lamprey populations from British Columbia, Washington State, Oregon, and California.
- The current project provided funding for five additional sites within California; seven additional sites in British Columbia, Washington State, Oregon, and California were included with funding from the Docker laboratory at the University of Manitoba.
- A total of 965 Pacific lampreys were genotyped from these 21 sites.
- A total of nine sites were included from California: the Trinity and Klamath rivers (Klamath drainage); the South Fork Eel River (Eel drainage); Mill and Tuolumne rivers (Sacramento drainage); Gualala River (Gualala drainage); Penitencia River (Coyote drainage); Arroyo Seco (Salinas drainage); and San Luis Obispo (San Luis Obispo drainage).
- Levels of genetic differentiation among locations were low, providing support for a lack of natal homing in Pacific lampreys. Although some pairwise F_{ST} values were significant and/or large, most were below 0.05. F_{ST} values for anadromous fish known for their homing abilities (e.g., sockeye salmon) generally are above 0.05.
- In the current study, the vast majority (93%) of the F_{ST} values above 0.05 involved comparisons among only five of the 21 sites: Chewuch (2008 and 2009), Salmon, and Duckabush rivers in Washington State, and Mill Creek in California.
- One possible explanation for the high degree of genetic differentiation between these sites and many of the other sites is a small number of spawning adults at these sites or water level fluctuations that wipe out all by a localized – and potentially – related number of ammocoetes. In these cases, differences in allele frequencies between locations would be due to sampling effects, not reproductive isolation.
- Temporal variation (between years and seasonally) was examined at three sites (Klamath, Willamette, and Chewuch rivers); for the Klamath and Willamette rivers, F_{ST} values were non-significant but for the Chewuch River, samples collected in 2008 and 2009 were significantly genetically differentiated with the maximum F_{ST} value observed among all comparisons (0.2569). As discussed above, variation between years in the Chewuch River may be due to small population sizes.
- It appears that Pacific lampreys from most of the sites examined in this study can be managed as one unit; hopefully, future investigations will indicate whether this is true for all sites.

Background

The Pacific lamprey, *Entosphenus tridentatus*, is an anadromous jawless fish that lives along the west coast of North America from Alaska to California (Morrow 1980; Rohde 1980; Ruiz-Campos and Gonzalez-Guzman 1996) and along the Pacific coast of Asia as far south as Japan (Scott and Crossman 1973). Like all lampreys, it spends

several years as a filter-feeding ammocoete larva in fresh water before undergoing metamorphosis (Pletcher 1963; Kan 1975; Richards 1980). After metamorphosis, Pacific lampreys migrate to the ocean and feed parasitically on fish. They remain in the ocean for several years before returning to fresh water to spawn and die (Beamish 1980).

In general, anadromous species of fish home to their natal streams (McDowall 2001). Anadromous salmon, for example, use olfactory cues to locate their natal streams. This leads to reproductive isolation of spawning populations (Dittman and Quinn 1996) and a high degree of genetic structure (Kitanishi *et al.* 2009). In contrast, there is increasing evidence that migratory lampreys do not home. Tagging studies have indicated that landlocked sea lampreys (*Petromyzon marinus*) in the Great Lakes do not return to their natal streams to spawn (Bergstedt and Seelye 1995). Anadromous lampreys such as the anadromous sea lamprey, the Southern Hemisphere pouched lamprey (*Geotria australis*), and the Arctic lamprey (*Lethenteron camtschaticum*) have low levels of genetic differentiation among locations (Bryan *et al.* 2005; Johnston *et al.* 1987; Docker 2006, respectively), likewise suggesting an absence of homing to natal streams.

Instead of migrating to natal streams, sea lampreys appear to migrate to streams currently containing ammocoete larvae. This is referred to as the "suitable river strategy" (Waldman *et al.* 2008). Sea lamprey larvae produce pheromones that attract adult lampreys during the migratory phase (Li *et al.* 1995; Vrieze and Sorensen 2001). Nine other species of lamprey from four genera, including the Pacific lamprey, also have been shown to produce and respond to larval pheromones (Fine *et al.* 2004). This sensitivity to larval pheromones seems to indicate that Pacific lampreys, like sea lampreys, migrate to streams containing larvae. As well, some genetic studies suggest the absence of natal homing in Pacific lamprey. Goodman *et al.* (2008) surveyed mitochondrial DNA (mtDNA) haplotype variation in Pacific lampreys from 81 locations from southern British Columbia to southern California and found a lack of genetic differentiation among locations, suggesting panmixia.

Debate continues, however, regarding the migratory strategies and resulting population structure of Pacific lampreys. Reports of size differences among different river systems (e.g., Beamish 1980; Kostow 2002), for example, have led to suggestions that there may be some local adaptation and reproductive isolation among Pacific lampreys from different locations. Large body size is observed in upstream-migrating Pacific lampreys from larger, more interior rivers like the Columbia River (where migration distances are long), whereas Pacific lampreys in coastal streams tend to be smaller (Kan 1975; Kostow 2002). Relatively large Pacific lampreys have also been reported in the Fraser and Skeena rivers of British Columbia (Beamish 1980). Furthermore, some genetic studies also have indicated the presence of significant genetic differentiation among locations. Beamish and Withler (1986) found small but significant differences in allozyme allele frequencies among Pacific lampreys from two rivers. Using amplified fragment length polymorphism (AFLP) analysis, Lin *et al.* (2008) likewise found significant differences in AFLP variation in Pacific lampreys from eight sites in the Pacific Northwest, Alaska, and Japan. In addition, although Goodman *et al.* (2008) concluded that there is high gene flow among Pacific lampreys along the west coast, they found a high (about 30%) but non-significant frequency of a rare haplotype in the Fraser River; this haplotype was found in less than 1% of the Pacific lampreys from other locations. This suggests that there may be some degree of reproductive isolation between lampreys spawning in the Fraser River and those spawning in other locations.

However, allozymes, AFLPs, and mitochondrial DNA may not provide the resolution required to sufficiently study population structure in Pacific lampreys. Microsatellites are the marker of choice for detecting population structure in closely related populations (Sunnucks 2000). Microsatellites combine information from many loci, which increases resolution and decreases error compared to mtDNA analysis with one or a few genome segments (Selkoe and Toonen 2006). Up until recently, however, Pacific lamprey microsatellite markers were not available for use. Microsatellite loci developed for the sea lamprey and the threespine stickleback (*Gasterosteus aculeatus*) generally failed to amplify in Pacific lamprey or had low polymorphism. Only two microsatellite loci had sufficient polymorphism to be used in Pacific lamprey, severely reducing the resolution of the analysis (Howard and Close 2003).

Purpose/Objectives

Nine polymorphic microsatellite loci have now been developed for the Pacific lamprey; these markers were developed in the Docker laboratory at the University of Manitoba with funding secured by Dr. Timothy Whitesel (USFWS–Columbia River Fisheries Program Office; Region 1). Erin Spice, an undergraduate Honours student at the University of Manitoba, applied these microsatellite loci to Pacific lampreys from nine sampling locations ranging from southern British Columbia to southern California (Spice 2010), using a subset of the lampreys previously used for mtDNA analysis by Goodman et al. (2008). The current project expands on this study by including Pacific lampreys from 12 additional sites; five more sites in California were included with funding from the current USFWS project and seven more sites in British Columbia, Washington State, Oregon, and California were included with funding from Docker's lab (Table 1).

Application of these higher-resolution markers will help to clarify the population structure of Pacific lampreys and inform any future management decisions. The presence or absence of homing to natal streams or other forms of reproductive isolation in Pacific lampreys may be inferred from the degree of genetic differentiation among lampreys from different locations. The null hypothesis for this study is that Pacific lampreys along the west coast of North America are a panmictic population; that is, individuals from all locations interbreed freely and there is no significant genetic differentiation at microsatellite loci among locations. The alternative hypothesis is that lampreys from different locations make up separate populations with significant genetic variation, indicating that there are barriers to gene flow among locations.

Significance of Research

Understanding the migratory strategy and population structure of Pacific lampreys is important in making conservation and management decisions. Since the 1960s, the number of Pacific lampreys observed at dams along the west coast of the United States has declined dramatically. In 2004, however, the United States Fish and Wildlife Service declined to list Pacific lampreys as endangered or threatened (United States Fish and Wildlife Service 2004), in part due to the lack of information on population structure in this species. Without information on population structure, it is impossible to determine whether Pacific lampreys should be subdivided into several management units along the Pacific coast, or managed as a single unit (United States Fish and Wildlife Service 2004). If all groups of Pacific lampreys

interbreed freely, managing Pacific lampreys as one unit is appropriate; if groups that spawn in different locations are reproductively isolated, it may be necessary to manage them as separate units.

Information on Pacific lamprey population structure is also important for restocking of streams where lamprey populations have declined, as any introduced lampreys must be able to interbreed freely with the existing population and should be locally adapted. Furthermore, knowledge of Pacific lamprey migratory strategies is important to understand how habitat loss is likely to affect Pacific lamprey populations. Dam construction and other forms of habitat disturbance contribute to the decline in Pacific lamprey populations (Close *et al.* 2002) by impeding migration and resulting in the loss of ammocoete habitat (e.g., through fluctuations in water levels) in a particular area (Moser and Close 2003; Luzier *et al*. 2009). If Pacific lampreys migrate to areas containing larvae based on pheromonal cues, they may migrate to another suitable area with larvae; however, if migration to natal streams occurs, habitat loss may result in the loss of spawning populations.

Methods

For this study, lampreys from 21 sites in British Columbia, Washington, Oregon, and California were collected by several sources (Table 1). These sites comprised a subset of the sites sampled by Goodman *et al.* (2008), as well as seven additional sites. Sites for analysis were chosen to cover a wide geographic area (Figure 1) and to include locations where previous studies had shown potential genetic differentiation (e.g., the Deadman and Deschutes rivers; Goodman *et al.* 2008 and Lin *et al.* 2008, respectively) and other key locations (e.g., the Klamath and Sacramento rivers).

DNA was extracted from all of the 965 samples for this study using the Wizard® Genomic DNA purification kit and following the manufacturer's instructions. Nine microsatellite primers developed or optimized for Pacific lamprey (Spice *et al.*, submitted to Genetic and Molecular Research; Table 2) were labeled with 6-Fam and Hex (Sigma Life Science) and Ned and Pet (Applied Biosystems) fluorescent dyes and used to amplify loci using PCR. Thermal cycler conditions were an initial denaturation at 94°C for two minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at temperatures ranging from 51 to 62°C (Spice *et al.* submitted) for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 5 minutes.

Microsatellite fragments were size fractionated using the ABI 3130 Genetic Analyzer (Applied Biosystems), and allele sizes were determined using GeneMapper v4.0 (Applied Biosystems).

GENEPOP v. 4.0.10 (Raymond and Rousset 1995; Rousset 2008) was used to enumerate alleles and calculate observed and expected heterozygosity for each locus. Tests of Hardy-Weinberg Equilibrium (HWE) were not performed due to the large number of samples; tests of HWE on a smaller number of samples can be found in Spice *et al.* (submitted). The genetic distance between locations was determined using GENEPOP to estimate Weir and Cockerham's (1984) $F_{ST.}$ FSTAT v. 2.3.9.2 (Goudet 2001) was used to test the significance of pairwise values of F_{ST} (Raymond and Rousset 1995). P-values were generated for each pairwise measure of F_{ST} and compared to a strict Bonferroni corrected p-value to determine significance (Rice 1989). An initial comparison was made between samples collected from the same site in different years. Samples were collected from the

Klamath River (site 14) in 2007 and 2008, from the Willamette River (site 8) in early and late 2008, and from the Chewuch River (sites 3 and 4) in 2008 and 2009. For the Klamath and Willamette Rivers, F_{ST} values were non-significant and below 0.05; samples collected from these rivers at different times were therefore considered as one group. For the Chewuch River, samples collected in 2008 and 2009 were significantly genetically differentiated with an F_{ST} value greater than 0.05; these samples were therefore considered as two groups. All further analysis was performed with the sample sites as listed in Table 1.

The program STRUCTURE v. 2.3.1 (Pritchard *et al.* 2000) was used to estimate the number of populations present along the west coast of North America. Probabilities of one to 25 populations being present among the entire sample set were calculated using the admixture model with allele frequencies correlated and with sampling location information included. The probability of one population being present represents the probability that all Pacific lampreys sampled belong to a single panmictic population (i.e., the probability that the null hypothesis is correct). Although only 21 sites were sampled, the probabilities of up to 25 populations being present were calculated in order to allow for the possibility that some sites sampled contained two or more reproductively isolated populations.

A neighbor-joining phylogenetic tree was constructed using POPULATIONS v. 1.2.30 (O. Langella, Centre National de la Recherche Scientifique, Laboratoire Populations, Génétique et Evolution, Gif sur Yvette, France, available from http://bioinformatics.org/~tryphon/populations/). The tree was constructed using 500 bootstrap replications and Cavalli-Sforza and Edwards' (1967) chord distance, which is an accurate method of constructing phylogenetic trees using microsatellite data from closely related groups (Angers and Bernatchez 1998). Bootstrap replications were performed on loci. This tree was visualized in TREEVIEW v. 1.6.6 (Page 1996).

Results

F_{ST} values between sites ranged from -0.0086 (essentially zero; between site 5, Pilchuck River, and site 10, Siletz River) to 0.2569 (between site 3, Chewuch River 2009, and site 4, Chewuch River 2008; Table 3). A total of 119 of the 210 F_{ST} values (57%) were statistically significant. For most pairwise comparisons, however, the F_{ST} values were low. Of the 210 pairwise comparisons, 135 (64%) had F_{ST} values below 0.05; 46 (22%) had F_{ST} values between 0.05 and 0.0999; 16 (7.6%) had F_{ST} values between 0.10 and 0.1499; and 13 (6.2%) had F_{ST} values above 0.15. Sites 3 and 4 (Chewuch), site 6 (Salmon), site 7 (Duckabush), and site 16 (Mill) each had several F_{ST} values above 0.05. Of the 75 F_{ST} values above 0.05, 70 (93%) were generated by comparisons with these five sites.

Within California, 18 of 36 (50%) pairwise comparisons were significant; six of these were generated by comparisons with Mill Creek. Pairwise F_{ST} values within California ranged from -0.0029 (essentially zero; between Gualala River, site 17, and Klamath and S.F. Eel river, sites 14 and 15, respectively) to 0.0982 (between Trinity River, site 13, and Mill Creek); however, when Mill Creek is excluded, the largest pairwise F_{ST} value is 0.0333 (between Arroyo Seco and San Luis Obispo rivers, sites 20 and 21). Comparing sites within California to sites outside of California, 56 of 108 (51.9%) of pairwise comparisons were significant, but F_{ST} values for 70 of 108 (64.8%) comparison were below 0.05. Pairwise F_{ST} values comparing sites within California to sites outside of California ranged from -0.0079

(between Salmon River, site 6, and Mill Creek) to 0.2245 (between Chewuch 2008, site 4, and Trinity River).

STRUCTURE analysis indicated that Pacific lampreys along the west coast of North America mostly likely form three population clusters (Table 4). Cluster 1 contains the majority of individuals at all sites except site 3 (Chewuch River) and site 5 (Pilchuck River). The individuals from Chewuch River 2009 were roughly evenly distributed between cluster 1 (43.8%) and cluster 2 (54.4%), with only a very small proportion (1.8%) in cluster 3. The majority of the individuals from Pilchuck River (81.9%) grouped in cluster 2, while 17.6% and 0.5% grouped in clusters 1 and 3, respectively. The only sites with more than 20% belonging to cluster 3 were 10, 11, and 15 (Siletz, S.F. Umpqua, and S.F. Eel rivers). Within California, the majority of individuals in all sites grouped in cluster 1, with percentages ranging from 64.5% (Penitencia Creek, site 19) to 97.6% (Tuolomne River, site 18).

An unrooted neighbor-joining tree showed no strong tendency for Pacific lampreys to group with geographically close populations (Figure 2). Only two groupings had greater than 50% bootstrap support: sites 6 and 16 (Mill Creek and Salmon River; 57%) and sites 2 and 7 (Deadman River and Duckabush River; 93%).

Discussion

Low genetic differentiation among most locations supports lack of natal homing in Pacific lamprey

The results of this study indicate that Pacific lampreys at most sites along the west coast of North America are not highly genetically differentiated from each other. F_{ST} values measure the genetic distance between two groups, and range from 0 to 1 (Selkoe and Toonen 2006). An F_{ST} value of 0 indicates a completely panmictic population with zero percent genetic divergence, whereas an F_{ST} value of 1 indicates complete reproductive isolation with fixation of different alleles (Balloux and Lugon-Moulin 2002). F_{ST} values for sockeye salmon, known for their homing ability (Dittman and Quinn 1996), within the Pacific Rim ranged from 0.027 to 0.160, with the majority of values above 0.05; values comparing sockeye salmon along the Pacific coast to those in Hokkaido Island, Japan, were as high as 0.310 (Beacham *et al.* 2006). In contrast, in the anadromous sea lamprey, which does not appear to home to natal streams (Waldman *et al.* 2008), pairwise θ_P (analogous to F_{ST}) values for locations along the Atlantic coast of North America ranged from below 0.01 to 0.02 (Bryan *et al.* 2005). Thus, for this study, we considered F_{ST} values below 0.05 to be low, values from 0.05 to 0.0999 to be moderate, values from 0.10 to 0.1499 to be high, and values 0.15 or greater to be very high. Although 57% of the pairwise comparisons for Pacific lamprey were statistically significant, the majority (64%) show low levels of differentiation, i.e. F_{ST} values less than 0.05. This supports the hypothesis that Pacific lampreys do not home to their natal streams, as homing results in significant reproductive isolation among groups spawning at different sites (Dittman and Quinn 1996).

However, five of 21 sites (four in Washington State and one in California) accounted for 93% of the F_{ST} values over 0.05, and require further analysis. The five sites that accounted for most of the F_{ST} values indicating moderate or high genetic differentiation were Chewuch River (2009, site 3 and 2008, site 4), Salmon River (site 6), Duckabush River (site 7), and Mill Creek (site 16). One possible explanation for the high degree of genetic differentiation between these sites and many of the other sites is a small number of spawning

adults at these sites. When the number of spawning adults is small, differences in allele frequencies between spawning locations are likely to be observed simply due to a sampling effect, not due to reproductive isolation (Allendorf and Phelps 1981). This would occur even when fish return to spawning areas "at random," with no correlation between natal site and spawning site. Similarly, water level fluctuations may destroy ammocoete habitat (Luzier *et al.* 2009) in all but a few localized areas; if ammocoetes surviving in these areas include the offspring of only a few parents, it could make Pacific lampreys from these sites appear to more genetically distinct from other sites than truly represents the group of spawners at that site. A low number of spawning adults or water level fluctuations may be attributable, at least in part, to the occurrence of hydroelectric dams on large rivers such as the Columbia River (Close *et al.* 2002; Moser *et al.* 2002). Low migration success caused by hydroelectric dams and/or low recruitment caused by water level fluctuations might account for the large genetic differentiation between lampreys sampled at Chewuch River in 2008 and those sampled in 2009; temporal variation was not observed at any other sites sampled in more than one year (e.g., the Trinity and mainstem Klamath rivers). Mill Creek, another of the sites which showed high and significant F_{ST} values when compared to other locations, is also far upstream in the Sacramento River. However, it is important to note that not all upstream sites (e.g., the Deschutes and Tuolomne rivers, sites 9 and 18) were highly genetically differentiated from other sites, and the Salmon and Duckabush rivers – although they were genetically differentiated from most other sites – are not affected by hydroelectric dams. Further investigation of the factors that may cause lampreys from some rivers to be particularly genetically distinct is necessary.

The results of the current study may correspond somewhat to those of Beamish *et al.* (1986). They examined allele frequencies in Pacific lampreys from two rivers in British Columbia using 17 allozyme loci. Pacific lampreys from the Fraser and Stamp rivers had significant differences in allele frequencies at two allozyme loci. Although these differences were significant, the difference in allele frequencies was not particularly large, and there were three loci at which allele frequency differences were not significant. It is difficult to compare microsatellite and allozyme data; however, Beamish and Withler's (1986) results seem to correspond to the slight but significant differences found for some pairwise comparisons in this study. In comparing the results of the current study with those of Lin *et al.* (2008) using AFLPs, most of the F_{ST} values are smaller in this study. In particular, both studies used samples from the Willamette, Deschutes, and Klamath rivers. For Lin *et al.* (2008), F_{ST} values for comparisons among these sites were 0.151 (Willamette and Deschutes), 0.05 (Willamette and Klamath), and 0.138 (Deschutes and Klamath). The corresponding values in this study were 0.0031, -0.0035 (essentially zero), and 0.001, all indicating very low genetic differentiation. In Lin *et al.* (2008), STRUCTURE analysis showed that these three sites grouped separately; in contrast, this study grouped all three sites in cluster 1. The different molecular techniques used to examine population structure in Pacific lamprey may provide an explanation for the differing results obtained. In particular, the use of presumably neutral markers (e.g., microsatellites) versus those that are potentially under selection (e.g., AFLPs) may affect estimates of genetic differentiation. AFLPs may give generally higher estimates of genetic differentiation than microsatellites. Some other species that have been studied using both AFLPs and microsatellites have had higher F_{ST} values with AFLPs: for example, the mosquito *Aedes aegypti* (Paupy *et al.* 2004) and the alpine sea holly plant *Eryngium alpinum* (Gaudeul *et al.* 2004). In addition, Campbell *et al.*

(2003) found that in the lake whitefish (*Coregonus clupeaformis*), a species with weak population structure, AFLPs were more accurate at assigning individuals to putative populations. As well, AFLPs are amplified from anywhere in the genome without prior knowledge of genomic sequence (Meuller and Wolfenbarger 1999; Meudt and Clarke 2007); thus, they have the potential to be functional loci. Some of the AFLP loci used by Lin *et al.* (2008) may have been under selection themselves or may have been linked to loci under selection. Functional loci may be able to demonstrate genetic differences in the lampreys that successfully migrated to spawning areas, or their offspring, due to selection on these loci without homing to natal streams. In contrast, allele frequencies at presumably neutral markers, such as mtDNA and microsatellites, are assumed to be determined only by gene flow.

STRUCTURE analysis indicated the likely presence of three population clusters. If Pacific lampreys returned to their natal streams to spawn with high fidelity, one would expect each location to form a separate breeding population, as in salmonids (Dittman and Quinn 1996). Since this does not appear to occur, it seems to indicate that Pacific lampreys do not return to their natal streams. However, the presence of more than one population cluster suggests that there are some barriers to gene flow among locations. Cluster 1 contained the majority of individuals from all sites except Chewuch River 2009 (site 3) and Pilchuck River (site 5). I suggest that cluster 1 represents the majority of the Pacific lamprey population, which appears to be panmictic. Clusters 2 and 3 may represent groups that have some barriers to gene flow with the main population and with each other. For example, in the Chewuch River, which is in the upper Columbia River drainage, it is possible that there are some freshwater-resident forms, although no such forms have been identified to date (John Crandall, Wild Fish Conservancy, pers. comm.). The lampreys grouping in cluster 2 at Chewuch and/or Pilchuck rivers represent other freshwater-resident derivatives of the Pacific lamprey; however, Pilchuck River did not have any F_{ST} values higher than 0.05 and very few pairwise comparisons were statistically significant, making the presence of a freshwater resident form unlikely. Cluster 3 did not contain a majority of individuals from any sites, and only three sites had more than 20% of the population grouped into cluster 3. It is uncertain what cluster 3 represents biologically. Further analysis, using different settings of the STRUCTURE program, may help to show whether or not these clusters are legitimate, consistent groupings.

The neighbor-joining tree showed no strong trend for geographically close sites to group together. Only Deadman (site 2) and Duckabush (site 7) rivers clustered closely together, with 93% bootstrap support. However, the nearby sites 5 and 6 (Pilchuck and Salmon rivers) did not group with sites 2 and 7. Goodman *et al.* (2008) found that 12 of 41 lampreys (29%) genotyped from Deadman River and two of 10 from Duckabush River (20%) had the rare RFLP haplotype 22. This haplotype was found in only four of the 1195 (0.3%) lampreys from other sites. Interestingly, none of the 10 lampreys genotyped from Pilchuck River or the 20 lampreys genotyped from Salmon River had this haplotype, illustrating congruence between the microsatellite and mictochondrial DNA analyses. It would be valuable to perform mictochondrial DNA analysis on the remaining lampreys sampled from Duckabush, Pilchuck, and Salmon rivers (and used in this study) to determine if this pattern remains when the sample size is increased. Both Deadman and Duckabush rivers eventually drain into the Strait of Juan de Fuca. It is possible that Vancouver Island is a barrier to gene flow, making it less likely that lampreys from rivers emptying into the Strait

of Juan de Fuca will enter the wider Pacific Ocean and, later, spawn in streams in other locations along the Pacific coast. Lampreys from Salmon River migrate downstream into the main Pacific Ocean and may be free to interbreed with lampreys from all other locations along the west coast. Pilchuck River also empties into the Strait of Juan de Fuca, but, as mentioned above, there may be a freshwater-resident form in Pilchuck River, or there may be another factor that causes it to group separately from other locations in the same area. Lampreys from different rivers in the same drainage were not grouped together on the neighbor-joining tree. Chewuch, Willamette, and Deschutes rivers (Columbia River drainage) did not group together, nor did Trinity and Klamath rivers (Klamath River drainage), nor Mill Creek and Tuolomne River (Sacramento River drainage). If Pacific lampreys homed to their natal streams, one would expect that sites that are geographically close or in the same drainage would group together. The lack of this trend provides further evidence that Pacific lampreys do not home to their natal streams.

Conservation and management implications

This study shows that there is little genetic differentiation among Pacific lampreys in different locations and suggests that Pacific lampreys do not home to their natal streams. Pacific lamprey numbers have declined dramatically since the 1960s (Close *et al.* 2002), and the absence of natal homing has several implications for conservation and management of this species.

The absence of natal homing indicates that a lamprey does not have to have hatched at a particular site to spawn successfully at that site. This bodes well for translocations; however, if juvenile lampreys are translocated to a particular site, there is no guarantee that they will return to that site to spawn. Larval pheromones appear to be the major factor influencing spawning site selection in lampreys. Translocations of ammocoetes to streams where lampreys have been extirpated may be important in establishing a spawning site: not because these ammocoetes will necessarily return to the site to spawn, but because they will produce pheromones that will attract adult lampreys. Larval pheromones have been used to attract invasive sea lampreys into traps (Wagner *et al.* 2006); it is possible that they may also be used to attract desired species such as the Pacific lamprey to potential spawning grounds. In an attempt to reintroduce Pacific lamprey to the Umatilla River, sexually mature adults were released at several locations within the river between 1999 and 2007 (Close *et al.* 2009). These adults successfully spawned and produced offspring. From 2004 to 2007, the number of upstream migrants entering the Umatilla River was higher than it was prior to reintroduction; however, fewer than 20 upstream migrants were caught in all years. This slight increase in upstream migrants could be interpreted as due to natural variation, natal homing of offspring, or attraction of adults by larval pheromones (Close *et al.* 2009); however, other studies indicating the importance of larval pheromones suggest that, after several years of reintroductions, larval density in the Umatilla River had reached a level sufficient to attract adults. This indicates that translocation of spawning-phase adults into suitable habitat may allow the re-establishment of natural spawning sites. Modifications to fishways to allow more efficient lamprey passage and control of water levels in important ammocoete habitat may also be valuable in conservation efforts.

In order to properly manage and conserve a species, units for management must be clearly defined. Moritz (1994) defines management units as "populations with significant divergence of allele frequencies at nuclear or mitochondrial loci." For most locations,

although some pairwise F_{ST} values were statistically significant, they never exceeded 0.05 (indicating low genetic differentiation). Some sites, notably Chewuch River, Salmon River, Duckabush River, and Mill Creek, had higher F_{ST} values, indicating moderate to high genetic differentiation. The reasons for higher genetic differentiation at these sites are still uncertain; thus, it would be valuable to re-sample these sites in other years and compare temporal and geographic variation. As well, genetic information should be combined with information on water levels, number of spawning adults, dams, and the possibility of freshwater-resident forms in these rivers. As STRUCTURE analysis grouped most of the sites analyzed in this study as one population, it is probably appropriate to manage Pacific lampreys as one unit. However, special attention should be paid to highly differentiated sites in order to ascertain that this is indeed the correct course of action.

Conclusions

This study supports the lack of natal homing in Pacific lampreys. Although some pairwise F_{ST} values were significant and/or large, most were below 0.05, and several "anomalous" sites were responsible for the vast majority of the high F_{ST} values. STRUCTURE analysis indicates that one population cluster contained the majority of individuals from 19 of 21 sites. A neighbor-joining tree indicated no strong trend for sites that are geographically close or in the same drainage to group together. Future studies should pay special attention to sites that were identified as highly genetically differentiated in this study. Pacific lampreys from most of the sites examined in this study can probably be managed as one unit; hopefully, future investigations will indicate whether this is true for all sites.

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Table 1. Locations and collection information of 21 sites where Pacific lampreys (*Entosphenus tridentatus*) were collected. S.F. = South Fork; W.F. = West Fork. Site numbers correspond to those in Figures 1 and 2. In addition to using lampreys collected for the mtDNA study by Goodman *et al.* (2008), specimens from other locations were provided by Eric Taylor (University of British Columbia), John Crandall (Wild Fish Conservancy), Benjamin Clemens (Oregon State University), Gregory Silver (USFWS), and Damon Goodman (USFWS). * indicates sites included in initial study by Spice (2010).

River	Drainage	State	Site number	Latitude	Longitude	Date(s) collected	Number	Life stage	Provided by
Nass	Nass	BC	1	54.978	-129.889	2008-06-14 to 2008-08-06	43	Adults	Eric Taylor
Deadman*	Thompson	BC	2	50.750	-120.917	1995-10-04 to 1995-10-07	30	Ammocoetes	Goodman *et al.* (2008)
Chewuch	Columbia	WA	3	48.640	-120.152	2009-08-25 to 2009-08-26	20	Ammocoetes	John Crandall
Chewuch	Columbia	WA	4	48.640	-120.152	2008-07-29 to 2008-09-08	29	Ammocoetes	John Crandall
Pilchuck*	Snohomish	WA	5	47.942	-122.075	2004-07-13	50	Ammocoetes	Goodman *et al.* (2008)
Salmon	Queets	WA	6	47.789	-123.608	2004-07-15	46	Ammocoetes	Goodman *et al.* (2008)
Duckabush*	Duckabush	WA	7	47.655	-122.948	2004-07-14	48	Ammocoetes	Goodman *et al.* (2008)
Willamette	Columbia	OR	8	45.353	-122.619	2008-04-30 to 2008-08-18	50	Adults	Benjamin Clemens
Deschutes	Columbia	OR	9	45.248	-121.046	2010	48	Ammocoetes	Gregory Silver
Siletz*	Siletz	OR	10	44.764	-123.915	2004-05-26	48	Ammocoetes	Goodman *et al.* (2008)
S.F. Umpqua*	Umpqua	OR	11	43.212	-123.349	2003-10-21	50	Ammocoetes	Goodman *et al.* (2008)
W.F. Illinois*	Rogue	OR	12	42.152	-123.660	2003-10-20	51	Ammocoetes	Goodman *et al.* (2008)
Trinity	Klamath	CA	13	41.583	-124.050	2004, 2005, 2010-07-01	78	Ammocoetes	Damon Goodman
Klamath	Klamath	CA	14	41.547	-124.084	2007, 2008	37	Adults	Benjamin Clemens
S.F Eel	Eel	CA	15	40.342	-123.941	2004-06-12	49	Ammocoetes	Goodman *et al.* (2008)
Mill	Sacramento	CA	16	40.046	-122.094	2005-03-14	48	Ammocoetes	Goodman *et al.* (2008)
Gualala*	Gualala	CA	17	38.774	-123.505	2004-06-21	50	Ammocoetes	Goodman *et al.* (2008)
Tuolumne	Sacramento	CA	18	37.647	-120.494	2004-11-24	50	Ammocoetes	Goodman *et al.* (2008)
Penitencia*	Coyote	CA	19	37.394	-121.833	2004-06-22	40	Ammocoetes	Goodman *et al.* (2008)
Arroyo Seco	Salinas	CA	20	36.281	-121.323	2004-06-25	50	Ammocoetes	Goodman *et al.* (2008)
San Luis Obispo*	San Luis Obispo	CA	21	35.280	-120.665	2004-06-23	50	Ammocoetes	Goodman *et al.* (2008)

Table 2. Characteristics of nine microsatellite loci developed for Pacific lamprey (*Entosphenus tridentatus*), and tested in 965 individuals from 21 sites along the west coast of North America. Lri primers were developed in western brook lamprey (*Lampetra richardsoni*) by Luzier *et al.* (2010) and optimized for use in Pacific lamprey by Spice *et al.* (submitted). Three of the 12 loci optimized for *Entosphenus* by Spice *et al.* (2010) were excluded from further analysis because it was difficult to establish allele sizes accurately.

Locus	GenBank Accession No.	Repeat motif	N_A	Size range (base pairs)	H_E	H_O
Etr-1	HM594248	Interrupted $[CA]_N$	4	223-229	0.173	0.175
Etr-2	HM594249	Interrupted $[CT]_N$	7	241-255	0.690	0.616
Etr-3	HM594250	Interrupted $[CA]_N$, $[CAA]_N$	15	131-183	0.637	0.655
Etr-4	HM594251	$N_{18}[CA]_5N_{58}[GGT]_5N_{29}$	7	159-177	0.609	0.691
Etr-5	HM594252	Interrupted $[GCT]_N$, $[GGT]_N$, $[GCG]_N$	10	162-189	0.688	0.704
Etr-6	HM594253	Interrupted $[CAT]_N$, $[CAG]_N$	17	268-319	0.693	0.711
Lri-3	HM594256	$[CA]_6G[CA]_9G[CA]_7G[CA]_5$	22	197-249	0.695	0.644
Lri-7	HM594260	$[CAT]_3TAT[CAT]_4$	9	136-181	0.644	0.707
Lri-9	HM594262	$[AAC]_5[CAC]_3[AAC]_3$	7	246-270	0.555	0.583

Table 3. Pairwise F_{ST} values for Pacific lamprey (*Entosphenus tridentatus*) collected from 12 locations along the west coast of North America. Site numbers correspond to the site numbers listed in Table 1 and Figure 1; sites 13-21 are within California. Values were calculated using GENEPOP v. 4.0.10 (Raymond and Rousset 1995; Rousset 2008). Significance of F_{ST} values after a strict Bonferroni correction (Rice 1989) was tested using FSTAT v. 2.9.3.2 (Goudet 2001), and significant genetic differentiation is indicated by *. Unshaded cells indicate F_{ST} below 0.0500; light gray, F_{ST} between 0.05 and 0.0999; mid-gray, F_{ST} between 0.10 and 0.1499; and dark gray, F_{ST} 0.15 or higher.

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0.0320*	0.0868*	0.2277*	0.0306	0.0888*	0.1063*	0.0118*	-0.0081	0.0336	0.0659*	0.0730*	0.0099	0.0033	0.0310*	0.1076*	0.0116*	0.0320*	0.0134*	0.0013	0.0489*
2		0.1131*	0.1394*	0.0186*	0.0420*	0.0142*	0.0209*	0.0166*	0.0123	0.0226*	0.0237*	0.0134*	0.0358*	0.0088*	0.0752	0.0150*	0.0037*	0.0420	0.0313	0.0244*
3			0.2569*	0.1037*	0.0882*	0.1657*	0.0601*	0.0806*	0.1040*	0.0992*	0.1814*	0.1020*	0.0940*	0.1405*	0.0865*	0.1006*	0.1346*	0.1271*	0.0685*	0.1348*
4				0.1603*	0.0533	0.0764*	0.1634*	0.2169*	0.1411*	0.0932*	0.1027*	0.2245*	0.2102*	0.1451*	0.0683*	0.1720*	0.1162*	0.1711*	0.2177*	0.1517*
5					0.0365*	0.0457*	-0.0020	0.0180	-0.0086	0.0058	0.0223	0.0081	0.0092	0.0037	0.0487*	-0.0006	0.0184	0.0140	0.0170	0.0002
6						0.0217*	0.0397	0.0772	0.0388	0.0000	0.0451*	0.0780	0.0820	0.0528	-0.0079	0.0531	0.0363*	0.0626*	0.0727*	0.0566*
7							0.0590*	0.0811*	0.0379*	0.0249*	0.0303*	0.0697*	0.0953*	0.0395*	0.0555*	0.0584*	0.0333*	0.0894*	0.0968*	0.0491*
8								0.0031	0.0012	0.0128	0.0378*	0.0006	-0.0035	0.0075	0.0476	-0.0053	0.0197*	0.0032	0.0052	0.0115*
9									0.0200	0.0476*	0.0573*	-0.0017	0.0010	0.0175	0.0974*	0.0015	0.0231*	0.0138*	0.0013	0.0338*
10										0.0022	0.0102	0.0086*	0.0089	-0.0039	0.0511	-0.0018	0.0120	0.0094	0.0175	-0.0065
11											0.0225	0.0350*	0.0424	0.0169	0.0059	0.0207	0.0239	0.0329	0.0395*	0.0138
12												0.0489*	0.0521*	0.0052	0.0685*	0.0247	0.0044	0.0319	0.0561	0.0129
13													0.0006	0.0080	0.0982*	-0.0026	0.0241*	0.0188*	0.0133*	0.0171*
14														0.0117	0.0954	-0.0029	0.0276	0.0024	0.0050	0.0129
15															0.0739*	-0.0029	0.0007*	0.0080*	0.0241*	-0.0005
16																0.0682*	0.0644*	0.0706	0.0822*	0.0715*
17																	0.0107*	0.0010	0.0079	0.0062
18																		0.0164*	0.0290*	0.0176*
19																			0.0062	0.0193
20																				0.0333*

Table 4. Results of STRUCTURE analysis for Pacific lampreys (*Entosphenus tridentatus*) from 21 sites along the west coast of North America. Site numbers correspond to those given in Table 1 and Figure 1; sites 13-21 are within California.

Site number	Cluster 1	Cluster 2	Cluster 3
1	0.973	0.019	0.009
2	0.882	0.115	0.004
3	0.438	0.544	0.018
4	0.948	0.050	0.003
5	0.176	0.819	0.005
6	0.931	0.062	0.007
7	0.978	0.019	0.003
8	0.898	0.077	0.026
9	0.918	0.076	0.006
10	0.442	0.167	0.391
11	0.758	0.027	0.215
12	0.941	0.028	0.030
13	0.775	0.173	0.052
14	0.879	0.105	0.015
15	0.737	0.058	0.206
16	0.912	0.075	0.013
17	0.793	0.099	0.109
18	0.976	0.019	0.005
19	0.645	0.332	0.022
20	0.696	0.292	0.012
21	0.925	0.034	0.042

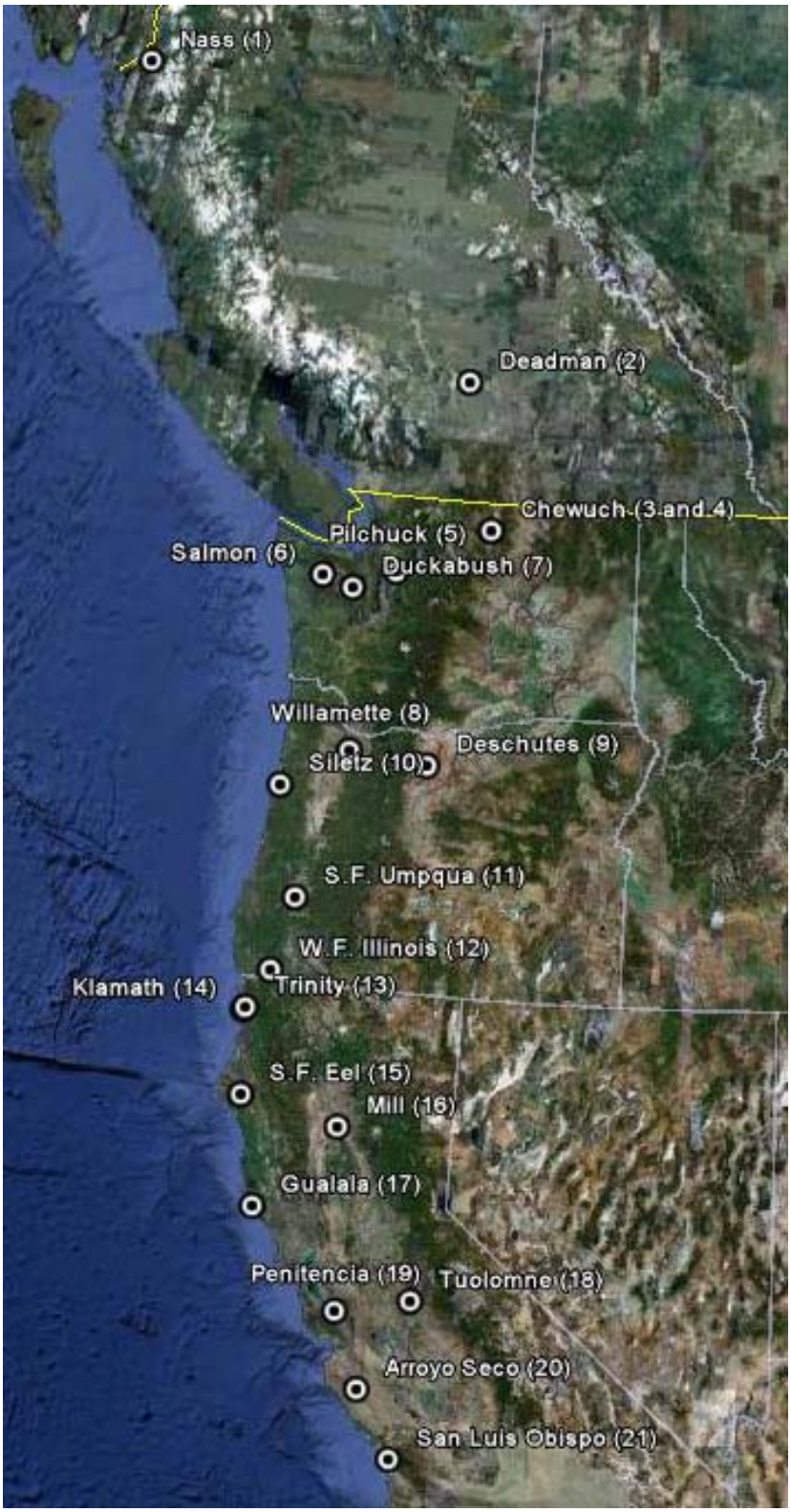

Figure 1. Google Earth map of collection sites for Pacific lamprey (*Entosphenus tridentatus*). Site names and numbers correspond to those given in Table 1.

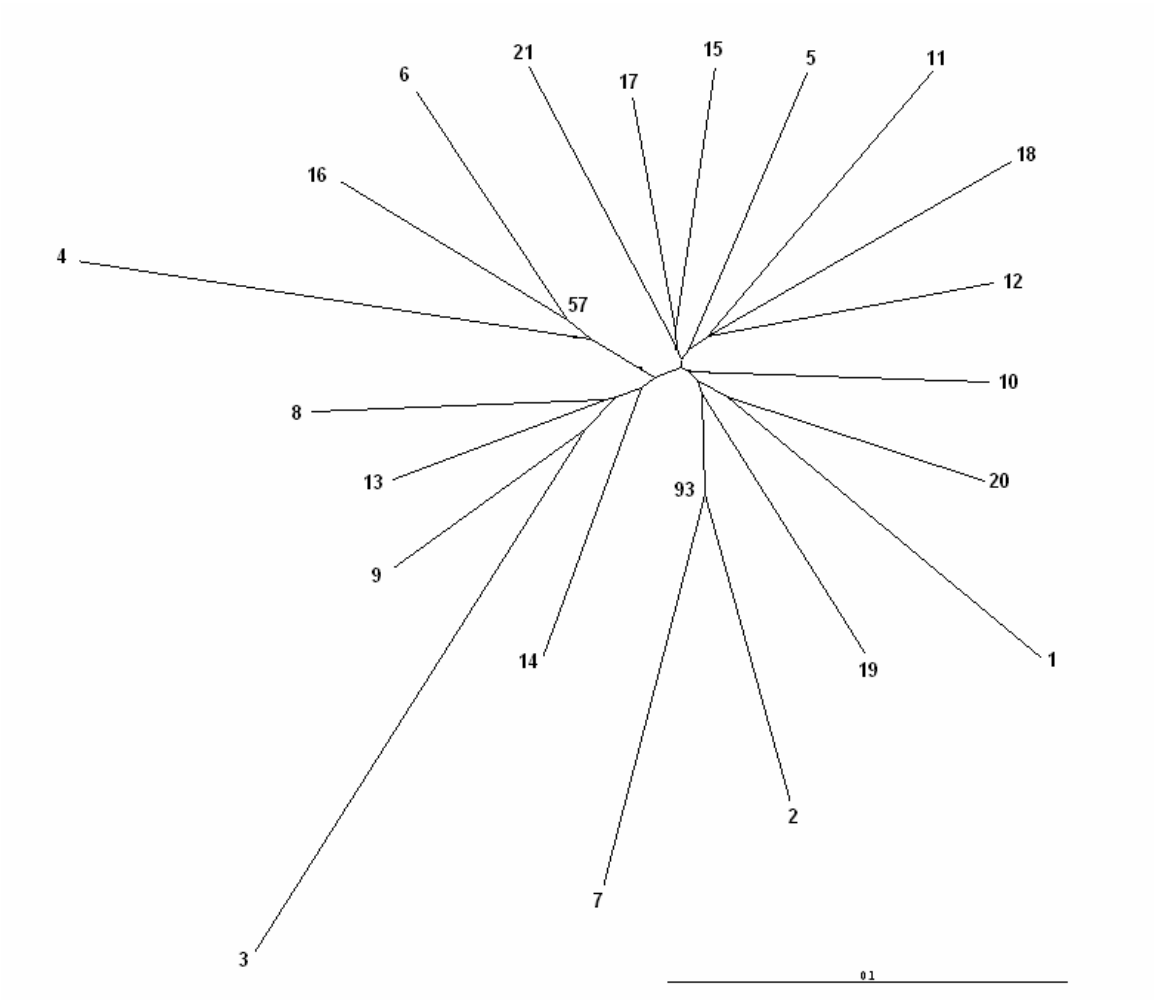

Figure 2. Neighbor-joining tree showing the relationships among Pacific lamprey collected from 21 sites along the west coast of North America. Site numbers correspond to those given in Table 1 and Figure 1; sites 13-21 are within California. Bootstrap values greater than 50% are shown.