

Microsatellite DNA analysis of parapatric lamprey (*Entosphenus* spp.) populations: implications for evolution, taxonomy, and conservation of a Canadian endemic

Eric B. Taylor, Les N. Harris, Erin K. Spice, and Margaret F. Docker

Abstract: Parapatric freshwater and anadromous parasitic lampreys (Petromyzontiformes) from southwestern Vancouver Island, British Columbia, have been described as distinct taxa (Vancouver lamprey (*Entosphenus macrostomus* (Beamish, 1982)) and Pacific lamprey (*Entosphenus tridentatus* (Richardson, 1836)), respectively), using morphology, life history, and physiology. We tested for genetic differentiation at microsatellite DNA loci between these taxa and similar freshwater parasitic lampreys from two other lakes. The number of alleles and expected heterozygosity averaged 3.8 and 0.50, respectively, across loci and populations, and anadromous populations were more variable than freshwater populations. Population subdivision was moderate ($F_{ST} = 0.096$, $P < 0.001$) and 3% of the total variation was found between taxa and 1.7% was found among populations within taxa (both $P < 0.001$). Parapatric freshwater and anadromous parasitic lampreys separated by a maximum of 40 km were more distinct (mean $F_{ST} = 0.042$) than were anadromous populations located 800 km from one another (mean $F_{ST} = 0.012$). Localities within lakes with parasitic freshwater lampreys, however, showed little differentiation ($F_{ST} = 0.0-0.08$). Our data support recognizing *E. macrostomus* and *E. tridentatus* as distinct species, but similar levels of differentiation between these taxa and other freshwater parasitic lampreys suggest a species complex where the taxonomy remains unclear.

Key words: Petromyzontidae, *Entosphenus tridentatus*, *Entosphenus macrostomus*, Pacific lamprey, Vancouver lamprey, microsatellites, parapatric populations.

Résumé : Des lamproies parasites (Pétromyzontiformes) parapatriques, dulcicoles et anadromes, du sud-ouest de l'île de Vancouver, Colombie-Britannique, ont été décrites comme des taxons différents, soit respectivement, la lamproie à grand disque (*Entosphenus macrostomus* (Beamish, 1982)) et la lamproie du Pacifique (*Entosphenus tridentatus* (Richardson, 1836)), d'après leur morphologie, leur cycle biologique et leur physiologie. Nous avons vérifié la différenciation génétique à des locus microsatellites d'ADN entre ces taxons et d'autres lamproies parasites d'eau douce semblables provenant de deux autres lacs. Le nombre d'allèles et l'hétérozygotie attendue sont en moyenne respectivement de 3,8 et de 0,50, pour l'ensemble des locus et des populations; les populations anadromes sont plus variables que les populations d'eau douce. La subdivision de la population est moyenne ($F_{ST} = 0,096$, $P < 0,001$); 3 % de la variation totale se retrouve entre les taxons et 1,7 % entre les populations à l'intérieur des taxons ($P < 0,001$ dans les deux cas). Les lamproies parasites parapatriques anadromes et dulcicoles séparées par un maximum de 40 km sont plus distinctes ($F_{ST} = 0,042$ en moyenne) que des populations anadromes situées à 800 km l'une de l'autre ($F_{ST} = 0,012$ en moyenne). Il y a cependant peu de différenciation ($F_{ST} = 0,0-0,08$) entre les sites dans un même lac contenant des lamproies parasites d'eau douce. Nos données appuient la reconnaissance d'*E. macrostomus* et d'*E. tridentatus* comme espèces distinctes; cependant, l'existence de niveaux similaires de différenciation entre ces taxons et d'autres lamproies parasites d'eau douce laisse croire à un complexe d'espèces dont la taxonomie reste à clarifier.

Mots-clés : Petromyzontidae, *Entosphenus tridentatus*, *Entosphenus macrostomus*, lamproie du Pacifique, lamproie à grand disque, microsatellites, populations parapatriques.

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Introduction

Geographic areas where one or more divergent populations come into contact have provided rich opportunities to study the ecological, genetic, and geographic factors that influence interactions. For instance, contact zones between whole communities have helped to illuminate large-scale, historical, geographic, or climatological factors influencing patterns of species diversity (Remington 1968; Lomolino et al. 2006). On a smaller scale, contact zones between divergent populations provide opportunities to understand how the genetic and ecological characteristics of interacting populations influence patterns of gene flow and genetic divergence between them (Endler 1977; Coyne and Orr 2004; Toews and Irwin 2008).

Fishes have provided rich model systems to study both of these aspects of biotic contact zones. In particular, there are several systems involving sympatric and parapatric populations of teleost fishes that have been exploited to understand the biogeography, genetics, and ecology of divergence and speciation (Coyne and Orr 2004; Taylor 2004). In still other cases, the study of natural and human-induced contact zones has had important implications for conservation of one or both interacting taxa (e.g., Allendorf et al. 2001; Taylor 2004). One of the most widely studied systems of contact zones in fishes involves postglacial parapatric or sympatric pairs of genetically, ecologically, and morphologically divergent, but taxonomically undescribed, populations that would seem to meet the definition of species under the biological species concept or its more recent derivatives (e.g., Hausdorf 2011). These populations are typically referred to as “species pairs” (e.g., McPhail 1984; reviewed by Taylor 1999) and, quite remarkably, this general evolutionary phenomenon characterizes fishes descended from lineages at the very base of the vertebrate tree of life—the lampreys (Petromyzontiformes)—to some of the most derived teleosts (e.g., Gasterosteiformes).

Lampreys exhibit a complex array of life-history types (see review by Docker 2009). Some species are ectoparasites of other fishes, feeding on their host’s surface tissue and bodily fluids; others are nonparasitic (i.e., nonfeeding) during their brief adult lives, but have extended filter-feeding larval stages. Another axis of differentiation includes anadromous (sea-run) and freshwater-resident life histories; although all nonparasitic lampreys are exclusively freshwater-resident, species of parasitic lampreys may be exclusively anadromous, entirely freshwater-resident, or both. In many instances, lamprey populations that are similar in gross morphology, but that differ in feeding mode, are parapatric or sympatric. In contrast to most other cases of closely related, but distinct ecological types in sympatry or parapatry in fishes, these different feeding types in lampreys have been recognized as distinct taxa (see review by Docker 2009). For instance, the anadromous parasitic European river lamprey (*Lampetra fluviatilis* (L., 1758)) and the freshwater-resident and nonparasitic European brook lamprey (*Lampetra planeri* (Bloch, 1784)) are found in broad sympatry in western Europe. Recent molecular data, however, indicate that *L. planeri* is polyphyletic and likely the result of multiple, independent episodes of divergence from *L. fluviatilis* across its range (Espanhol et al. 2007).

Another example of closely related, but ecologically distinct species occurring in parapatry is that of the Pacific lamprey (*Entosphenus tridentatus* (Richardson, 1836)), which is parasitic and anadromous throughout much of the North Pacific basin, and the parasitic, but freshwater-resident Vancouver lamprey (*Entosphenus macrostomus* (Beamish, 1982)) (formerly known as *Lampetra macrostoma* Beamish, 1982; see Renaud et al. 2009). These two taxa are similar morphologically, but Beamish (1982) described the Vancouver lamprey as a distinct taxon owing to its smaller size and slight, but detectable differences in body proportions (notably oral disc size), pigmentation, physiology, and spawning time and location. The Vancouver lamprey was described as endemic to the Cowichan Lake system on southeastern Vancouver Island, British Columbia (B.C.), Canada, where it resides within the interconnected Cowichan and Mesachie lakes and feeds on salmonids (genera *Oncorhynchus* Suckley, 1861 and *Salvelinus* Richardson, 1836). The outlet of Cowichan Lake is the Cowichan River, which supports a native population of anadromous Pacific lamprey. Despite being described as distinct taxa, mitochondrial DNA (mtDNA) sequence data from a small number of specimens failed to document any differences between the Vancouver and the Pacific lampreys (Docker et al. 1999), and given that the two forms do not appear to be strictly sympatric within Cowichan Lake itself, their status as distinct biological species remains uncertain (McPhail 2007). Notwithstanding some uncertainty as to the veracity of its status as a distinct taxon, the Vancouver lamprey is recognized as a “designatable unit” (DU) for conservation purposes within the context of Canada’s *Species at Risk Act* where they are currently listed as Threatened (COSEWIC 2009). Assessing the level of genetic distinctiveness of *E. macrostomus*, however, would provide a more robust test of their status as a DU distinct from *E. tridentatus*.

Finally, there are reports of parasitic, freshwater *E. tridentatus* like lampreys in at least three other, disjunct, locations in southwestern B.C.—West Lake on Nelson Island, Village Bay Lake on Quadra Island, and two adjacent lakes (Ruby and Sakinaw lakes) on the Sechelt Peninsula (Beamish 2001; COSEWIC 2009). This suggests either that the Vancouver lamprey may have a broader distribution than previously appreciated or that this feeding type has evolved independently multiple times, which would complicate the current taxonomy of the Vancouver lamprey (McPhail 2007).

In this study, we assayed samples of Vancouver and Pacific lampreys from the Cowichan Lake system at eight microsatellite DNA loci to provide a more sensitive test of genetic distinctiveness than possible with mtDNA (Selkoe and Toonen 2006). In addition, we included lampreys collected from two lakes on the adjacent mainland (Ruby and Sakinaw lakes on the Sechelt Peninsula) to assess their similarity to Vancouver lamprey from the Cowichan Lake system. If freshwater parasitism evolved once, rather than independently, in these two systems, we expect these populations to be more similar to each other than either is to intervening populations of the Pacific lamprey, their putative ancestor.

Materials and methods

Sample collection

We initially focused on sampling adults to avoid possible complications of higher genetic relatedness associated with sampling juveniles that might represent a small number of families. Consequently, fieldwork was conducted from April to August 2007 and 2008 to coincide with the suspected time of Vancouver lamprey spawning when adults are more easily captured (COSEWIC 2009; Beamish and Wade 2010). Sampling was conducted using backpack electrofishing units (Smith Root Inc., model 12-B POW) in areas of clean gravel on beaches and in streams that are characteristic of good spawning areas (Beamish 1982; Beamish and Wade 2010). We also used Fyke nets set perpendicularly to shore and in the inlet to Mesachie Lake (Halfway Creek) to capture adult lampreys en route to upstream spawning locations. The nets were checked daily and in some circumstances twice each day. Despite extensive search effort, we were only able to sample five adults from Cowichan Lake, all of which were clearly identifiable as *E. macrostomus* using the characters in Beamish (1982). Beamish and Wade (2010) reported capturing only a single adult Vancouver lamprey with even more sustained trapping effort. Consequently, we sampled larval Vancouver and Pacific lampreys from multiple locations within the Cowichan, Ruby, and Sakinaw lake systems (Fig. 1). These locations typically consisted of areas of slow water, downstream of gravel spawning areas, and with abundant organic debris deposition (L.N. Harris and E.B. Taylor, unpublished data). We attempted to sample lampreys from a variety of areas per location and across multiple size classes to minimize the chances of sampling larvae from a limited number of families. Age–size relationships are not known for larval Vancouver or Pacific lamprey, but Docker and Beamish (1994) reported that larval least brook lamprey (*Lampetra aepyptera* (Abbott, 1860)) over a size range of 20–180 mm from two streams in the eastern United States represented ages 1–5 years. Our samples typically ranged in size from 40 to 160 mm total length (supplementary Fig. S1).¹

Specimens captured by any of the abovementioned methods were euthanized in an overdose of buffered MS-222 and then preserved in 95% (v/v) ethanol. In total, we collected approximately 30 lamprey from each of five localities within Cowichan Lake, two localities in the adjoining Mesachie Lake, two localities in the Cowichan River, two localities in Ruby Lake (Sechelt Peninsula), and one locality in Sakinaw Lake (Sechelt Peninsula). All of the adults captured were confirmed to be *E. macrostomus* morphologically using the key in Beamish (1982). Larvae of the different species of lampreys are much more difficult to identify morphologically and representative samples could not be distinguished between *E. macrostomus* and *E. tridentatus* using the key in Richards et al. (1982). Beamish and Wade (2010) reported similar identification problems for larvae, but when they collected representative samples of larvae from Cowichan Lake, transported them to the laboratory, and allowed them to metamorphose, all Cowichan Lake lampreys were identified as *E. macrostomus*, whereas those collected from the Cowi-

chan River transformed into *E. tridentatus* or western brook lamprey (*Lamperta richardsoni* Vladykov and Follett, 1965) (Beamish and Wade 2010). Sakinaw Lake may have an anadromous Pacific lamprey population in its outlet stream that empties into the ocean, but adults of similar size to Vancouver lamprey and which are known to parasitize fishes in fresh water exist both in Sakinaw and Ruby lakes (McPhail 2007; E.B. Taylor, personal observation). The lampreys from these lakes have mtDNA cytochrome *b* sequences ($N = 5$ from each lake; M.F. Docker, unpublished data) that are identical to those of both *E. tridentatus* and *E. macrostomus*. The freshwater form in Sakinaw and Ruby lakes, however, is morphologically different from *E. macrostomus* (see COSEWIC 2009). Consequently, we consider the parasitic lampreys from Ruby and Sakinaw lakes to be *E. tridentatus* until such time as they are examined closely taxonomically.

We also sampled 30 adult parasitic, anadromous Pacific lamprey from one locality on the Nass River, some 700–850 km north on the northwest coast of B.C. (Fig. 1). The Nass River fish were captured at a fish wheel at Gitwinksihlkw on the mainstem Nass River (55°11'N, 129°13'W) during their upstream spawning migration.

DNA extraction and microsatellite screening

Lamprey DNA was extracted from muscle tissue obtained from whole specimens stored in 95% (v/v) ethanol using Qiagen DNeasy extraction kits, following the manufacturer's instructions. Subsequent to the final wash step, DNA was eluted with 150 μ L of AE buffer provided by the manufacturer and stored at –20 °C until analysis.

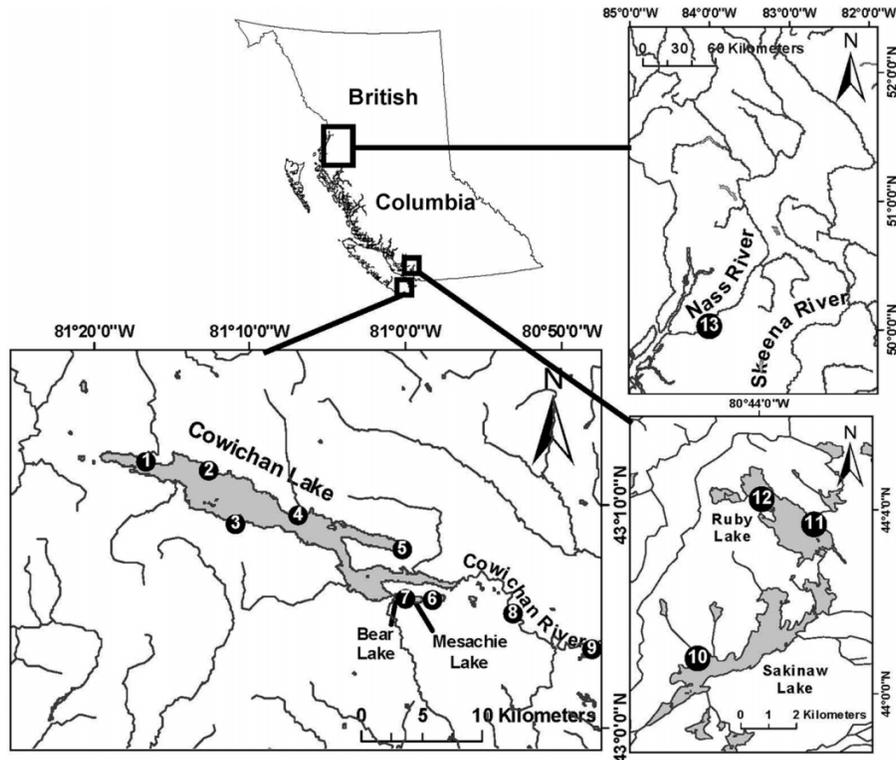
Samples were assayed at eight microsatellite DNA loci developed from *L. richardsoni* ($N = 3$; *Lri-3*, 7, and 9; Luzier et al. 2010) and *E. tridentatus* ($N = 5$; *Etr-1*, 2, 3, 5, and 6; Spice et al. 2011). These loci were amplified from the DNA extractions by polymerase chain reaction (PCR) using forward primers 5'-end labeled with 6-Fam or Hex (Sigma Life Science) or Ned or Pet (Applied Biosystems) fluorescent dyes. Fragment size analysis was performed on an ABI 3130 Genetic Analyzer, and allele sizes were determined using Genemapper version 4.0 (Applied Biosystems) as detailed in Spice et al. (2011).

Statistical analyses

We used MICRO-CHECKER (van Oosterhout et al. 2004) to check our data for evidence of scoring errors, large allele drop-out, and (or) null alleles. Genetic variation was summarized in each sample by calculating observed (H_O) and expected (H_E) heterozygosity, number of alleles (N_A), and allelic richness (A_R) using FSTAT version 2.9.3 (Goudet 1995, 2001). As a quantitative measure of the direction and extent of any population deviation from Hardy–Weinberg equilibrium, Weir and Cockerham's (1984) estimator f of the inbreeding coefficient, F_{IS} , was estimated at each locus using FSTAT. The Fisher's exact test assessed genotypic linkage disequilibrium (LD) among pairs of loci within each sample using a Markov chain method in GENEPOP version 3.3 (Raymond and Rousset 1995, 2001). Differences in all measures of genetic variation were tested between freshwater-resident samples ($N = 10$ locations) and anadromous

¹Supplementary Fig. S1 and Table S1 are available with the article through the journal Web site (<http://nrcresearchpress.com/doi/suppl/10.1139/z11-135>).

Fig. 1. Map of collection localities for Vancouver lamprey (*Entosphenus macrostomus*) (normal type) and Pacific lamprey (*Entosphenus tridentatus*) (italic type) in British Columbia (B.C.). Underlined localities represent anadromous samples. 1, Cowichan Lake 1 (west of the mouth of Little Shaw Creek); 2, Cowichan Lake 2 (mouth of McKay Creek); 3, Cowichan Lake 3 (mouth of unnamed Creek west of Croft Creek); 4, Cowichan Lake 4 (mouth of Cottonwood Creek); 5, Cowichan Lake 5 (mouth of Meade Creek); 6, Mesachie Lake 1 (Mesachie Lake outlet); 7, Mesachie Lake 2 (Mesachie Lake inlet); 8, Cowichan River 1 (at Stoltz Pool); 9, Cowichan River 2 (below Skutz Falls near Duncan, B.C.); 10, Kokomo Creek (Sakinaw Lake); 11, Klein Creek (mouth of Klein Creek in Ruby Lake); 12, Ruby Lake (unnamed beach location in Ruby Lake); 13, Nass River.



samples ($N = 3$ locations) using the permutation procedure in FSTAT ($N = 1000$ permutations).

Because the majority of our samples consisted of larvae, we assessed levels of genetic relatedness within each sample to guard against using samples from a small number of families. We used the program IDENTIX version 1.1 (Belkhir et al. 2002) to calculate mean Identity and its variance as a measure of consanguinity of larvae within a sample. The observed distribution of both the mean and the variance of pairwise Identity coefficients within each (I_{xy}) were compared with a null distribution of 1000 multilocus genotypes expected under panmixia generated by random resampling across alleles from the original data (Belkhir et al. 2002; cf. Small et al. 2009). Even when mean I_{xy} does not vary from the null expectation, indicating that individuals within a sample are no more genetically related than expected in a randomly mating population, a significantly higher variance in the observed I_{xy} can indicate that the sample is composed of several independent groups of related individuals, where pairwise comparisons involve either related or unrelated individuals (Belkhir et al. 2002; Small et al. 2009).

We used our data to derive estimates of effective population size (N_E), a parameter of central importance in population and conservation genetics owing to its role in influencing fluctuations in allele frequencies and loss of heterozygosity across generations (Allendorf and Luikart

2007). There are a number of methods available to infer N_E from genetic data (e.g., see Waples 2005; Palstra and Ruzante 2008). Two general kinds of methods are those that estimate these parameters from a single time sample (e.g., methods based on LD or sibship assignments; Hill 1981; Wang 2009) and those that compare allele frequencies between two temporally spaced samples (i.e., so-called temporal methods; Waples 1989; Jorde and Ryman 1995). As our samples consisted of single time points, we used the sibship assignment method implemented by COLONY version 2.0.1.1 (Wang 2009; Jones and Wang 2010) to estimate N_E for each sample point. This method infers the contemporary N_E from estimated sibship frequencies, drawing on the idea that a smaller population will result in a higher proportion of sibs in any given random sample. Importantly in our instance, the sibship procedure can be applied to subpopulations experiencing immigration and nonrandom mating (Wang 2009). We ran two medium-length runs in COLONY (results were identical between runs and longer runs on a subset of the sample points produced similar values), under a polygynous mating system with prior unknown allele frequencies. We compared these sibship estimates to those derived from examination of LD using the program LDNe (Waples and Do 2008) to generate estimates of contemporary N_E and their 95% confidence intervals (95% CI). The LD method operates on the principle that departures from random

association between alleles across loci (LD) or the correlation between alleles between pairs of loci will be inversely proportional to the N_E . Given that lampreys spend multiple years as larvae and that we sampled a diversity of size classes, our data probably represent multiple cohorts per locality so that our estimates of N_E are more properly thought of as estimates of N_B , the effective number of breeders that produced the sampled larvae (Waples 2005).

To examine genetic structure, pairwise multilocus F_{ST} was estimated by θ (Weir and Cockerham 1984) between all samples and the significance of genetic differentiation was tested using a procedure implementing 1000 permutations in FSTAT. We tested the significance of hierarchical population structure using the analysis of molecular variance approach (AMOVA) employed in ARLEQUIN version 3.0 (Excoffier et al. 2005), which partitions the total genetic variance into covariance components associated with different levels of genetic structure: within populations across the entire study system, between sample localities within lakes (Cowichan–Mesachie and Ruby–Sakinaw) or rivers (Nass and Cowichan rivers) and among lakes and rivers. Ten thousand permutations of individual genotypes among samples (either between or within groups), or whole samples among groups, tested the significance of each index of differentiation.

We used the model-based Bayesian clustering analysis within STRUCTURE (Pritchard et al. 2000) to assess population structure spatially. We used the admixture model with correlated allele frequencies and a burnin of 50 000 iterations followed by an additional 150 000 iterations, replicated five times. We ran simulations with hypothesized numbers of populations (K) ranging from $K = 1$ to $K = 18$ (5 more than the total number of locations sampled). We assessed confidence in the number of genetic clusters from the likelihood scores and from the rate of change of these scores following the procedure of Evanno et al. (2005) as processed by STRUCTURE HARVESTER (Earl 2011). Finally, 100 bootstrap replicates of the allele frequency matrix were generated using SEQBOOT in PHYLIP version 3.6 (Felsenstein 2005). These matrices were then used to calculate 100 Cavalli-Sforza's chord genetic distance (Cavalli-Sforza and Edwards 1967) matrices (using GENDIST from PHYLIP). Similarity among samples was summarized by generating 100 neighbor-joining trees (Saitou and Nei 1987) using NEIGHBOR (from PHYLIP) and subjecting these to majority-rule consensus tree analysis using NEIGHBOR and CONSENSE (from PHYLIP).

For all analyses involving multiple, simultaneous hypothesis tests, significance criteria for each group of tests were determined using the false discovery rate procedure outlined by Narum (2006).

Results

Variation within populations

Seven lampreys assayed exhibited allele sizes at *Etr-1* and *Etr-6* characteristic of the genus *Lampetra* (Spice et al. 2011). These seven individuals were all found at the Cowichan River site located below Skutz Falls (Fig. 1) and were removed from all subsequent analyses except for their use as an outgroup in the neighbor-joining tree analysis.

Across the eight loci, the number of alleles assayed ranged from 2 (*Etr-1*) to 12 (*Etr-6*) and expected heterozygosity

from 0.03 (*Etr-1*) to 0.65 (*Lri-7*; Table 1 and supplementary Table S1¹). MICRO-CHECKER indicated that a null allele(s) was suspected at *Etr-3* in four populations: two in Cowichan Lake, one in the Cowichan River, and the Nass River sample. Estimates of null allele frequency and corrected genotype frequencies were calculated using the van Oosterhout estimator, as there was no indication of null allele homozygotes at this locus (van Oosterhout et al. 2004). After employing this correction, the mean number of alleles across populations and loci ranged from 2.5 (Klein Creek) to 5.3 (Cowichan River site 1), expected heterozygosity from 0.40 (Klein Creek) to 0.59 (Cowichan River site 1), and allelic richness (adjusted to 23 individuals) from 2.5 (Klein Creek) to 5.0 (Cowichan River site 1) (Table 1). In 21–28 pairwise comparisons between loci within populations (*Etr-1* was monomorphic in all but three samples), there were three instances of evidence of LD; *Lri-3* and *Etr-5* in Klein Creek, the same two loci in Kokomo Creek, and *Lri-7* and *Etr-5* in Kokomo Creek (maximum $P = 0.006$). When the lampreys from the three anadromous samples were compared with the 10 freshwater-resident samples, anadromous lampreys had significantly higher allelic richness (4.8 vs. 3.0; $P = 0.008$) and expected heterozygosity (0.56 vs. 0.48; $P = 0.01$), but lower F_{ST} (0.008 vs. 0.086; $P = 0.014$).

Estimates of F_{IS} within populations ranged from -0.10 (Mesachie Lake 2) to 0.19 (Kokomo Creek) and three samples had overall F_{IS} values that were significantly different from 0 (maximum $P = 0.005$). The *Lri-3* locus deviated from Hardy–Weinberg equilibrium as summarized by F_{IS} within two samples, both of which were associated with deficiencies of heterozygotes (Table 1). One sample exhibited evidence of genetic relatedness being higher than expected in random samples from a panmictic population; the Nass River sample had significantly higher variance in identity ($I_{xy}(\text{variance}) = 0.022$ versus the mean of the null distribution = 0.018; $P < 0.0001$).

The sibship and LD methods produced reasonably concordant estimates of N_B for a number of samples. Estimates from sibship analysis ranged from 21 to 54 with defined and relatively narrow upper and lower 95% confidence values (Table 1). By contrast, while estimates from the LD method were comparable in a few cases, many estimates yielded negative point estimates and upper 95% confidence values of infinity (Table 1).

Variation among populations

Across all populations, F_{ST} was estimated as 0.096 (95% CI, 0.034–0.113) and ranged from values of 0 (between the two Cowichan River samples; between two samples from Ruby Lake; between Mesachie Lake inlet and one Cowichan Lake sample) to 0.19 (between one Sakinaw Lake and Cowichan Lake sample) (Table 2). Most pairwise comparisons were statistically significant even after incorporating false discovery rate adjustments; those that were not significant included comparisons within Cowichan Lake, between the two Cowichan River samples, between two samples from Ruby and Sakinaw lakes (Sechelt Peninsula), and between the two Cowichan River samples and the Nass River sample (Table 2). All comparisons between the Cowichan Lake system and Sakinaw–Ruby lakes, and between these samples and the anadromous lampreys from the Nass River, were signifi-

Table 1. Measures of genetic variation and effective number of breeders in samples of Vancouver lamprey (*Entosphenus macrostomus*) (CowL1–MesL2; normal type) and Pacific lamprey (*Entosphenus tridentatus*) (CowR1–Nass R; italic type) assayed at eight microsatellite DNA loci.

	Sample													
	CowL1	CowL2	CowL3	CowL4	CowL5	MesL1	MesL2	<i>CowR1</i> *	<i>CowR2</i> *	<i>KokomoC</i>	<i>KleinC</i>	<i>RubyL</i>	<i>Nass R</i> *	
Map No.	1	2	3	4	5	6	7	8	9	10	11	12	13	
<i>N</i>	27	29	29	26	28	30	30	29	30	23	30	30	43	
<i>N_A</i>	3.25 (1.2)	3.02 (1.2)	3.14 (1.2)	2.88 (1.1)	2.88 (1.1)	3.38 (1.6)	3.25 (1.3)	5.25 (2.5)	4.75 (2.1)	3.25 (1.2)	2.50 (1.1)	3.50 (1.2)	5.13 (2.2)	
<i>A_R</i> †	3.33 (1.6)	3.16 (1.4)	2.96 (1.2)	2.87 (1.1)	3.21 (1.3)	2.92 (1.2)	2.83 (1.0)	4.96 (2.3)	4.72 (2.1)	2.49 (1.1)	3.36 (1.1)	3.17 (1.1)	4.67 (1.8)	
<i>H_O</i>	0.52 (0.26)	0.47 (0.22)	0.50 (0.22)	0.52 (0.29)	0.49 (0.29)	0.45 (0.24)	0.54 (0.27)	0.56 (0.22)	0.53 (0.18)	0.36 (0.27)	0.50 (0.22)	0.42 (0.19)	0.45 (0.17)	
<i>H_E</i>	0.54 (0.23)	0.49 (0.21)	0.48 (0.22)	0.49 (0.21)	0.49 (0.20)	0.49 (0.20)	0.49 (0.21)	0.59 (0.20)	0.58 (0.17)	0.38 (0.24)	0.46 (0.22)	0.46 (0.25)	0.53 (0.19)	
<i>F_{IS}</i>	0.05 (0.24)	–0.01 (0.21)	0.02 (0.14)	0.09 (0.23)	0.03 (0.18)	–0.04 (0.18)	–0.10 (0.24)	0.04 (0.11)	<u>0.09 (0.07)</u>	–0.09 (0.10)	0.19 (0.35)	<u>0.10 (0.13)</u>	<u>0.14 (0.09)</u>	
<i>N_B(SA)</i>	29 (14–75)	29 (15–63)	30 (17–60)	31 (16–72)	23 (12–46)	33 (17–85)	32 (17–69)	30 (18–57)	51 (26–150)	21 (11–41)	35 (17–91)	27 (11–53)	54 (29–124)	
<i>N_B(LD)</i>	– (30–∞)	– (54–∞)	– (18–∞)	– (31–∞)	34 (10–∞)	82 (16–∞)	52 (14–∞)	50 (19–∞)	– (103–∞)	52 (12–∞)	64 (23–∞)	43 (15–∞)	– (115–∞)	

Note: *N*, sample size; *N_A*, mean number of alleles; *A_R*, mean allelic richness; *H_O*, mean observed heterozygosity; *H_E*, mean expected heterozygosity; *F_{IS}*, mean inbreeding coefficient; *N_B(SA)*, effective number of breeders from sibship assignment; *N_B(LD)*, effective number of breeders from linkage disequilibrium (LD). Mean values are calculated across loci. Values in parentheses for *N_A*–*F_{IS}* are standard deviations, whereas those for *N_B* estimates are 95% confidence limits. Underlined values of *F_{IS}* are significantly different from 0. A “–” value for *N_B(LD)* represents negative point estimates (see text). Map No. refers to locations in Fig. 1. CowL1–CowL5, Cowichan Lake 1 – Cowichan Lake 5; MesL1–MesL2, Mesachie Lake 1 – Mesachie Lake 2; *CowR1*–*CowR2*, Cowichan River 1 – Cowichan River 2; *KokomoC*, Kokomo Creek; *KleinC*, Klein Creek; *RubyL*, Ruby Lake, *Nass R*, Nass River.

*Anadromous populations.

†Adjusted to a minimum sample size of 23 individuals.

Table 2. Matrix of pairwise *F_{ST}* values (θ) between samples of Vancouver lamprey (*Entosphenus macrostomus*) (normal type) and Pacific lamprey (*E. tridentatus*) (italic type) estimated from variation across eight microsatellite DNA loci.

	MesL2	CowL1	CowL2	CowL3	CowL4	CowL5	<i>CowR1</i> *	<i>CowR2</i> *	<i>RubyL</i>	<i>KleinC</i>	<i>KokomoC</i>	<i>Nass R</i> *
MesL1	0.02492	0.02686	<u>–0.00244</u>	<u>0.01670</u>	0.06509	0.07380	0.04933	0.05673	0.10980	0.11806	0.11477	0.07300
MesL2		0.04487	<u>0.02732</u>	<u>0.03103</u>	<u>0.01819</u>	0.02739	<u>0.01584</u>	<u>0.01920</u>	0.10543	0.12016	0.10976	0.02831
CowL1			0.02344	<u>0.01585</u>	0.07603	0.08843	0.06803	0.05594	0.17653	0.19023	0.17984	0.07811
CowL2				<u>0.00852</u>	0.04644	0.05487	0.03658	0.05098	0.12060	0.13401	0.12550	0.05507
CowL3					0.07248	0.06974	0.06535	0.07159	0.15439	0.17053	0.16341	0.07181
CowL4						0.01107	0.03075	0.04121	0.12718	0.13175	0.13347	0.02859
CowL5							0.02258	0.06161	0.14763	0.17099	0.14202	0.04763
<i>CowR1</i>								<u>0.00842</u>	0.08233	0.12372	0.08009	<u>0.01438</u>
<i>CowR2</i>									0.10074	0.14354	0.11137	<u>0.01351</u>
<i>RubyL</i>										0.02418	<u>–0.00177</u>	0.09473
<i>KleinC</i>											<u>0.04173</u>	0.13425
<i>KokomoC</i>												0.09312

Note: All values are significantly different from 0 ($P > 0.0098$), controlling for the false discovery rate following Narum (2006), except those that are underlined. Sample codes are defined in Table 1.

*Anadromous populations.

cant. Twelve of the 14 comparisons between samples from Cowichan–Mesachie lakes and the Cowichan River anadromous samples were statistically significant (Table 2).

The AMOVA analysis indicated that a larger percentage of the total variation in allele frequencies was resolved among lakes–rivers (6.4%; $P < 0.0001$) than resolved among localities within lakes–rivers (1.6%; $P = 0.0002$). The remaining variation was found within individual localities (92.0%; $P < 0.0001$). When the samples were grouped by *E. macrostomus* (Cowichan–Mesachie lakes only, $N = 7$) and anadromous *E. tridentatus* ($N = 3$), 2.7% ($P = 0.008$) of the variation was attributable to differences between taxa, 1.7% ($P = 0.0001$) to variation between samples within taxa, and 95.6% ($P = 0.0001$) to variation within individual samples. When the Sakinaw–Ruby lakes samples were added to the *E. tridentatus* group, the amount of variation between taxa (3.4%; $P < 0.001$) was slightly less than that among populations within taxa (4.2%; $P < 0.0001$).

The STRUCTURE analysis consistently identified four genetic populations as the most likely population structure among the 13 samples (Table 3; Fig. 2). There were variable levels of admixture within each of the four genetic groups among the 13 localities (Fig. 3), but they generally clustered into two groups of Cowichan Lake samples, the Cowichan River and Nass River samples, and the Sechelt Peninsula samples (Ruby and Sakinaw lakes). These groupings were largely resolved in the neighbor-joining tree as well (Fig. 4); there were two groups consisting of (1) samples from Cowichan–Mesachie lakes that clustered together and separately from all others (96% bootstrap support) and (2) the samples from the Sechelt Peninsula that clustered together and separately from all others (71%). The samples of anadromous Nass River *E. tridentatus* and the samples of anadromous Cowichan River *E. tridentatus* were intermediate to these samples of freshwater-resident *E. macrostomus* from Cowichan Lake and the Sechelt Peninsula lakes (Fig. 4).

Discussion

Divergence within and among populations

Our microsatellite DNA data provided relatively consistent measures of genetic variation within sample localities, but for all variables, the anadromous samples were characterized by higher levels of variation. The higher variation in anadromous populations is consistent with freshwater versus anadromous comparisons within and between other species of fishes, and is typically thought to stem from the greater long-term effective population sizes within anadromous populations and species (reviewed in DeWoody and Avise 2000). Indeed, our estimates of the effective numbers of breeders (N_B) tended to be higher in the anadromous samples of *Entosphenus*. Our estimates of N_B are strictly applicable to samples that constitute a single cohort because both methods we employed utilize either sibship estimates or measures of LD that stem from matings within the previous parental generation. Given that lampreys spend several years as larvae (McPhail 2007) and that we sampled a wide size range of larvae, it is likely that our samples consist of several cohorts. This could lead to an upward bias in N_B from the sibship analysis, given that sibs should be less frequent in samples consisting of multiple larval cohorts, and a downward bias in

Table 3. Statistics from STRUCTURE analysis (Pritchard et al. 2000) of population structure across samples of Vancouver lamprey (*Entosphenus macrostomus*) and Pacific lamprey (*Entosphenus tridentatus*) inferred from variation across eight microsatellite DNA loci.

K	LnP(K)		ΔK
	Mean	SD	
1	-6204.1400	0.0548	NA
2	-5937.0000	7.6217	21.304141
3	-5832.2333	7.3050	2.965996
4	<u>-5749.1333</u>	<u>7.0741</u>	<u>22.942746</u>
5	-5828.3333	1.2858	14.828409
6	-5888.4667	17.2816	0.194812
7	-5951.9667	37.0526	4.186843
8	-6170.6000	311.8629	1.225859
9	-6006.9333	72.0930	5.140581
10	-6213.8667	74.0443	1.817380
11	-6286.2333	4.4523	1.864188
12	-6350.3000	225.4364	0.245006
13	-6469.6000	105.9290	0.123353
14	-6601.9667	190.4324	1.150890
15	-6515.1667	222.5920	NA

Note: Shown are the mean and standard deviations (SD) of the log-likelihood values (LnP) for different hypothesized numbers of genetic populations (K) in the total sample of lamprey across five replicate analyses. Also shown is the mean value of ΔK , the ad hoc statistic of Evanno et al. (2005) used to summarize the second-order rate of change of LnP(K). The underlined value of $K = 4$ represents both the most likely value of K and that with the greatest rate of change of LnP(K). NA, not applicable given that ΔK cannot be calculated for these values of K .

N_B from the LD method, as genetic differences between cohorts could increase LD (Luikart et al. 2010). In addition, the LD method produced many negative point estimates that can be attributed to situations when the signal from the genetic data arises exclusively from sampling error, which itself results when either the population size is very large or the genetic data provide inadequate information (Waples and Do 2010). Despite these difficulties, which are common in studies of genetic estimates of effective population size (see Luikart et al. 2010), convergence of estimates from different analytical methods is often a guide to the most reliable estimates (e.g., Luikart et al. 2010; Phillipsen et al. 2011). In several cases (e.g., Cowichan Lake site 5, Ruby Lake), point estimates of N_B between methods were comparable and relatively low at between ~20 and 60. Although such apparently low values for N_B (which should also reflect relatively low value of N_E) might be interpreted as a cause of some conservation concern (e.g., Luikart et al. 2010; Phillipsen et al. 2011), they are perhaps not surprising given the relatively small areas that were sampled to obtain each collection of larvae (i.e., a few 10s of square metres of habitat). In addition, low to no genetic differentiation observed among many samples from within the two lake systems suggests that genetic exchange occurs among areas within lakes which reduces concerns that result when populations are small and isolated from each other.

We were unable to confirm the identity of our larval samples in the Cowichan River system as either *E. macrostomus* or *E. tridentatus* using morphology given the very subtle differences between these species (Richards et al. 1982). Our

Fig. 2. Admixture coefficient (Q) for individual Vancouver lamprey (*Entosphenus macrostomus*) (normal type) and Pacific lamprey (*Entosphenus tridentatus*) (italic type) assayed at eight microsatellite DNA loci. Localities with anadromous lampreys are underlined. Each individual is represented by a thin vertical line where the height of each of four coloured portions is equal to the proportional contribution of each of four genetic groups (red, yellow, blue, and green) as determined by the STRUCTURE analysis (Pritchard et al. 2000). Samples are grouped by sample locality: 1–7, Cowichan–Mesachie lakes (*E. macrostomus*); 8–9, Cowichan River (*E. tridentatus*); 10–12, Sakinaw–Ruby lakes (presumptive *E. tridentatus*); 13, Nass River (*E. tridentatus*).

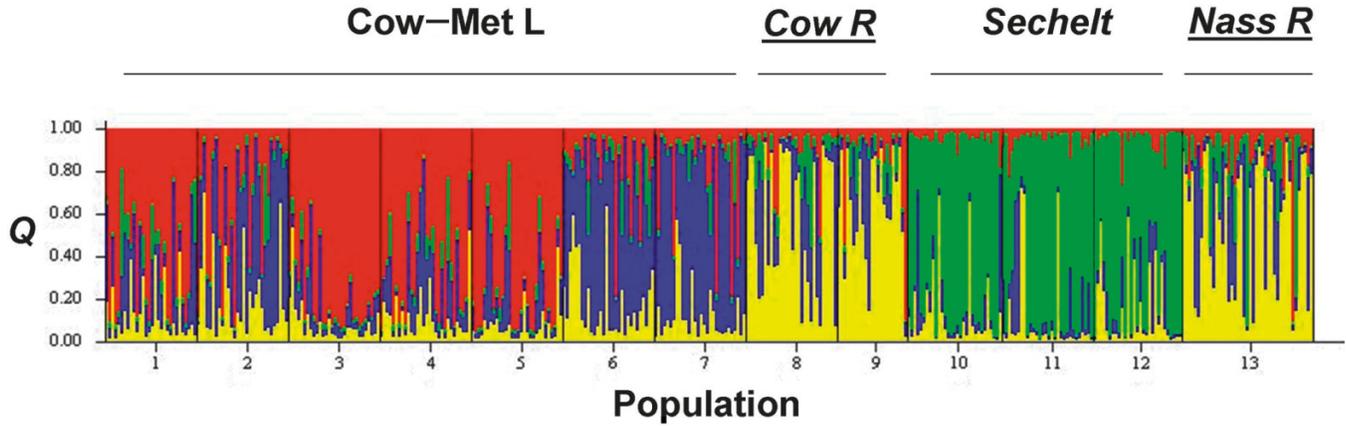
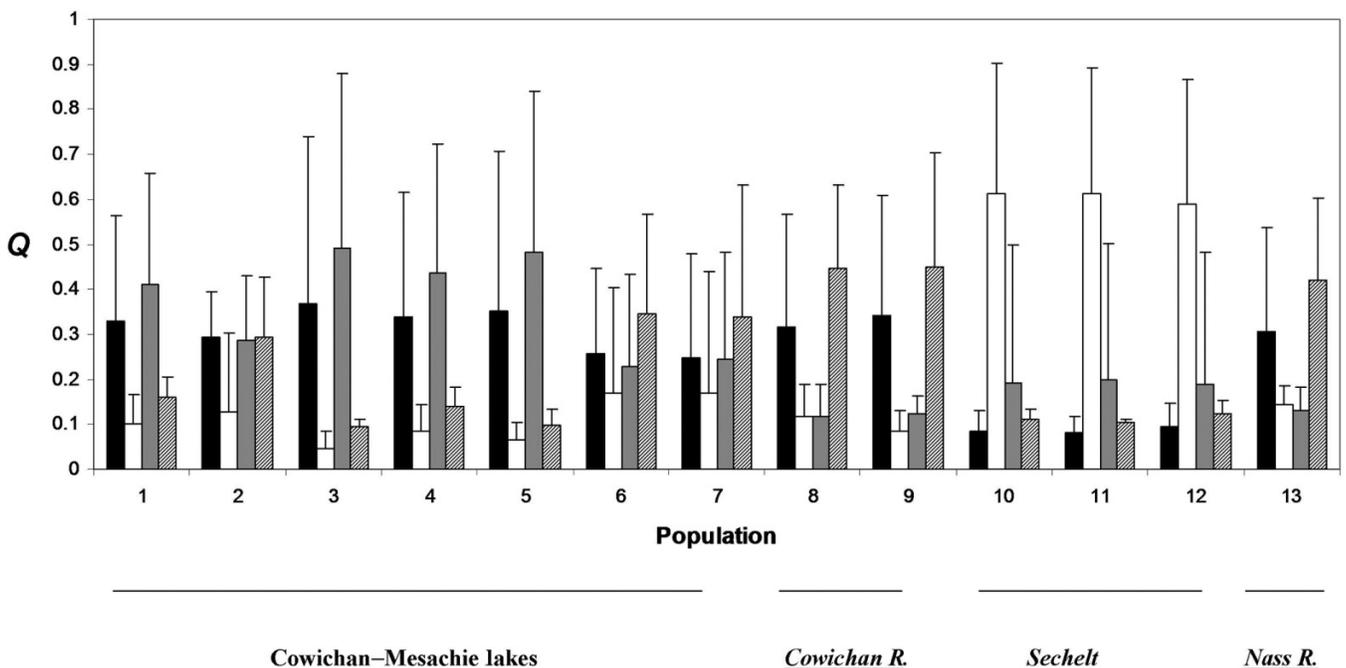


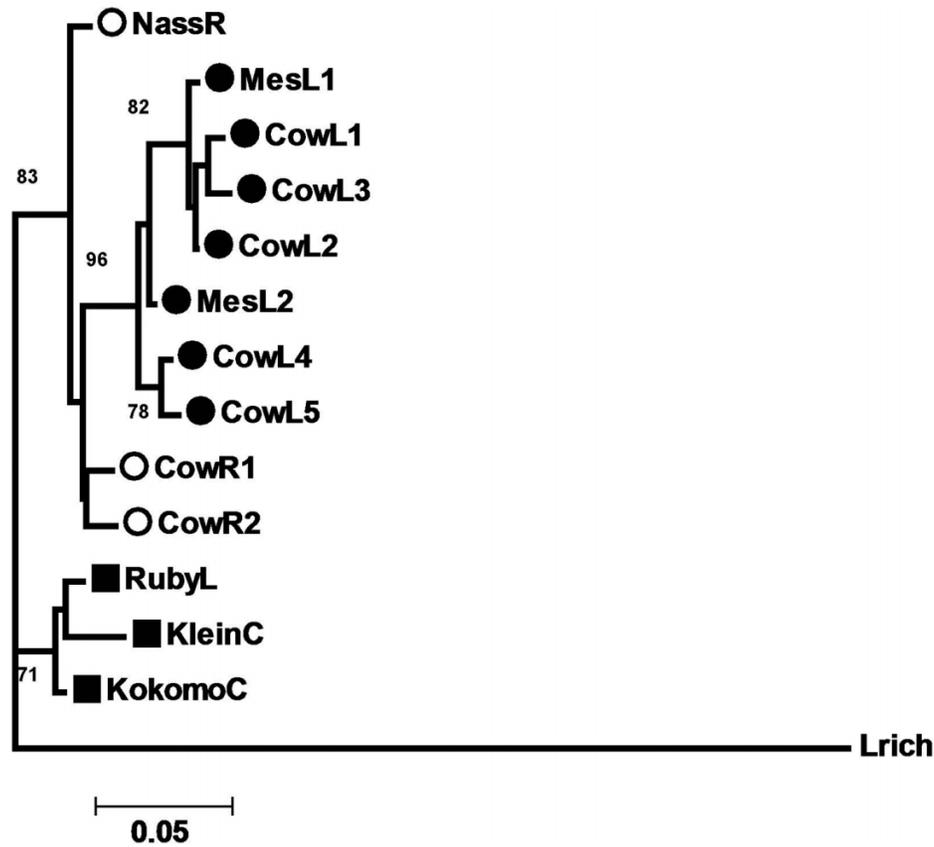
Fig. 3. Mean (+SD) proportional admixture of four genetic groups identified by five replicate STRUCTURE analysis (Pritchard et al. 2000) across 13 sample localities of Vancouver lamprey (*Entosphenus macrostomus*) (normal type) and Pacific lamprey (*Entosphenus tridentatus*) (italic type) assayed at eight microsatellite DNA loci. Localities with anadromous lampreys are underlined. Populations are grouped by sample locality: 1–7, Cowichan–Mesachie lakes (*E. macrostomus*); 8–9, Cowichan River (*E. tridentatus*); 10–12, Sakinaw–Ruby lakes (presumptive *E. tridentatus*); 13, Nass River (*E. tridentatus*). The bars from left to right within each population grouping are as follows: solid black bars, genetic group 1; white bars, genetic group 2; grey bars, genetic group 3; diagonal-hatched bars, genetic group 4; the quartet of bars within each population grouping represents the red, green, blue, and yellow genetic groups, respectively, shown in Fig. 2.



sampling, however, was focused on areas previously characterized as having adult *E. macrostomus* or *E. tridentatus*, respectively (Beamish 1982; Beamish and Wade 2010), and our samples of suspected anadromous *E. tridentatus* were genetically indistinguishable from our sample of known anadromous *E. tridentatus* from the Nass River. Consequently, we are confident that our assignment of samples to the two putative taxa is accurate.

Our data are the first genetic evidence that the Vancouver lamprey and parapatric Pacific lamprey represent distinct gene pools and, therefore, supports the recognition of the former as a distinct taxon (Beamish 1982). The F_{ST} values between *E. macrostomus* from Cowichan–Mesachie lakes and *E. tridentatus* in the Cowichan River averaged about four times those observed between two populations of *E. tridentatus* (Cowichan River and Nass River) that are located over

Fig. 4. Neighbour-joining tree of pairwise Cavalli-Sforza chord genetic distances generated from assays of eight microsatellite DNA loci in Vancouver lamprey (*Entosphenus macrostomus*) and Pacific lamprey (*Entosphenus tridentatus*). Numbers at branch points represent bootstrap percentage scores from 100 bootstrap pseudoreplicates. The tree is rooted using samples of western brook lamprey (*Lampetra richardsoni*) (“Lrich”). ●, Cowichan–Mesachie lake samples (*E. macrostomus*); ○, anadromous samples (*E. tridentatus*); ■, Sakinaw–Ruby lakes samples (presumptive *E. tridentatus*). Sample codes are defined in Table 1.



800 km from each other. The lack of differentiation between such distant anadromous populations is consistent with modest levels of genetic differentiation among Pacific lamprey populations from across wide areas of the Pacific basin (Goodman et al. 2008; Lin et al. 2008). Given that the greatest distance between Cowichan–Mesachie lake samples of *E. macrostomus* and Cowichan River samples of *E. tridentatus* is approximately 40 km and that there are no complete migration barriers between these areas (at least in a downstream direction), our data clearly imply that there must be substantial restrictions in gene flow between the two species in the Cowichan Lake–River system promoted by means other than physical distance alone. Although a fishway was constructed at Skutz Falls on the Cowichan River in 1955 to facilitate the upstream passage of salmon and trout, the falls were probably not a complete barrier to upstream movements by anadromous fishes including lampreys (e.g., Lill et al. 1975). The falls constitute a series of rapids and small chutes that drops 5.5 m over a horizontal distance of 90 m, but native populations of *E. tridentatus*, which are typically larger than *E. macrostomus* (Beamish 1980, 1982), have penetrated hundreds of kilometres upstream postglacially in large rivers such as the Fraser, Columbia, and Skeena rivers through similarly and undoubtedly more difficult passage areas (McPhail 2007; Beamish and Wade 2010). It is impossible to discount the possibility that anadromous lamprey occasionally enter Cow-

ichan Lake to spawn in the lake and its inlet tributaries, but those habitats are at the very least dominated by *E. macrostomus*. Interestingly, Cowichan Lake also contains a population of freshwater-resident sockeye salmon (“kokanee”, *Oncorhynchus nerka* (Walbaum in Artedi, 1792)). Although anadromous sockeye salmon are occasionally reported from the Cowichan River, this life-history type is very rare in the system (Neave 1949). Consequently, for whatever reason, the Cowichan Lake system appears to be a favourable one for the development of freshwater-resident life-history forms of some species.

Should *E. macrostomus* and *E. tridentatus* occasionally come into contact, several other aspects of their biology may minimize the potential for gene flow. First, the aforementioned differences in size may limit reproductive interactions, as mate selection is strongly size-dependent in lampreys (Hardisty and Potter 1971; Beamish and Neville 1992). Second, *E. macrostomus* in Cowichan Lake appears to spawn largely on submerged beach areas, an unusual behaviour in lampreys, whereas *E. tridentatus* favours tributary streams, as well as the mainstem Cowichan River, and they spawn earlier than *E. macrostomus* (Beamish 1982; Beamish and Wade 2010). Finally, the two species differ in physiological traits related to osmoregulation consistent with differences in their life history; *E. tridentatus* appears to be unable to persist in fresh water after metamorphosis, whereas *E. macrostomus* is capable of gradual acclimation to full strength

seawater (Beamish 1982). These differences in physiology strongly suggest that selection against hybrids between the species could also minimize gene flow between them in nature (cf. Salewski 2003).

Within each lake system, levels of genetic differentiation among locations were absent (Ruby–Sakinaw lakes) to modest (Cowichan–Mesachie lakes). The lack of genetic differentiation within lakes is perhaps not surprising given the small spatial scale of our sampling, especially within the Ruby–Sakinaw lakes, and the modest population structure apparently exhibited by *E. tridentatus* (Goodman et al. 2008; Lin et al. 2008; this study). Still, some significant differences were found between localities within Cowichan Lake, raising the possibility that genetically and perhaps demographically distinct populations of *E. macrostomus* exist within this system. Repeated sampling and analysis, however, are needed to determine if specific differences resolved in the current study are consistent across time and might be explained by factors such as variation in reproductive ecology (e.g., spawning in inlet streams versus lakeshore beaches).

Origin of freshwater parasitic lampreys

Our data and that of Docker et al. (1999) are consistent with a model of evolution of freshwater parasitic lampreys in Cowichan Lake from ancestral populations of anadromous *E. tridentatus*. This is suggested by the close similarity in morphology of the two species relative to other species (Beamish 1982), the lack of differentiation between them in mtDNA (Docker et al. 1999), and the formation of Cowichan Lake postglacially (i.e., <15 000 years ago; Halstead 1968). The formation of freshwater populations of fishes in coastal lakes from anadromous founders is a common inference in a variety of species complexes (reviewed in Behnke 1972; Taylor 1999). In addition, and again as is reminiscent of patterns in other taxa, our data suggest that the evolution of freshwater parasitic lampreys in lakes on the Sechelt Peninsula has occurred independently from that in the Cowichan Lake drainage. The lampreys in Ruby and Sakinaw lakes were clearly distinct both from anadromous *E. tridentatus* and from freshwater *E. macrostomus*, and the two groups of freshwater lampreys were reciprocally monophyletic and separated from each other by the anadromous Pacific lamprey samples. Espanhol et al. (2007) used a mtDNA phylogeny to infer multiple origins of the freshwater nonparasitic *L. planeri* from the parasitic *L. fluviatilis* in western Europe. The derivation of nonparasitic, freshwater lampreys from parasitic ancestral forms is a common occurrence in the Petromyzontidae and has resulted in a number of “paired” or “satellite” species in most genera (Zanandrea 1959; reviewed in Salewski 2003 and Docker 2009).

By contrast, *E. macrostomus* is one of the relatively few instances of a freshwater parasitic lamprey that is thought to have been derived from an anadromous parasitic form (*E. tridentatus*). Other freshwater parasitic derivatives of anadromous lampreys are known from relatively few areas: the Klamath Basin of Oregon and California where two other freshwater-resident parasitic lampreys in the genus *Entosphenus*, the Klamath lamprey (*Entosphenus similis* Vladykov and Kott, 1979 = *Lampetra similis* (Vladykov and Kott, 1979)) and the Miller Lake lamprey (*Entosphenus minimus* Bond and Kan, 1973 = *Lampetra minima* (Bond and Kan, 1973)),

are found; the Great Lakes of eastern North America where once anadromous sea lampreys (*Petromyzon marinus* L., 1758) have diverged into freshwater parasitic forms after their invasion of those systems via human-constructed shipping canals; *L. fluviatilis* in landlocked Lake Ladoga (Russia) and Loch Ness (Scotland); and the Arctic lamprey (*Lethenteron camtschaticum* (Tilesius, 1811)) in the Naknek River system (Alaska), Great Slave Lake (Northwest Territories), and elsewhere (see Docker 2009). Whereas the evolution of non-parasitic species is thought to result from the selection favouring abandonment of the feeding phase in the face of high costs associated with migration and predation risk during the adult feeding phase (Salewski 2003; Docker 2009), the relatively restricted distribution of these freshwater parasitic forms suggests that some specific ecological conditions are required for their evolution or persistence. Specific ecological conditions (a simplified fish community) are thought to have contributed to the localized evolution of benthic and limnetic species pairs of threespine stickleback (*Gasterosteus aculeatus* L., 1758) from ancestral marine forms (Ormond et al. 2011); presumably a minimum level of host fish forage base is critical for the evolution of freshwater parasitic lampreys. Alternatively, Beamish (1985) and Salewski (2003) argued that parasitic freshwater lampreys represent an intermediate phase in the transition from anadromous parasitic to freshwater nonparasitic forms (but see Docker 2009), thus suggesting that the parasitic freshwater form is evolutionarily unstable and lacks persistence relative to anadromous parasitic and freshwater nonparasitic forms.

The evidence for independent evolution of freshwater parasitic forms of *Entosphenus* that we have presented adds to the growing examples of parallel evolution in fishes (reviewed in Taylor 1999) and the potential role of ecology in divergence in lampreys (Salewski 2003). Finally, it is interesting to note that although studies have shown that *E. tridentatus* appears incapable of surviving in fresh water throughout its feeding phase (Beamish 1982; Clarke and Beamish 1988), it appears to have independently given rise to freshwater-resident parasitic derivatives at least three times (i.e., at least once in the Klamath basin, once in the Cowichan Lake system, and once on the Sechelt Peninsula).

Taxonomic and conservation implications

Beamish (1982) described *E. macrostomus* from a single lake system on Vancouver Island in southwestern B.C. and considered the species to be endemic to that system. Our data support the taxonomic distinction between *E. macrostomus* and *E. tridentatus* that was originally based on morphological, life history, and physiological data (Beamish 1982). Furthermore, our data indicate that even greater genetic differences occur between the Cowichan–Mesachie lakes *E. macrostomus* and a similar phenotype in Ruby and Sakinaw lakes on the Sechelt Peninsula than between either of these freshwater parasitic lampreys and *E. tridentatus*. There has, however, been no systematic phenotypic comparison of *E. macrostomus*, *E. tridentatus*, and other putative freshwater instances of *E. tridentatus* that have been reported from at least three other lakes in southwestern B.C. (McPhail 2007; Beamish and Wade 2010). Consequently, and until such a comprehensive comparison that includes the Sechelt and other populations has been completed, their taxonomic



status, and by extension that of *E. macrostomus*, retains some uncertainty. Should these other occurrences of freshwater parasitic lampreys show similar genetic relationships to each other and to the *Entosphenus* taxa that we have examined, it would appear that the group as a whole fits the model of “species complexes” described for other temperate fishes (Hagen and McPhail 1970; reviewed by Behnke 1972 and Taylor 1999) with important implications for current taxonomy (cf. Espanhol et al. 2007; Docker 2009).

Notwithstanding the taxonomic complications of multiple allopatric occurrences of parasitic freshwater lampreys derived from *E. tridentatus*, our data strongly support the recognition of these freshwater and anadromous parasitic lampreys as separate designatable units (Green 2005) within the context of Canada’s *Species at Risk Act*; they are discrete in terms of genetic traits and the differentiation in migratory life history, size at maturity, and associated morphological and physiological traits are consistent with adaptive differences that are significant to the evolutionary legacy and persistence of the *Entosphenus* complex.

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