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SHORT COMMUNICATION

Population genetic structure of the salmon louse, *Lepeophtheirus salmonis* (Krøyer) on wild and farmed salmonids around the Pacific coast of Canada

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The population genetic structure of the salmon louse (*Lepeophtheirus salmonis*: Caligidae) is of considerable interest because of indirect evidence suggesting transmission of this ectoparasite between wild salmon and farmed salmon (reviewed by Boxaspen 2006). Consequently several studies have looked for farm-specific molecular markers that would allow the path of transmission to and from wild fish to be traced. Unfortunately, an early report of RAPD markers that were specific to Scottish salmon farms (Todd, Walker, Wolff, Northcott, Walker, Ritchie, Hoskins, Abbott & Hazon 1997) was not found in a second RAPD study (Dixon, Shinn & Sommerville 2004) or in a more extensive study using six microsatellite markers (Todd, Walker, Ritchie, Graves & Walker 2004). Nevertheless, interest in finding farm-specific molecular markers continues. It is still not possible to follow the salmon lice larvae during the 4–14-day period they spend in the plankton before they infect a host (Johnson & Albright 1991). We therefore tested the research hypothesis that population genetic structure existed among salmon lice populations collected from different geographical regions of British Columbia, Canada, because of restricted gene flow.

Samples of the salmon louse, *L. salmonis*, were obtained from three different wild Pacific salmon species by sports fishing and during creel (sports fishery catch) surveys and from farmed Atlantic salmon by netting (Table 1). Sea lice were removed from each salmon host with forceps and preserved in 95% ethanol. To test for population genetic structure with the largest sample size possible, we decided to use a fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) (Folmer, Black, Hoeh, Lutz & Vrijenhoek 1994) that has been extensively used for DNA barcoding (Hebert, Cywinska, Ball & deWaard 2003). This allowed us to make use of a large number of existing salmon louse COI sequences and to easily compare the sequence divergence that we observed among different clades with that of other species of animals (Hebert *et al.* 2003).

Template DNA was extracted from individual salmon lice using modified proteinase K methods. A $1\,mm^3$ piece of ethanol-preserved tissue was obtained from each specimen and placed directly into 96-well plates containing lysis buffer and proteinase K. Subsequent DNA extraction used a glass fibre protocol (Ivanova, deWaard & Hebert 2006). A 710-bp fragment of the COI gene was polymerase chain reaction (PCR) amplified using the primer pairs LCO1490 and HCO2198 (Folmer *et al.* 1994) or LepF1 and LepR1 (Hebert, Penton, Burns, Janzen & Hallwachs 2004) and visualized in a 96-well E-Gel (Invitrogen Canada, Burlington, ON, Canada). The PCR mix included 6.25 μL of 10% trehalose, 1.25 μL 10 × PCR buffer, 0.625 μL (50 mM) $MgCl_2$, 0.125 μL (10 μm) of each primer, 0.0625 μL (10 mM) dNTPs, 0.0625 μL Platinum

Table 1 Collection data, BOLD IDs and GenBank accession numbers for 175 new *Lepeophtheirus salmonis* (Krøyer) samples

Region	Exact site	Latitude (°N)	Longitude (°W)	Host species	Date collected	No. of lice	No. of hosts	Process ID	Sample ID	GenBank
Barkley Sound	7 mile	48.8	−125.3	*Oncorhynchus tshawytscha*	17 August 2007	5	1	SLOBC119-07 to SLOBC115-07	2007-BCSL-023 to 2007-BCSL-019	FJ411843 to FJ411847
Barkley Sound	7 mile	48.8	−125.3	*Oncorhynchus gorbuscha*	17 August 2007	6	1	SLOBC114-07 to SLOBC109-07	2007-BCSL-018 to 2007-BCSL-013	FJ411848 to FJ411853
Barkley Sound	Diploc	48.9	−125.1	*Oncorhynchus tshawytscha*	17 August 2007	6	1	SLOBC126-07 to SLOBC121-07	2007-BCSL-030 to 2007-BCSL-025	FJ411837 to FJ411842
Barkley Sound	Diploc	48.9	−125.1	*Oncorhynchus tshawytscha*	18 August 2007	7	1	SLOBC154-07 to SLOBC148-07	2007-BCSL-058 to 2007-BCSL-052	FJ411811 to FJ411817
Barkley Sound	Flemming Island	48.9	−125.1	*Oncorhynchus tshawytscha*	18 August 2007	14	2	SLOBC146-07 to SLOBC127-07	2007-BCSL-050 to 2007-BCSL-031	FJ411818 to FJ411836
Barkley Sound	Kirby Point	48.9	−125.2	*Oncorhynchus tshawytscha*	18 August 2007	6	1	SLOBC160-07 to SLOBC155-07	2007-BCSL-064 to 2007-BCSL-059	FJ411805 to FJ1810
Barkley Sound	The Wall	48.8	−125.1	*Oncorhynchus tshawytscha*	18 August 2007	5	1	SLOBC100-07 to SLOBC099-07	2007-BCSL-004 to 2007-BCSL-003	FJ411860 to FJ411861
Barkley Sound	The Wall	48.8	−125.1	*Oncorhynchus tshawytscha*	17 August 2007	2	1	SLOBC138-07 to SLOBC133-07	2007-BCSL-042 to 2007-BCSL-037	FJ411826 to FJ411830
Barkley Sound	Whittlestone	48.8	−125.2	*Oncorhynchus tshawytscha*	17 August 2007	6	1	SLOBC108-07 to SLOBC103-07	2007-BCSL-012 to 2007-BCSL-007	FJ411854 to FJ11859
Broughton Archipelago	Blackfish	50.6	−126.8	*Oncorhynchus kisutch*	23 July 2006	5	1	SLOBC192-07 to SLOBC188-07	2007-BCSL-096 to 2007-BCSL-092	FJ411862 to FJ411866
Broughton Archipelago	Blackfish	50.6	−126.8	*Oncorhynchus gorbuscha*	25 July 2006	9	1	SLOBC169-07 to SLOBC161-07	2007-BCSL-073 to 2007-BCSL-065	FJ411796 to FJ411804
Broughton Archipelago	Fife	50.8	−126.6	*Oncorhynchus gorbuscha*	27 July 2006	17	3	SLOBC187-07 to SLOBC170-07	2007-BCSL-091 to 2007-BCSL-074	FJ411862 to FJ411795
Broughton Archipelago	Farm 1	50.7	−126.3	*Salmo salar*	1 November 2005	7	7	SLOBC007-06 to SLOBC001-06	2006-BCSL-007 to 2006-BCSL-001	FJ411970 to FJ411976
Broughton Archipelago	Farm 2	50.7	−126.5	*Salmo salar*	1 November 2005	7	7	SLOBC014-06 to SLOBC008-06	2006-BCSL-014 to 2006-BCSL-008	FJ411963 to FJ411969
Broughton Archipelago	Farm 4	50.6	−126.6	*Salmo salar*	1 November 2005	7	7	SLOBC028-06 to SLOBC022-06	2006-BCSL-028 to 2006-BCSL-022	FJ411949 to FJ4111955
Broughton Archipelago	Farm 9	50.6	−126.7	*Salmo salar*	1 November 2005	7	7	SLOBC059-06 to SLOBC053-06	2006-BCSL-059 to 2006-BCSL-053	FJ411918 to FJ411924
Broughton Archipelago	Farm 10	50.8	−126.3	*Salmo salar*	1 November 2005	7	7	SLOBC066-06 to SLOBC060-06	2006-BCSL-066 to 2006-BCSL-060	FJ411911 to FJ411917
Broughton Archipelago	Farm 13	50.6	−126.7	*Salmo salar*	1 November 2005	7	7	SLOBC087-06 to SLOBC081-06	2006-BCSL-087 to 2006-BCSL-081	FJ411890 to FJ411896
Broughton Archipelago	Farm X	50.8	−126.6	*Salmo salar*	1 March 2005	2	1	SLOBC096-06 to SLOBC95-06	2006-BCSL-096 to 2006-BCSL-095	FJ411881 to FJ411882
Campbell River	Farm 3	50.2	−125.1	*Salmo salar*	1 November 2005	7	7	SLOBC021-06 to SLOBC015-06	2006-BCSL-021 to 2006-BCSL-015	FJ411956 to FJ411962
Campbell River	Farm 6	50.3	−125.3	*Salmo salar*	1 November 2005	7	7	SLOBC039-06 to SLOB033-06	2006-BCSL-039 to 2006-BCSL-033	FJ411938 to FJ411944
Campbell River	Farm 7	50.2	−125.2	*Salmo salar*	1 November 2005	6	6	SLOBC045-06 to SLOBC040-06	2006-BCSL-045 to 2006-BCSL-040	FJ411932 to FJ1937
Campbell River	Farm 8	50.4	−125.3	*Salmo salar*	1 November 2005	7	7	SLOBC052-06 to SLOBC046-06	2006-BCSL-052 to 2006-BCSL-046	FJ411925 to FJ411931
Campbell River	Farm 11	50.2	−125.1	*Salmo salar*	1 November 2005	7	7	SLOBC073-06 to SLOBC067-06	2006-BCSL-073 to 2006-BCSL-067	FJ411904 to FJ411910
Campbell River	Farm 12	50.3	−125.1	*Salmo salar*	1 November 2005	7	7	SLOBC080-06 to SLOBC074-06	2006-BCSL-080 to 2006-BCSL-074	FJ411897 to FJ411903
Port Alice	Farm 5	50.5	−127.8	*Salmo salar*	1 November 2005	4	4	SLOBC032-06 to SLOBC029-06	2006-BCSL-032 to 2006-BCSL-029	FJ411945 to FJ411948
Prince Rupert	Farm 14	52.8	−128.4	*Salmo salar*	1 November 2005	7	7	SLOBC094-06 to SLOBC088-06	2006-BCSL-094 to 2006-BCSL-088	FJ411883 to FJ411889

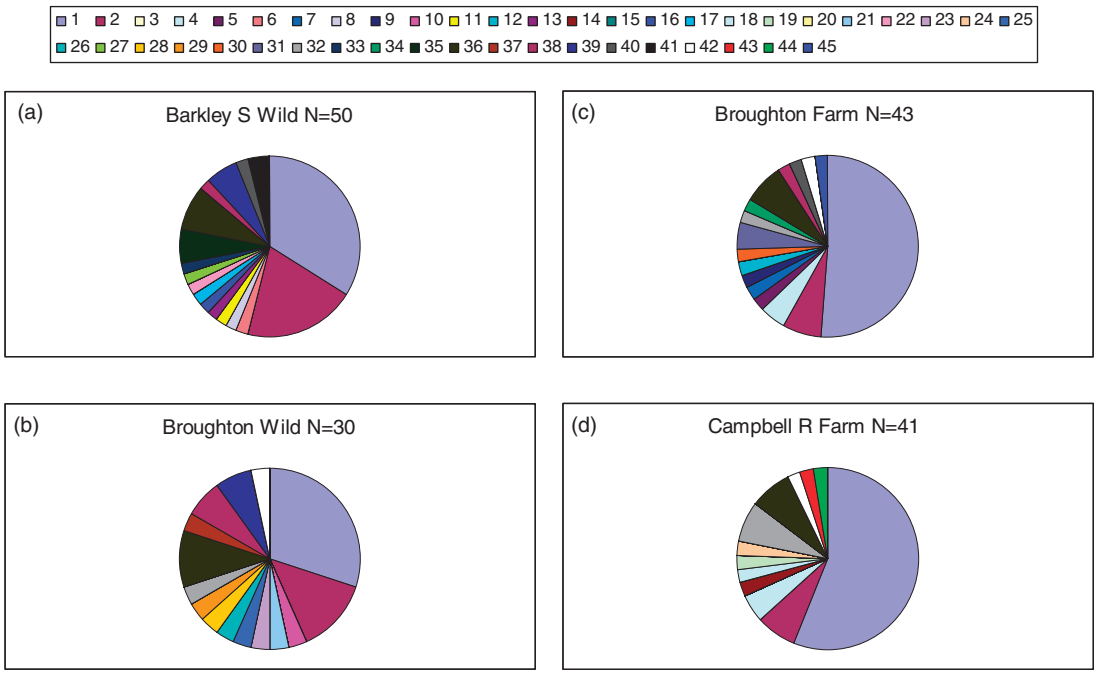

Figure 1 Pie diagrams of mtDNA haplotype frequencies in two farmed populations and two wild populations of salmon from British Columbia that had the largest sample sizes (Appendices S1 and S2).

Taq polymerase (Invitrogen), 2 μL of H_2O and 2 μL of template DNA for a total volume of ~12.5 μL. Polymerase chain reactions were run under the following thermal cycle conditions: 2 min at 94 °C followed by, 35 cycles of 40 s at 94 °C, 40 s at 54 °C and 1 min at 72 °C, and finally 10 min at 72 °C. Polymerase chain reaction products producing single clear amplicons were sequenced (following deWaard, Ivanova, Hajibabaei & Hebert 2008) in both directions using the PCR primers and BIGDYE version 3.1 on an ABI PRISM 3730 capillary sequencer (Applied Biosystems, Mississauga, ON, Canada). DNA sequences (see Table 1 for GenBank accession numbers) and photographs of all the salmon louse specimens have been deposited in the Barcode of Life Database (BOLD; http://www.barcodinglife.org).

For the population genetic structure analysis, all full-length sequences without any ambiguous sites were aligned manually using MEGA 4.0 (Tamura, Dudley, Nei & Kumar 2007) and truncated to the same length of 581 base pairs. We then exported only the parsimony informative sites to a new data file using MEGA. This removed the singleton haplotypes from our dataset, which increased our statistical power to detect population structure with the sample sizes that were available. We then used TCS 1.21 (Clement, Posada & Crandall 2000) to construct a haplotype network. To estimate spatial population structure, we performed exact tests of population differentiation, which is powerful when the sample sizes are small relative to the number of alleles (Raymond & Rousset 1995) using ARLEQUIN 3.01 (Excoffier, Laval & Schneider 2005).

A total of 194 COI sequences in the combined dataset between our 175 new samples and 19 previously published from Vancouver Island (Yazawa, Yasuike, Leong, von Schalburg, Cooper, Beetz-Sargent, Robb, Davidson, Jones & Koop 2008) were included in our population structure analysis. The dataset contained 29 variable sites that were present in at least two salmon lice and comprised 45 haplotypes (supporting information, Appendices S1–S3). Haplotype 1 was the most common and made up between 30 and 56% of the lice from four host populations that we had the largest samples from (Fig. 1). Haplotype 2 was the second most common and the network analysis with TCS suggests that it is likely ancestral to all other haplotypes (Fig. 2).

To our surprise, we observed considerable population structure not only between lice from wild salmon hosts caught on the east and west coasts of Vancouver Island (Barkley Sound Wild versus Broughton Wild) but also between lice from wild and farmed salmon hosts within a region (Broughton

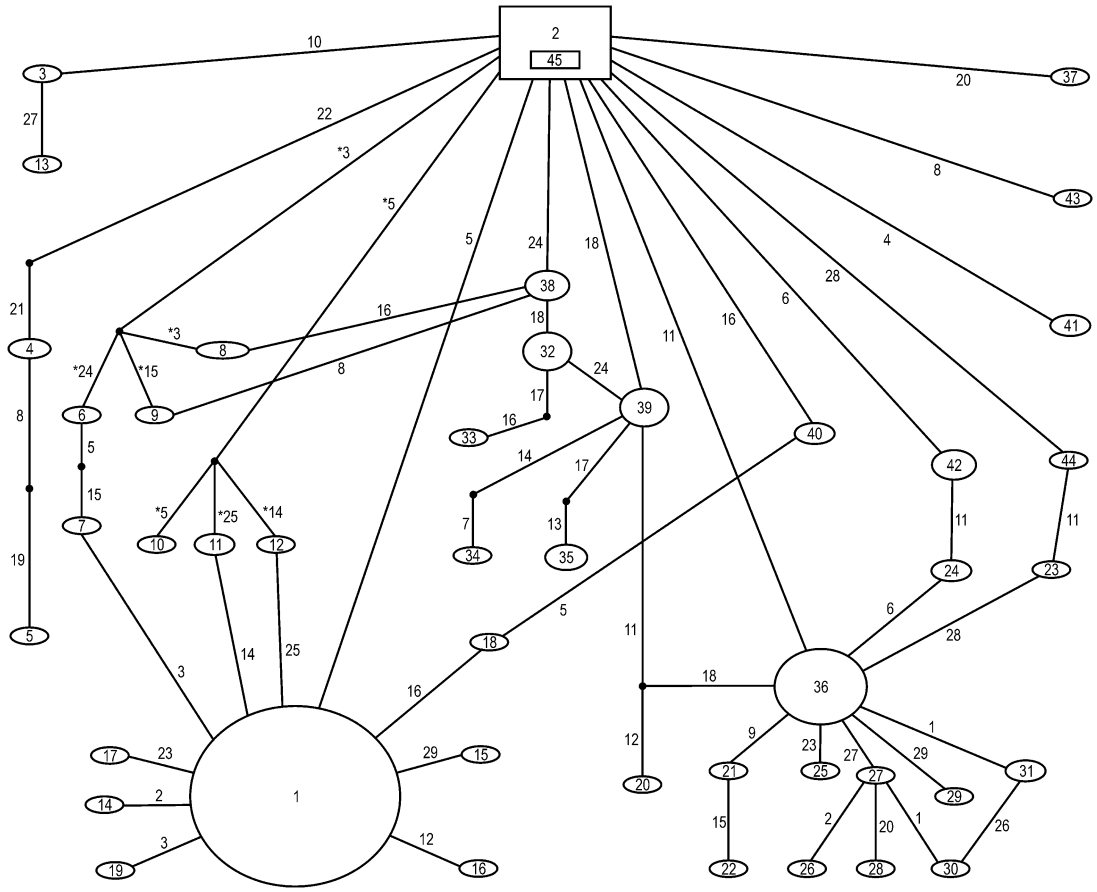

Figure 2 Number of mutation steps between all 45 cytochrome *c* oxidase subunit 1 mitochondrial haplotypes observed in this study as estimated by the program TCS. The size of the polygon represents the relative abundance of a particular haplotype. The rectangle refers to a haplotype (#2) that is likely ancestral to the others (Appendix S2). Nodes for intermediate haplotypes that were not observed in our sample are shown with a dot. Numbers along the branches refer to the number of the base pair where the substitution between the two haplotypes took place. Those with an asterix had more than two different kinds of nucleotides that were substituted.

Table 2 Above diagonal: F_{st} values between pairs of four large host populations of *Lepeophtheirus salmonis* calculated using conventional *F* statistics. Below diagonal: probability values from exact test of sample differentiation based on haplotype frequencies (Raymond & Rousset 1995)

	Barkley Sound Wild	Broughton Wild	Broughton Farmed	Campbell R. Farmed
Barkley Sound Wild		0.13**	0.21**	0.24**
Broughton Wild	0.00000 ± 0.0000		0.19**	0.22**
Broughton Farmed	0.00000 ± 0.0000	0.00000 ± 0.0000		0.29**
Campbell R. Farmed	0.00000 ± 0.0000	0.00000 ± 0.0000	0.00000 ± 0.0000	

**All F_{st} values were very highly significant even after a Bonferroni correction.

Wild versus Broughton Farmed; Table 2). This later result suggests that migration between the Broughton farmed and Broughton wild populations was low enough to allow the two populations to maintain different haplotype frequencies. Table 2 shows that the probability of observing this distribution of haplotypes under the null hypothesis of panmixia, given those predicted by the marginal values in the table,

is <0.00001. We believe that our detection of significant population structure with moderately small sample sizes is the result of using an exact test method of analysis of only the parsimony informative sites. We used this method to analyse the same 658 base pair 'BIO barcoding' portion (Folmer *et al.* 1994) of the salmon louse COI sequences first published by Tjensvoll, Glover and Nylund (2006) and we detected significant population structure between all pairs of their six Atlantic Ocean sites. Unlike their previous AMOVA analysis where they found 'little evidence to suggest that *L. salmonis* is divided into discrete populations . . . [except for a] weak degree of sub-division Canada and the Northeast Atlantic . . . ' our technique detected significant pairwise population structure between the salmon louse *L. salmonis* collected from Atlantic salmon (*Salmo salar*) from three Norwegian salmon farms (Appendix S4).

Analysis of 185 of our new COI sequences from British Columbia, combined with all previously published COI sequences for *L. salmonis*, using the Kimura two-parameter distance method (Kimura 1980) and the neighbour-joining tree-building algorithm implemented on BOLD shows two different clades (Fig. 3; Appendix S5) with considerable sequence divergence (4.8–7.7%) between samples collected from the Pacific and samples collected from the Atlantic. This is more than the 3% sequence divergence for this COI fragment that is typical among sibling species of animals (Hebert *et al.* 2003). Our larger dataset confirms previous estimates of divergence between the Pacific and Atlantic *L. salmonis* based on COI sequences from 63 salmon louse sampled from sites throughout the northern Pacific Ocean as well as the pattern of divergence shown for another mitochondrial gene (16S) and for nuclear EST sequence data (Yazawa *et al.* 2008). These combined results strongly suggest that *L. salmonis* from the Pacific coast should be described as a new species once detailed morphological work can be performed. We also compared our sequences with COI sequences published for the genus *Caligus* (Øines & Heuch 2005) and confirmed that none of our sequences were from that genus.

We have shown that it is feasible to use a highly repeatable molecular marker and high-throughput 'barcoding' methods (deWaard *et al.* 2008) to detect population genetic structure in the salmon louse. The presence of population structure among salmon louse from different regions of British Columbia supports our hypothesis that gene flow is restricted during the salmon louse larva's 4–14-day planktonic period. However, we would like to caution our readers that our current study contains too few lice sampled from too few hosts to make conclusive statements about the frequency of transmission of lice from wild fish to farm fish. We observed significant differences in haplotype frequencies between the

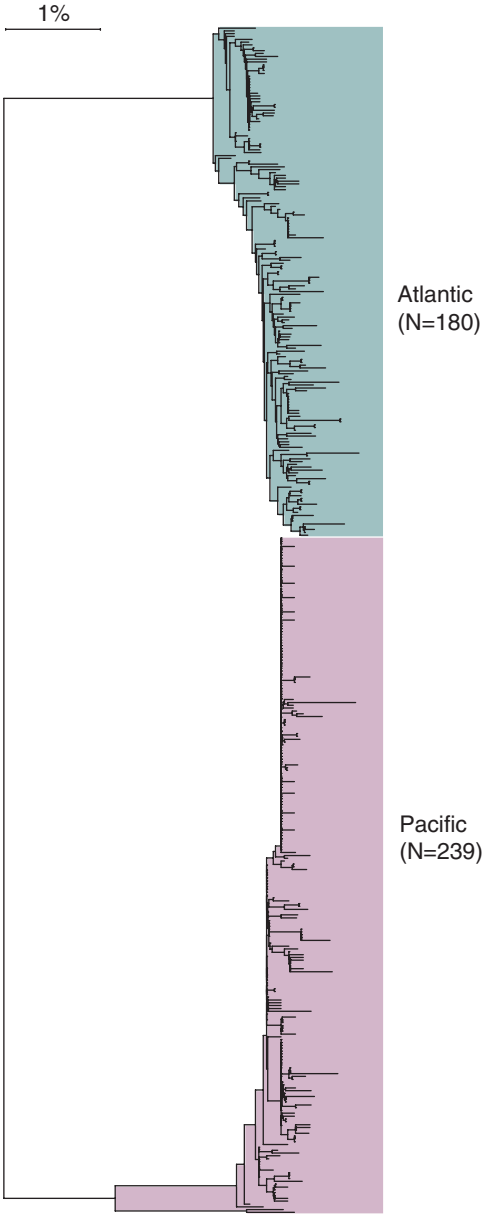

Figure 3 A neighbour-joining tree (Kimura two-parameter distance) of cytochrome *c* oxidase subunit 1 haplotypes from samples of the salmon louse, *Lepeophtheirus salmonis* (Krøyer), from the Northern Pacific and Northern Atlantic oceans (see Appendix S5 for larger version of tree with full population labels).

Broughton Archipelago farm fish and the Broughton Archipelago wild fish, which could be caused by a severe genetic bottleneck when lice from a small number of parents are transmitted between the farm fish and the wild fish. However, an alternative explanation is that the differences are the result of temporal variation in haplotype frequencies – the wild samples were collected in 2005 and the farm samples were collected in 2006. Temporal variation in haplotype frequencies can result from the sweepstakes reproductive success common in marine organisms with planktonic larval stages, whereby the next generation can be produced by a very few individuals (Hedgecock 1994) who are likely to be related because of spatial aggregation of relatives during settlement (Lee & Boulding 2007). Our preliminary results suggest that transmission of lice between the two populations is complex but could be better understood by collecting very large samples of adult salmon lice from both farm fish and wild fish over a period of several years. We recommend that several salmon lice from each host fish be preserved and genotyped for the 'BIO barcoding' COI fragment. This will enable the patterns of genetic variation within a host fish to be separated from those among host fish.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Abundances of 45 different COI haplotypes of the salmon louse sampled from nine different geographical locations (Table 1). Populations with a y suffix (Online Appendix 3).

Appendix S2. Sequence of COI haplotypes of the 45 haplotypes used in our population structure analysis. The haplotype number and sequence at bottom of a group applies to all of the sample IDs just above it.

Appendix S3. Correspondence between BIO Process IDs and GenBank accession numbers and names for 20 salmon lice samples from Vancouver Island, British Columbia from Yazawa et al. (2008). EU288204.1 was not used in the TCS haplotype network analysis because it has such a high sequence divergence that we suspect that it may be a different taxon.

Appendix S4. Exact Test of Sample Differentiation Based on Haplotype Frequencies using Parsimony informative sites within 658 bp "BIO barcoding" portion of COI sequences published by Tjensvoll et al. 2006

Appendix S5. A neighbour-joining tree (Kimura two parameter distance) of COI haplotypes labelled with the collection locations of individual salmon louse, *Lepeophtheirus salmonis* (Krøyer). The DNA sequence from each salmon louse is indicated by a BOLD Processing Sample Labels with their GenBank accession number following the vertical line. Sample labels beginning with SLOBC are new sequences from the current study (Table 1). The 54 samples prefixed with GGBSL are from throughout the Pacific Ocean (for map see Figure 1 in Yazawa et al 2008) comprise: EU288201-EU288216, EU288244-EU288246, EU288250-EU288251 from British Columbia, Canada (N = 20, see Online Appendix 3); EU288218-EU288230 Bering Sea (N = 9); EU288231-EU288243, EU288247-EU288249 Alaska, United States (N = 13); EU288252-EU288263 Hokkaido, Japan (N = 12). The 180 Samples prefixed with GGBCX are from the Atlantic Ocean (Tjensvoll et al., 2006) and comprise: AY602587-AY602616 from Finmark, Norway (N = 30); AY602617-AY602646 from Sogn og Fjordane, Norway (N = 30); AY602647-AY602676 from Øst-Agder, Norway (N = 30); AY602677-AY602706 from the Isle of Skye, Scotland (N = 30); AY602707-AY602736 are from the Republic of Komi, Russia (N = 30), and AY602737-AY602766 from New Brunswick, Canada (N = 30).

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Keywords: cytochrome *c* oxidase, mitochondrial DNA, sports fishing surveys, sweepstakes hypothesis

