

Supplementary Information

Collection and Maintenance of Fish

Some of our study populations were already well researched by other investigators but at other sites only minimal background information was available. We therefore chose, wherever possible, stream-resident populations in which all or nearly all individuals possessed only anterior plates, to ensure that we were studying stream-resident fish rather than anadromous sticklebacks or their immediate hybrids. The vast majority of individuals in anadromous populations typically have bony plates extending unbroken, or nearly so, along the sides of the body, whereas in many stream populations there are only anterior plates¹. Stream and anadromous fish bred sympatrically at three of the sites from which we collected females for the main mating tests; they were allopatric for the remaining populations (Supplementary Table 1).

Alaskan stream and anadromous sticklebacks were collected from a stream leading into Resurrection Bay, near Seward, Alaska. British Columbia stream and anadromous fish were collected in Langley from a tributary of the Salmon River. Icelandic stream-resident stickleback came from a stream at Eyrarbakki, southwestern Iceland, from which we collected only two anadromous individuals in two years of trapping. Norwegian stickleback were collected from a stream near Holsnoi which drains a small lake into the sea. There they occur in sympatry with spawning anadromous fish (P. Jakobsen, pers. comm., April 2001), although anadromous fish had not yet arrived when we made our collections and were not included in our study. We also excluded from our study any conspicuously keeled (the keel is an extension from the tail stock and is formed of modified lateral plates) individuals encountered at this site, in case they were hybrids between stream-residents and more heavily plated anadromous stickleback. Scottish stickleback were collected from two small streams leading into the River Tyne, near Dunbar. Stream-resident fish were collected from a high density site at which anadromous fish were not present. Anadromous fish (sympatric with low densities of stream-resident fish) were collected from another small stream directly off the main channel of the Tyne. Scottish anadromous males were used extensively in this study but because few females of this population were available for mating trials, they were tested only against Scottish males and are not included in analyses of reproductive isolation except within regions.

All Japanese anadromous fish were collected from streams near Kushiro on the East coast of Hokkaido Island and belonged to the Pacific, rather than Japan Sea, clade. It was necessary to collect from different streams in each of the three years of the study, owing to inter-year variation in run sizes and other logistical constraints. These streams lacked stream-resident stickleback, although a freshwater-resident population is present in a lake upstream from one of the sites. These anadromous populations were similar phenotypically (e.g., female mean SL's ranged only from 81.7-83.3mm among populations, mean male red scores from 3.5-3.8) and genetically (note that genetic data were available only for 2000, 2001) and produced similar results, so their mating pattern data were pooled in all analyses. In regression analyses involving genetic distance, we used average distances. Data from the 2000 study population were used for the AMOVA and phylogenetic analyses and are provided in Supplementary Table 2. Japanese stream fish were collected from the headwaters of Nakagawa Creek, a tributary of the Ibi River system, Honshu island.

All fish were held in mixed-sex tanks with members of their own populations.

Experimental Protocols

Females to be used in experimental trials were placed in aquaria held at the same temperature as experimental aquaria, 15-19°C depending upon the date, for at least 3 hours prior to testing. If the female entered the male's nest (swimming all the way into it) the trial was terminated and she was pushed through before she could release her eggs. Males were tested with up to two females (three in the case of Alaskan males, which were in short-supply), always of different populations, or raising treatments in the size-manipulation study. Nest inspection, the penultimate stage in successful courtship, was defined as the female inserting her snout into the nest-entrance.

Females were tested only once. In red-scoring, males were scored while a same-population gravid female was held in his tank in a 1 l flask. They were scored on a three-level scale at five anatomical landmarks and these scores summed. A subset of males were further assessed using spectrophotometry, which gave similar results (McKinnon and Aurit, unpublished).

Population-combinations in mating trials are summarized in Supplementary Table 1.

Overall, we tested an average of 11.3 females from each of 10 populations with an average of 7.5 male populations for a total of 850 trials. The slopes presented in Fig. 2 correspond to the following populations, from right to left along the top margin then top to bottom along the y axis: Japan anadromous, British Columbia anadromous, Scottish stream, Alaska anadromous, Norway stream, Iceland stream, British Columbia stream, Japan stream, Alaska stream.

Size manipulations

Field-collected sticklebacks were used as parents for all crosses except that parents of the small-treatment Japan anadromous females were laboratory-raised. All fish were kept at approximately 18°C from May through October, 12-15°C in the winter.

Genetic Distance and Phylogenetic Analyses

We estimated how long populations had been isolated using time (in generations, approximately years) = $\delta\mu^2/2*u$ where u = mutation rate, both the human mutation² rate, $5*10^{-4}$; and the sheep-chicken rate³, $1.2*10^{-4}$. Mean genetic distances and estimates of divergence time are given in Supplementary Tables 2-3. AMOVA's were calculated using Arlequin⁴.

A neighbor-joining tree based on $\delta\mu^2$, which takes allele length into account, groups populations mainly by geography rather than by ecotype, as suggested by the AMOVA's and as assumed in our analyses of mating compatibility (Supplementary Fig. 1). We compared this tree to other trees using maximum likelihood and the Kishino-Hasegawa test⁵ implemented in the program CONTML of PHYLIP⁶, which takes only allele frequency into account, not allele length. Our $\delta\mu^2$ tree was found to differ significantly from a tree allowing just a single origin of each ecotype (diff ln L = -76.56, SD=36.61, $p<0.05$), consistent with our assumptions and the AMOVA analyses. Our $\delta\mu^2$ tree did not differ significantly from the maximum likelihood (ML) tree calculated from allele frequencies only (consensus CONTML tree based on 500 bootstraps of the allele frequency matrix using the SEQBOOT program of PHYLIP, allele frequencies calculated using GENEPOP⁷: diff ln L = -9.36, SD=7.14, $p>0.05$). Nor did our tree differ from a tree minimally modified to possess unequivocally independent origins of the stream populations (diff ln L = -5.57, SD=5.22, $p>0.05$; this adjustment was limited to the four stream populations for which we have data from the adjacent anadromous populations). The topology of the Delta μ^2 tree is similar to the independent origins tree except that it groups anadromous populations from BC and Alaska together rather than with their adjacent stream populations (Supplementary Fig. 1). This arrangement, if correct, might result from low levels of gene flow between Alaska and

British Columbia anadromous populations via anadromous populations between them. Even so, the possibility of genetic interdependence of these two anadromous populations would have little effect on our analysis of mating compatibility because evolutionary history was found not to influence mating patterns significantly. In addition, these two anadromous populations differ significantly in body size ($p < 0.0001$, two sample t-test: Alaska anadromous females are more than 15% larger than British Columbia anadromous females), which our analyses consistently suggest is very important to mating patterns.

In the interests of caution, however, we have conducted additional analyses that assumed not only that these anadromous populations are not independent but also that the British Columbia stream and Alaska stream populations share a common origin and that the Scottish stream and Norway stream populations share a common origin; this is the most conservative scenario for these populations that is at all plausible. Following this logic, we have averaged mating results for each of these new regions (Western North America and Western Europe excluding Iceland) and conducted new tests of assortative mating by ecotype with fewer degrees of freedom. The test for parallel reproductive isolation remains significant ($p = 0.023$, t-test, 1-tailed, $n = 6$). In an analogous, conservative reanalysis, the test for assortative mating by size also remains significant ($p = 0.0024$, t-test, 1-tailed, $n = 6$).

Supplementary References

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Supplementary Table 1: Population combinations tested.

Female Population	Male Population									
	Alaska Anad	Alaska Stream	BC Anad	BC Stream	Iceland Stream	Japan Anad	Japan Stream	Norway Stream	Scotland Anad	Scotland Stream
Alaska Anad	Sy,SR (6)	-	Al,DR (3)	Al,DR (7)	-	-	Al,DR (2)	Al,DR (1)	-	-
Alaska Stream	Sy,SR (5)	Sy,SR (5)	Al,DR (6)	Al,DR (9)	Al,DR (4)	Al,DR (1)	Al,DR (1)	Al,DR (3)	-	-
BC Anad	Al,DR (10)	-	Sy,SR (13)	Sy,SR (14)	Al,DR (6)	Al,DR (21)	Al,DR (18)	Al,DR (8)	-	-
BC Stream	Al,DR (10)	Al,DR (3)	Sy,SR (13)	Sy,SR (14)	Al,DR (14)	Al,DR (24)	Al,DR (21)	Al,DR (10)	Al,DR (15)	Al,DR (13)
Iceland Stream	Al,DR (9)	Al,DR (4)	Al,DR (12)	Al,DR (14)	Sy,SR (14)	Al,DR (9)	Al,DR (14)	Al,DR (9)	Al,DR (13)	Al,DR (9)
Japan Anad	Al,DR (11)	-	Al,DR (29)	Al,DR (33)	Al,DR (14)	Sy,SR (29)	Al,SR (26)	Al,DR (9)	Al,DR (10)	Al,DR (8)
Japan Stream	Al,DR (10)	Al,DR (4)	Al,DR (16)	Al,DR (17)	Al,DR (11)	Al,SR (19)	Sy,SR (18)	Al,DR (9)	Al,DR (8)	Al,DR (8)
Norway Stream	Al,DR (2)	-	Al,DR (8)	Al,DR (9)	Al,DR (10)	Al,DR (6)	Al,DR (11)	Sy,SR (13)	-	-
Scotland Anad	-	-	-	-	-	-	-	-	Sy,SR (16)	Sy,SR* (14)
Scotland Stream	-	-	Al,DR (9)	Al,DR (11)	Al,DR (11)	Al,DR (11)	Al,DR (11)	-	Al,SR* (16)	SI,SR (16)

Anad indicates anadromous, Sy indicates sympatry, Al indicates Allopatry, SR indicates same region, DR indicates different region, - indicates no trials. Sample size is given in brackets.

*The Scottish stream population did not, to the best of our knowledge, co-occur with anadromous sticklebacks. The Scottish anadromous population resided alongside an apparently low density of stream-resident sticklebacks which were likely closely related to the nearby Scottish stream population; thus one Scottish population is considered allopatric with the opposite ecotype and the other is considered sympatric.

Supplementary Table 2. Pairwise mean genetic distances for three common distance measures.

	Alaska Anad	Alaska Stream	BC Anad	BC Stream	Iceland Stream	Japan Anad	Japan Stream	Norway Stream	Scotland Anad
Alaska Stream	3.765	*							
	0.49	*							
	0.359	*							
BC Anad	0.572	3.589	*						
	0.343	0.47	*						
	0.085	0.307	*						
BC Stream	7.393	1.205	5.178	*					
	0.421	0.49	0.41	*					
	0.224	0.439	0.193	*					
Iceland Stream	12.01	9.261	9.507	7.817	*				
	0.555	0.609	0.527	0.522	*				
	0.501	0.750	0.442	0.418	*				
Japan Anad	7.942	3.607	6.547	3.342	14.6	*			
	0.638	0.639	0.622	0.612	0.754	*			
	0.625	0.774	0.541	0.468	1.050	*			
Japan Stream	49.614	40.789	49.851	42.588	45.352	33.026	*		
	0.868	0.864	0.833	0.819	0.771	0.864	*		
	2.011	1.922	1.767	1.729	1.428	1.978	*		
Norway Stream	19.984	18.285	17.805	16.591	5.885	23.24	35.113	*	
	0.57	0.687	0.568	0.599	0.409	0.785	0.846	*	
	0.528	0.895	0.580	0.652	0.241	1.335	1.787	*	
Scotland Anad	12.427	14.092	11.344	14.13	2.341	18.35	40.93	6.307	*
	0.491	0.639	0.487	0.516	0.322	0.711	0.789	0.405	*
	0.341	0.736	0.344	0.362	0.098	0.822	1.582	0.245	*
Scotland Stream	12.129	10.938	9.753	9.755	4.455	16.986	57.296	16.078	9.088
	0.711	0.719	0.672	0.715	0.543	0.806	0.879	0.658	0.601
	0.670	0.844	0.555	0.719	0.341	1.310	2.140	0.611	0.444

For each pair of populations, mean genetic distances were calculated using *Microsat* with 1000 bootstraps. The top value in each cell is $\delta\mu^2$, the middle value is (1-proportion of shared alleles), and the bottom value is Nei's D. Anad indicates anadromous.

Supplementary Table 3. Estimates of divergence time based on $\delta\mu^2$.

	Alaska Anad	Alaska Stream	B.C. Anad	B.C. Stream	Iceland Stream	Japan Anad	Japan Stream	Norway Stream	Scotland Anad
Alaska Anad	0								
Alaska Stream	3362±1594 15688±7438	0 0							
B.C. Anad	511±374 2383±1746	3204±1275 14954±5950	0 0						
B.C. Stream	6601±3525 30804±16450	1076±539 5021±2517	4623±2267 21575±10579	0 0					
Iceland Stream	10723±5111 50042±23850	8269±3043 38588±14200	8488±4372 39613±20404	6979±3133 32571±14621	0 0				
Japan Anad	7091±3485 33092±16263	3221±974 15029±4546	5846±3131 27279±14613	2984±1180 13925±5508	13036±4755 60833±22192	0 0			
Japan Stream	44298±17712 206725±82654	36419±16449 169954±76763	44510±19796 207713±92379	38025±17445 177450±81408	40493±18403 188967±85879	29488±13788 137608±64342	0 0		
Norway Stream	17843±10058 83267±46938	16326±5535 76188±25829	15897±8294 74188±38704	14813±4887 69129±22804	5254±1983 24521±9254	20750±7252 96833±33842	31351±10036 146304±46833	0 0	
Scotland Anad	11096±7514 51779±35067	12582±6318 58717±29483	10129±8037 47267±37504	12616±5803 58875±27079	2090±997 9754±4654	16384±7036 76458±32833	36545±16953 170542±79113	5631±3415 26279±15938	0 0
Scotland Stream	10829±3287 50538±15338	9766±4613 45575±21529	8708±2527 40638±11792	8710±4369 40646±20388	3978±1613 18563±7525	15166±6742 70775±31463	51157±20785 238733±96996	14355±4390 66992±20488	8114±4305 37867±20092

Time is in generations based on $\delta\mu^2$ distances (see Supplementary Table 2). For each pair of populations, two estimates \pm SE are given: the upper value is based on a mutation rate from humans; the lower value is based on a mutation rate from chickens or sheep. Anad indicates Anadromous.

Figure Legends

Supplementary Fig. 1. Consensus unrooted neighbor-joining tree based on $\delta\mu^2$, generated using PHYLIP's "Neighbor," for the ten study populations. Values at nodes represent level of support for 1000 replicate bootstrap analyses (*Microsat*). The first one-two letters of each label denote region (A, Alaska; BC, British Columbia; I, Iceland; J, Japan; N, Norway; S, Scotland) whereas the final letter indicates ecotype (A, anadromous; S, stream). Trees constructed with other distance measures or by continuous maximum likelihood produced similar topologies with comparable bootstrap support.

