Mitochondrial DNA in Gasterosteus and Pleistocene glacial refugium on the Queen Charlotte Islands, British Columbia

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Abstract: Biotic refugia during the Pleistocene have been postulated for the Queen Charlotte Islands, British Columbia, although geological data suggest complete ice coverage. Based on restriction endonuclease map variation in mitochondrial DNA, we show that morphologically endemic freshwater populations of threespine stickleback (Gasterosteus aculeatus) contain two lineages of haplotypes. One is widely distributed and closely related to marine haplotypes (0.9% maximum sequence divergence), consistent with a postglacial origin. A second is restricted to the Argonaut Plain and is highly distinctive (2.09% minimum and 2.84 maximum sequence divergence from marine haplotypes) indicative of prolonged isolation in freshwater. This is the first molecular genetic evidence that a refugium may have persisted on the archipelago throughout the Pleistocene.

The Queen Charlotte Islands, 90 km off the northwest coast of North America, were extensively glaciated in the Pleistocene but the presence of plant species with globally disjunct distributions suggests that parts of the archipelago, such as mountain tops, remained permanently ice-free (1). Morphological endemism, generally at the sub-specific level, in angiosperms, crustaceans, birds and mammals has also been cited as supporting evidence for refugia (2) yet post-glacial colonization and differentiation have not been excluded. Recently, discovery of a continuous pollen profile over the last 16 000 years have led to a proposal of a refugium in the Argonaut Plain on the north-eastern region of the archipelago (3). Geologically, this area seems an unlikely candidate since it is low elevation, underlain with glacial outwash gravels, and from recent investigations of deep-sea cores, was probably inundated from mainland and local icesheets (4). We have undertaken an analysis of mitochondrial DNA (mtDNA) to determine whether there are genetic markers consistent with long term continuity of lineages in this region. Evolutionary studies of the threespine stickleback (Gasterosteus aculeatus) in freshwater lakes of the archipelago show the occurrence of morphological traits that are highly derived from the putative marine ancestors (5). Thus, these are logical candidates for molecular analyses. Results from a preliminary mtDNA study on three of these freshwater populations on the southern border of the Argonaut Plain indicate high similarity with marine stickleback, consistent with rapid evolution in postglacial periods (6). We

characterize here the mtDNA from four northeastern Argonaut populations, five populations from elsewhere on the Queen Charlotte Islands, one from the adjacent mainland of British Columbia and one from the Atlantic Ocean near Nova Scotia (Figure 1). Our data demonstrate the presence of two distinctive mtDNA lineages in the archipelago, one of which may have persisted in a freshwater refugium on the Argonaut Plain throughout the Pleistocene.

Total cellular DNA (nuclear and mitochondrial) was extracted from 147 individuals. Samples were digested with 10 restriction endonucleases (HinFI, BglI, EcoRI, HindIII, PstI, PvuII, SstI, SstII, SalI, and HincII)(7). Resulting fragments were separated by electrophoresis through 1% agarose gels and transferred to nylon membranes by Southern blotting techniques (8). MtDNA from threespine stickleback cloned into the plasmid pUC19 (9) was radioactively labelled (10), and then hybridized to membrane-bound DNA at 65 degrees C in a solution of 7% SDS, 1.0mM E.D.T.A. and 0.263M Na_2HPO_4, pH8.0 (11). Membranes were washed (12) and then exposed to X-ray film for 1 to 3 days.

Restriction site maps of all enzymes used in the survey, except HinFl, which recognizes a tetranucleotide sequence, were constructed using double digestion and partialling procedures (13); of the 40 sites located, 18 were polymorphic (Figure 2a). Since a large number of fragments were generated by HinFl, these restriction sites were not mapped. Nucleotide diversity between pairs of haplotypes was estimated from only mapped site differences using the maximum likelihood method of Nei and Tajima (14). Haplotypes were arranged in

a hand-drawn parsimony network and also clustered with UPGMA using sequence divergence. As the topologies were congruent, only the former is presented.

We identified 11 mtDNA haplotypes from the Pacific region. These include a common form (43% of individuals sampled), designated as A, four haplotypes with moderate (12-17%) frequencies and six rare (<7%) haplotypes. These were grouped into two lineages based on site changes and distinctive HinF1 restriction fragment patterns (Figure 2b). The first is composed of 9 haplotypes of which all but one can be derived from the closest haplotype by a single site change. The haplotypes were widespread, occurring in marine waters, in the freshwater locality from the mainland of British Columbia and those from the Queen Charlotte Islands south of the Argonaut Plain. Localities include morphologically diverse stickleback such as three allopatric populations with gigantism in body size (5). The similarity of the haplotypes in the freshwater populations to the marine haplotypes is consistent with a postglacial origin of the freshwater populations. This assemblage of related haplotypes will be referred to as the Marine lineage. A second, highly distinctive lineage was composed of two haplotypes (K, L) which were at least seven sites removed from the Marine lineage. Since these were restricted to the Argonaut Plain, the two haplotypes will be referred to as the Argonaut lineage. Most localities surveyed had only a single lineage but three localities, all within Argonaut Plain, contained both Marine and Argonaut lineages.

Haplotype frequencies and nucleotide divergence estimates within and

among these populations are shown in Table 1. Pairwise estimates between Argonaut and marine haplotypes averaged 2.49% (2.09-2.84%). Using the standard "molecular clock" of 2% sequence divergence per million years calibrated for mammals (15), the Argonaut and Marine lineages would have diverged 1.2 million years B.P. near the beginning of the Pleistocene. Uncertainties in calibration and differential rates of mtDNA evolution (16) limit confidence in this estimate, but even if the rates were an order of magnitude higher, the mean time for divergence of the Argonaut and Marine lineages would be over 100 000 years B.P., well before the last glacial advance. The absence in marine waters of the Argonaut haplotypes or any of the seven intermediate haplotypes between the Argonaut lineage and marine haplotypes strongly suggests that the lineage has been isolated in a freshwater refugium throughout the last two glacial advances and perhaps throughout much of the Pleistocene. Furthermore, the presence of both lineages in three populations on the Argonaut Plain, the confinement of the divergent lineage to the Argonaut Plain and the absence of intermediate haplotypes suggest secondary contact after prolonged isolation (17,18). Phylogenetic discontinuities of the magnitude observed here are rare within geographically small regions but when present have been associated with independent evidence for prolonged periods of isolation and secondary contact (19). Morphological data do not substantiate evidence for current introgression between marine stickleback and the distinct forms found in these populations nor have marine stickleback been observed in the lakes or outflow channels (20). Genetic exchange may have occurred during the elevated sea

levels about 8000 years B.P. when submerged river valleys on the Argonaut Plain (21) would have provided closer proximity of marine stickleback to these endemic lake populations.

Characterization of the Atlantic mtDNA haplotype (J) accentuates the divergence observed in the Queen Charlotte localities. Currently, the northern extent of G. aculeatus within the Pacific is the Bering Strait; in the western Atlantic, the species occurs to the northern region of Hudson Bay (22). There are no extant populations of stickleback across the central Arctic and time of last contact between Pacific and Atlantic stickleback is unknown. In spite of the 8000 km presently separating these populations, the Atlantic haplotype is less divergent from the Pacific marine haplotypes (1.15% divergence) than the Pacific marine haplotypes are from the Argonaut lineage (2.09% minimum divergence) suggesting that the Atlantic and Pacific Gasterosteus haplotypes have diverged more recently than the Argonaut and Pacific lineages (23).

An alternative interpretation to the postulated Pleistocene origin of the Argonaut populations is postglacial colonization from marine stickleback with a subsequently increased rate of differentiation in the Argonaut Plain populations relative to those elsewhere in the archipelago. The substitution rates of non-neutral mutations in small populations that have undergone repeated bottlenecks can be more rapid than in those with a very large population size (24). In Drosophila, rates of mtDNA evolution were three times higher in small than in large populations (25). The highest frequencies of the L haplotype were found in the two smallest lakes (approximately 1 ha), where

bottlenecks could have occurred. However, the L haplotype is found in two watersheds separated by marine waters. For this bottleneck hypothesis to be correct would require parallel evolution of the L haplotype, concomitant loss of intermediate haplotypes and an evolutionary rate several orders of magnitude higher than those reported in the literature (26). Postglacial colonization would also be implicated and the present hypothesis refuted if, with further surveys, L or K haplotypes were found in marine waters. Such a restricted spatial distribution of mtDNA haplotypes in continuously distributed marine habitat would be unusual (21,27), and would furthermore require the enigmatic loss in marine waters of the seven intermediate haplotypes which connect K to the nearest marine haplotype.

The stickleback population of Rouge Lake was the only one surveyed on the Argonaut Plain that did not contain any marine mitochondrial types in the sample. While this could be sampling error, the differentiation in the mtDNA is consistent with several other anomalies observed at this site. Morphologically, the fish exhibit some of the most pronounced reduction in body armour and spines observed in the species and contain a unique symbiotic association with a new taxon of dinoflagellate which appears to have no counterpart elsewhere in the circumboreal distribution of either group (28). Furthermore, all of the populations with the divergent mtDNA lineage lack bony lateral plates, which represents a highly derived feature in this species. When taken in concert with the predicted divergence time from the mtDNA, an extended preglacial history in the Argonaut Plain is strongly indicated. If

refugia were large enough to support freshwater fish throughout the Pleistocene, then other associated biota such as invertebrates, anadromous fishes (i.e. Oncorhynchus) and terrestrial vertebrates may also have persisted during this period (29). A highly divergent haplotype in any of the remaining endemics would greatly strengthen the hypothesis of a Pleistocene refugium in the archipelago.

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Table 1. Haplotype composition, within population divergence, and distance from nearest Pacific marine haplotype for Gasterosteus populations. N = individuals surveyed. Localities as in Figure 1.

Location (N)	Haplotype (%)	Maximum Within Population Divergence (%)	Divergence from Nearest Marine Haplotype (%)
Rouge (10)	L (100)	0	2.455
Harelda (16)	A (56) C (13) L (31)	2.744	2.455
Serendipity (15)	A (40) C (7) L (53)	2.744	2.455
Imbre (15)	A (20) B (13) C (47) K (7) L (13)	2.837	2.091
Drizzle (12)	A (100)	0	0
Boulton (9)	A (78) D (11) G (11)	0.812	0.564
Mayer (11)	A (28) H (72)	0.564	0.564
Skidigate (12)	A (33) B (8) H (50) I (8)	0.868	0.868
Van (11)	C (9) F (91)	0.268	0.268
Shrimp (12)	A (67) H (33)	0.564	0.564
Masset (8)	A (38) C (62)	0.276	-----
Sheldon (13)	A (62) C (31) E (8)	0.565	-----
Nova Scotia (3)	J (100)	0	1.151

Figure 1. Collection localities for Gasterosteus. Marine populations- Masset Inlet (MI), Nova Scotia (NS) and Sheldon's Lagoon (SL). Freshwater populations-Boulton Lake (BO), Drizzle Lake (DR), Harelda Lake (HA), Imbre Lake (IM), Mayer Lake (MY), Rouge Lake (RO), Serendipity Lake (SE), Shrimp Lake (SR), Skidigate Lake (SK) and Van Lake (VA). Hatched area denotes glacial outwash plain (Argonaut Plain). Deglaciation at south-east edge of plain occurred 16 000 y BP (3).

Figure 2a. Positions of monomorphic (outside circle) and polymorphic (inside circle) restriction sites for the enzymes BglI (B), EcoRI (E), HincII (H), HindIII (D), PstI (P), PvuII (U), SalI (A) SstI (S), and SstII (T). Numerical subscripts associated with each variable site are referenced in Figure 2c for defining composite site maps of all haplotypes observed. HincII recognizes a subset of the hexanucleotide sequence identified by SalI, and the two corresponding sites often changed together; in such cases SalI was bracketed (A), and not incorporated into the analyses. A_1, however, behaved independently of the corresponding H_8, and was therefore treated as an additional site.

Figure 2b. MtDNA topological network, distribution and frequency of haplotypes, and presence/absence for all sites mapped for Gasterosteus aculeatus from Queen Charlotte Islands, British Columbia. Locality acronyms as in Figure 1.

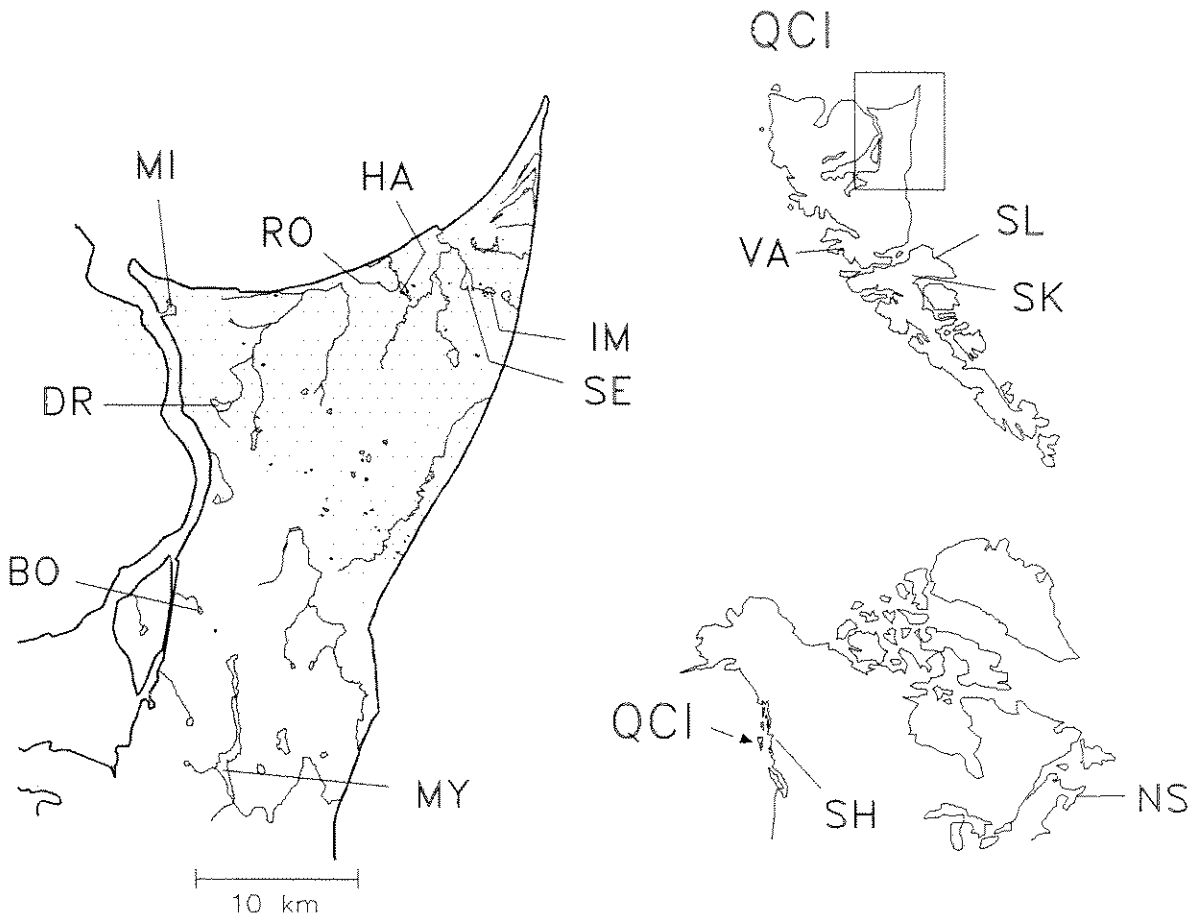
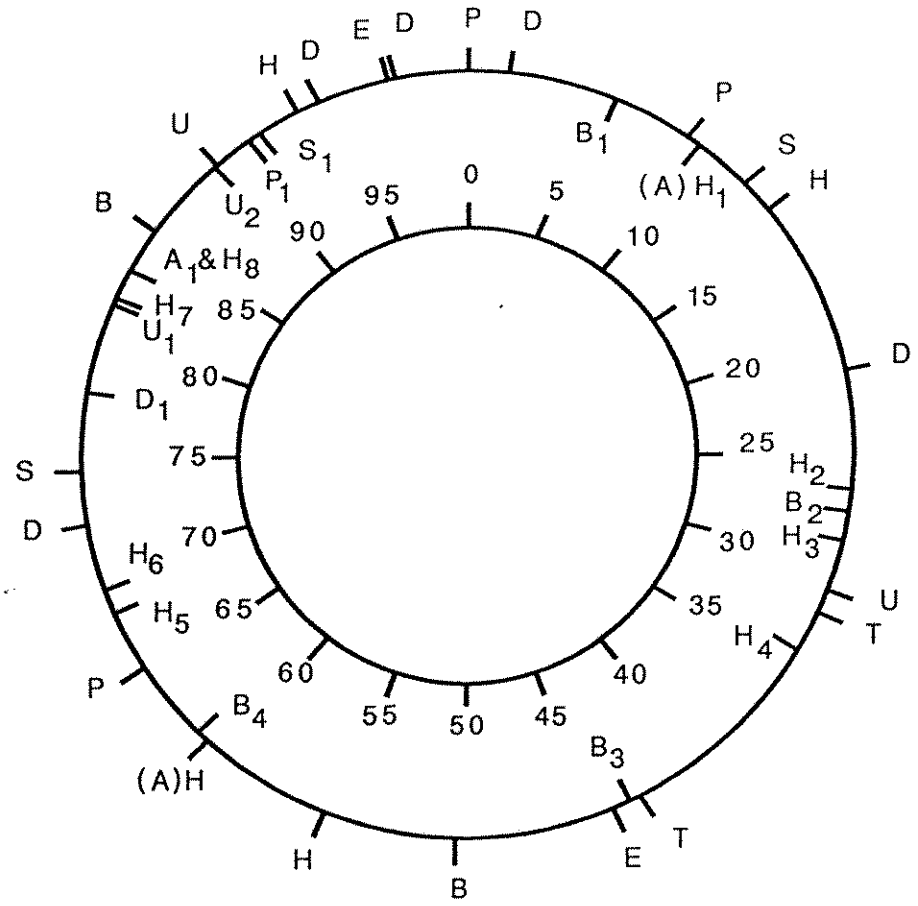


Fig 1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H D
U
S1
P1
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H
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E
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B3
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U
T
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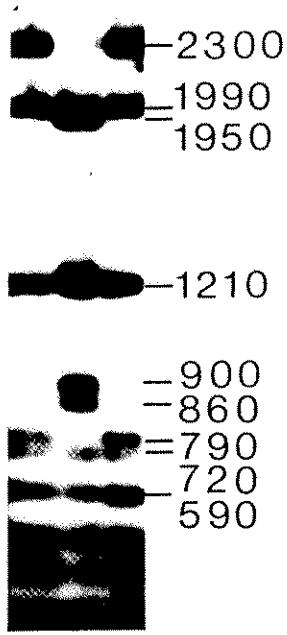
2300
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1950
1210
900
860
790
720
590

32 54

Table 1. Composite restriction site and restriction fragment profiles of mtDNA haplotypes.

	Type	Location(s)	Freq (%)	Restriction Site Pattern: Bgl1 (B) B_1	B_2	B_3	B_4	Hinc2 (H) H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	Hind3 (D) D_1	Pst1 (P) P_1	Pvu2 (U) U_1	U_2	Sst1 (S) S_1	Sal1 (A) A_1	Restriction Fragment Pattern: Hinf1
marine	J	Ns	2	-	+	-	-	+	+	-	+	+	-	+	+	-	-	-	-	+	+	I
	I	Sk	1	-	+	-	-	+	+	-	+	-	-	-	+	+	-	-	-	+	+	I
	H	My,Sk,Sr	12	-	+	-	-	+	+	-	+	-	-	-	+	+	-	+	-	+	+	I
	B	Im,Sk	2	-	+	-	-	+	+	-	+	-	-	-	+	+	-	+	-	+	-	I
	E	Sl	1	-	+	-	-	+	+	-	-	+	-	-	+	+	-	+	-	+	-	I
	A	Bo,Dr,Ha,Im,Mi	43	-	+	-	-	+	+	-	+	+	-	-	+	+	-	+	-	+	-	I
	D	My,Se,Sk,Sl,Sr Bo	1	-	+	-	+	+	+	-	+	+	-	-	+	+	-	+	-	+	-	I
	C	Ha,Im,Mi,Se	14	-	+	-	-	+	+	-	+	+	+	-	+	+	-	+	-	+	-	I
	F	Sl,Va Va	7	-	+	-	-	+	+	+	+	+	+	-	+	+	-	+	-	+	-	I
	G	Bo	1	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	I
argonaut	K	Im	1	+	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	+	-	II
	L	Ha,Im,Ro,Se	17	+	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	II