Associations between single nucleotide polymorphisms in candidate genes and growth rate in Arctic charr (*Salvelinus alpinus* L.)

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We tested for associations between single nucleotide polymorphisms (SNPs) in five candidate genes allied with the growth hormone axis and the age-specific growth rate of Arctic charr (*Salvelinus alpinus* L.: Salmonidae). Two large full sib families ($N = 217$ and 95) were created by backcrossing males that were hybrids between two phenotypically divergent populations from Labrador, Canada and from Nauyuk Lake, Canada to females that were from Nauyuk Lake. Measures of individual growth rate (wet weight and fork length) were made three times during a 420-day period after the juveniles were transferred from 4 to 11°C. We then identified SNP markers in 10 proposed candidate genes known to be related to the growth hormone axis. Comparative alignments of amino-acid sequences and nucleotide sequences from other fish species were used to design PCR primers that would amplify 0.5–3 kb DNA regions of the candidate genes. All the individuals in the two backcross families were genotyped for these SNP markers using either polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) or bidirectional amplification of specific alleles (Bi-PASA) approaches. A significant association between a particular SNP allele and early growth was found for the locus containing the growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide genes (GHRH/PACAP2, $P = 0.00001$). We argue that using comparative sequence information to design PCR primers for candidate genes is an efficient method for locating quantitative triat loci in nonmodel organisms.
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Introduction

Genome scans using anonymous molecular markers have proved to be effective as a means of detecting quantitative trait loci (QTLs) in model organisms that have a linkage map giving high-resolution coverage of their genome. Genomic scans are often performed by crossing genetically diverse strains and following the coinheritance of phenotypic traits and anonymous markers in different families (Lynch and Walsh, 1997; Rothschild *et al*, 2000). However, the genome scanning approach has its drawbacks for nonmodel organisms with large genome sizes like Arctic charr. The Arctic charr (*Salvelinus alpinus*) has a large genome size resulting from a tetraploidization event in a common ancestor of the salmonid fishes living about 60 million years ago (Allendorf and Thorgaard, 1984). Woram (2001) has developed a partial linkage map for Arctic charr using microsatellite (STR) markers. However, her map has a relatively low level of resolution and does not cover all the linkage groups, so it would be difficult to go from mapping to identifying the QTLs.

Candidate gene markers can be used similarly to anonymous markers, but they are more precisely targeted and can detect variation in, or close to, genes of known or inferred function that relates directly or indirectly to the trait of interest (Lynch and Walsh, 1997). Markers are usually chosen in genes known to regulate the metabolic network controlling a particular quantitative trait (Lynch and Walsh, 1997). A statistical association between specific molecular haplotypes (alleles) of a candidate gene and the trait of interest is taken as evidence that the gene is either directly involved in the genetic control of the trait or that the functional polymorphism is sufficiently close to the marker so that the two loci are in linkage disequilibrium (Lynch and Walsh, 1997).

Growth rate is a quantitative polygenic trait, which has a moderate heritability and is influenced by both prenatal and postnatal maternal effects (Falconer, 1989). Components of the growth hormone axis play a key role in regulating the growth process of animals; therefore, human and animal geneticists have sought to find alleles of genes in the growth hormone axis that are associated with low (and high) growth phenotypes (reviewed by Sellier, 2000). Functional genomic regions known to affect growth in better-studied vertebrates can be explored as candidate genes for growth in teleost fishes.

Growth hormone (GH) is a polypeptide hormone synthesized in the somatotrophic cells of the pituitary. GH plays a major role in stimulating somatic growth in teleosts, including salmonid fishes (Chen *et al*, 1994; Shepherd *et al*, 1997; Devlin *et al*, 1995), and also has an osmoregulatory function to efficiently promote fresh water fish to adapt to seawater (Sakamoto *et al*, 1991).

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Table 1 Summary of SNPs in candidate genes of the GH axis found in Canadian Arctic charr strains

Gene	Investigated PCR fragment	Sire[a] of each family heterozygous for SNP?	Method used to detect polymorphism	Polymorphic region	SNP alleles
GH1	**A**: Promoter to exon 2	Yes	RFLP NlaIV	Promoter	G/C
			RFLP NlaIV	Intron A	T/C
GH1	**B**: Exon 2 to exon 3	Family 1	RFLP Mnl	Intron B	C/T
GH1	**C**: Exon 4 to exon 5	Yes	RFLP BlpI	Exon[c] 5	G/A
GH2	**B**: Exon 4 to exon 5	Yes	Bi-PASA	Intron D	G/T
IGF1	Promoter to exon 1	Yes	RFLP NlaIV	Promoter	G/T
Pit1	**A**: Exon 1 to exon 2	Yes[b]	RFLP BamHI	Intron A	A/G
Pit1	**B**: Exon 4 to exon 6	No	—	—	—
Pit2	Exon 1 to exon 2	No	—	—	—
GHRH/PACAP1	Exon 4 to exon 5	No	—	—	—
GHRH/PACAP2	Exon 4 to exon 5	Family 1	RFLP Tsp509I	Intron 4	A/G
CRH	Gene	No	—	—	—
IGFR-α	Unknown	No	—	—	—
IGFR-β	Unknown	No	—	—	—

[a]And dam homozygous at SNP marker.
[b]However, dam of family 2 was also heterozygous, so family 2 was not analysed.
[c]The SNP 'G/A' was located at the third position of the sixth codon (the 18th nucleotide) in exon 5 of GH1 and represents a synonymous substitution because both GTA and GTG code for valine.

Recent studies have documented that the level of hormones in the growth hormone axis can be used as a marker of growth performance in teleost fish (Perez-Sanchez and LeBail, 1999).

The 10 candidate genes selected for investigation in this study are all known to be related to the growth hormone axis in other vertebrate species (Tables 1 and 2). Two different growth hormone genes, *GH1* and *GH2*, are present in all salmonid fishes and may have different expression patterns in different sexes and stages (Yang *et al*, 1997). A third GH gene *GHΨ* is present as a sex-linked pseudogene in male chinook and male coho salmon (Du *et al*, 1993). Insulin-like growth factor I (*IGF-1*) is an endocrine mediator of the action of growth hormone and is able to stimulate DNA synthesis and subsequent cell division in many cell types; *IGF*-1 binds to the receptor *IGFR-α*, whereas *IGFR-β* spans the plasma membrane and is responsible for communicating the signal for growth to the cell (Luskey, 1988). *GHRH* (= *GRF*) acts on the pituitary to stimulate the release of growth hormone from somatotrophic cells (Mayo *et al*, 1995). Pituitary adenylate cyclase activating polypeptide gene (*PACAP*) is a gene that is found on the same mRNA precursor as the *GHRH* gene in fish (Sherwood *et al*, 1994). PACAP hormone stimulates the release of GH, prolactin, adrenocorticotrophic hormone (ACTH) and luteinizing hormone (LH), (Miyata *et al*, 1989). *PIT*-1 is a pituitary-specific activator of *GH* (Tuggle and Trenkle, 1996). Corticotropin-releasing hormone (CRH) plays a major role in the hypothalamic control of adrenocortical function (Ando *et al*, 1999). Allelic variation in the structural or regulatory sequences of these genes might directly or indirectly affect age-specific growth rate in Arctic charr.

Two Canadian aquaculture strains of Arctic charr were established in the early 1980s from a small number of wild fish: the first strain was collected from the Fraser River in Labrador and the second from Nauyuk Lake in the Northwest Territories (Tabachek and de March, 1990). These two wild populations represent different evolutionary lineages (Brunner *et al*, 2001) and show genetic differences in several life history characters. In a common hatchery environment, most females of the Nauyak strain became sexually mature at 5–6 years of age whereas most females of the Labrador strain become sexually mature at 3 years of age (Tabachek and de March, 1990). The two strains have not yet been the target of a selective breeding program; however, growth is significantly heritable in other populations suggesting that selection for increased juvenile growth rate would be feasible (Nilsson, 1994). Growth rate in Arctic charr has a large environmental component (Nilsson, 1994; de March, 1997), so genetic markers that allowed juveniles with a high breeding value for growth rate to be identified and set aside as broodstock would be useful.

In this study we test for associations between single nucleotide polymorphisms (SNPs) in eight candidate genes and age-specific growth rate or condition in Arctic charr. The families used were the result of a backcross of a hybrid (Labrador × Nauyuk) male to a Nauyuk female. This design takes advantage of the low rates of crossover in salmonid males (Sakamoto *et al*, 2000), and ensures that our design will likely detect any large QTL that is on the same chromosome arm as one of the SNP markers (Lynch and Walsh, 1977). The SNP markers were genotyped in two full sib families totaling more than 300 fish which allowed us to investigate the association of these genes with age-specific weight, condition factor and an index of sexual maturity. We found a highly significant association between an allele at our GHRH/PACAP2 SNP marker and early juvenile growth.

Materials and methods

Backcross Arctic charr segregating for growth rate
Two strains of Arctic charr were used in this study. The Nauyuk strain was originally obtained from Nauyuk Lake, NWT (68°22′ N, 107°43′ W) in 1980, whereas the Labrador strain was originally obtained from the Fraser River in Labrador, Canada (56°39′ N, 63°10′ W) in 1984 (Tabachek and de March 1990; de March 1997). Eggs

Table 2 PCR primers used for analysis of SNP in 10 candidate genes of the GH axis and resulting Arctic charr DNA sequences

Amplified fragment		Primers for PCR of Arctic charr DNA fragment			Designed[a] using GenBank	Arctic charr[b] GenBank
Fragment	Length (bp)	Name	Position	Sequence (5′-3′)	Accession nos	Accession nos
GH1A	1012	GH1PRO	Promoter	TATTGKKGYTAAT-AAAGAAGC	L04688, U14551	AY219405
		GHEX2R	Exon 2	CGATGTTGAAGAG-CCGTTGGTTT	J03797	
GH1B	498	GH1EX2F	Exon 2	CTCTCTTTTAGTGTT-TCTGCTGTT	Charr	AY219406
		GHEXR	Exon 3	TAAGGTGAATAGCA-GAGTGGAACG	AF005921	
GH1C	1165	GHEX4F	Exon 4	GCTCAGCGACCTCA-AAGTGGGCAT	M19999, J03797	AY219407
		GHEX5R	Exon 5	GGTTCTGGTAGTAG-TTCCCGTAGG	U14551, X61938	
GH2B[c]	1296	GHEX4F	Exon 4	GCTCAGCGACCTCA-AAGTGGGCAT	M19999, J03797,	AY219408
		GHEX5R	Exon 5	GGTTCTGGTAGTAG-TTCCCGTAGG	U14551, X61938	
IGF1	634	IGF1PRO	Promoter	ACTAAACACTCAAA-TATTCTAGT	S74130	AY219409
		IGF1EX1	Exon 1	ATGCCACTGGAAKA-AATGACCGCT		
PIT1A	≅1600	PITEX1	Exon 1	CTTCACCACCCTGG-CAGGAGACT	D10444	AY219411
		PITEX2	Exon 2	TGTTTGCTCCGTTGA-CCATGTTGG	X98992	
PIT1B	644	PIT1EX4	Exon 4	CTCTGGCTGCGGTGC-ATGGCTCA	X98992	AY219411
		PIT1EX6	Exon 6	CCTTGGTCAGGTAGG-AGCTGTGGT	D10444	
PIT2	≅1700	PIT1EX1	Exon 1	CTTCACCACCCTGGC-AGGAGACT	X98992	AY219412
		PIT1EX2	Exon 2	TGTTTGCTCCGTTGA-CCATGTTGG	D10444	
GHRH/PACAP1	829	GHRHEX	Exon 4	TTAATAAAGCCTACA-GGAAAGCGC	X73233	AY219414
		PACAPEX5	Exon 5	GGCGTCCTTTGYTTCT-ATATCTCT	AF217251	
GHRH/PACAP2	792	GHRHEX	Exon 4	TTAATAAAGCCTACAG-GAAAGCGC	X73233	AY219415
		PACAPEX5	Exon 4	GGCGTCCTTTGYTTCT-ATATCTCT	AF217251	
CRH		CRH1	Exon 5	AAGCTCAATTTCCTCG-TCACCACC	AF098629,	AY219413
	585	CRHR1	5′END	GATGGAAAAGCAGCA-CTATGGTA	(Ando et al, 1999).	
IGFR-α		IGFR1F	5′END	AGTCARGGACGAGCCA-GAGACGTGT	AF021044	AY219416
	≅1100	IGFR1R	Unknown	GCGTTGAGATAAGACAT-CCCGTCAG	AF062499	
GFR-β		IGFR2F		CACTGTCTACCGCATCGA-CATCCAC		
	≅2500	IGFR2R	Unknown	GGGGCAGAACCTGGCTG-GAYGTGTT	AF021045	AY219417

Bi-PASA primers used for detecting the polymorphism in GH2 intron D[c]

Bi-PASA1F	5′-GGGGCGGGCGAAAGT-GAAATACAACTAG-3′	Specific to amplify allele with G
GH2EX5R	5′- GGTTCTGGTAGTAGTT-CCCGTAGG-3′	
Bi-PASA2F	5′-GGGGCGGGCGTTCCT-AGTTAGAAAGCA-3′	Specific to amplify allele with T
GH2EX4F	5′- GCTCAGCGACCT-CAAAGTGGGCAT-3	

[a]Accession numbers of GenBank DNA and amino-acid sequences used to design PCR primers.
[b]Accession numbers in GenBank for resulting Arctic charr DNA sequence fragments.
[c]GH2 SNP needed to be genotyped with Bi-PASA using the two additional internal primers shown.

from females of the Nauyuk strain and sperm from males that were F1 hybrids of crosses between females from the Labrador strain and males from the Nauyuk strain were obtained from Coldwater Fisheries in Coldwater, Ontario, Canada on 18 November 1998 and placed on ice. Back at the University of Guelph's Hagen Aqualab, the sperm from hybrid F$_1$ males was used to fertilize eggs from pure Nauyuk females to create backcross, full sib families. Eggs were hatched in Heath trays at 4°C in the Aqualab and the juveniles were transferred out of the trays on 20 March 1999. Two large families were chosen: family one consisted of the full sib offspring of 'hybrid sire 13' and 'Nauyuk dam 4', and family two consisted of the full sib offspring of 'hybrid sire 4', and 'Nauyuk dam 3'. The juveniles were maintained in floating containers at 4°C with excess food for 8 months. On 17 November 1999, the juveniles were distributed almost equally into seven tanks (mean = 54.0 fish per 113l tank) in a controlled environment aquarium system (11±1°C), and hand-fed an excess of food (3% of their body weight per day) to maximize their growth rate.

The initial measurements were taken on 14 June 2000, 7 months after the fish were transferred to a higher temperature room (11±1°C) when they were large enough (>10.00 g wet weight, >10 mm fork length) to be passive integrated transponder (PIT) tagged for individual identification. Fish were anaesthetized for 5–10 min with 0.025% 2-phenoxyethanol solution, fin clipped and then injected with PIT tags. The PIT tag number for each fish was recorded along with its fork length, measured to 0.1 cm with a metre stick, and its blotted wet weight, determined to 0.01 g with an electronic balance. We recorded the PIT tag number and measured the fork length and wet weight for the second time 11 months after transfer to the higher temperature room, and for the third time 13 months after the transfer. At the third measurement period, all fish were killed by overdosing them with 2-phenoxyethanol. The gonads were then dissected out of the carcasses so that sex and wet gonad weight could be determined. For each fish, gonad weight was divided by body weight to estimate a gonadosomatic index at measurement time 3 (GSI3) that indicated how sexually mature the fish was. Since all fish were of the same age when measured, we used wet weight (W) as a measure of age-specific growth performance and the cube root of wet weight divided by fork length as a measure of 'condition index (C)' or the 'fatness' of the fish.

Gene dissection, oligonucleotide design, polymerase chain reaction (PCR) and sequencing

The 10 proposed candidate genes that we screened for SNP markers are listed in Table 1. No DNA sequences for genes in the growth hormone axis were then available for Arctic charr in the GenBank database administered by NCBI (Benson *et al*, 2000) except for *GH*1 intron C (AF005921) and *GH*2 intron C (AF005909). Consequently, we downloaded all amino-acid sequences of these 10 candidate genes that were available for other vertebrates in GenBank and aligned them to find the conserved regions. We then designed partially degenerate oligonucleotides 'PCR primer pairs' in these conserved regions using DNA sequence information from other fish species and other vertebrates. We positioned the forward and reverse primers so that we would amplify 0.5–3 kb DNA regions of the candidate genes in Arctic charr, including at least one intron with approximately 100 bp of exon at each of the two ends (Table 2). The presence of the exons allowed us to identify the resulting PCR products by using BlastX (Altschul *et al*, 1997) to compare their DNA sequences with those in GenBank.

We obtained tissue for DNA extraction by cutting a 2 mm wide section off the distal margin of the caudal fin of anaesthetized fish. DNA was extracted from the fin clip using a slight modification of the method of Bardakci and Skibinski (1994).

The PCR amplification reactions were performed in a RoboCycler™ 96 (STRATAGENE) thermal cycler with a hot lid that was programmed for 3 min at 94°C and then 35 cycles consisting of 45 s at 94°C, 1 min at 57°C, and 1 min at 72°C. The program finished with a final extension of 7 min at 72°C. The reactions had a final volume of 20 µl composed of 0.5 µM of the forward and 0.5 µM of the reverse primer, 150 µM dNTPs (GIBCO) and 2.0 mM MgCl$_2$, to which we added 0.4 U of Taq DNA polymerase (GIBCO), 2.0 µL 10 × PCR reaction buffer (GIBCO) and 20–50 ng of Arctic charr genomic DNA.

PCR products were separated on 2.0% of agarose gel containing 0.5 µg ml^1 ethidium bromide. PCR fragments of the predicted size were cut out of agarose gels and purified with a Sephaglass™ kit (Pharmacia) before carrying out the sequencing reactions. DNA samples were cycle sequenced by using one of the two primers used in the original PCR reaction and the BigDye™ Terminator RR Mix Kit (Perkin Elmer) as recommended by the manufacturer. To detect allelic SNP in candidate gene regions, we sequenced these PCR fragments for our two sires, the two dams, and at least five individuals randomly chosen from each of the Nauyuk and the Fraser Arctic charr strains.

SNP detection and genotyping

SNPs were found by aligning DNA sequences for a particular gene from individual fish with the web-based multiple alignment program Clustal W Multiple Sequence Alignment Program, version 1.8. (Thompson *et al*, 1994). SNPs between the Nauyuk and the Labrador populations or within the populations were found in all 10 gene regions investigated. However, only a total of eight SNPs in five of our 10 candidate genes had alleles segregating in the progeny of at least one of our two backcross families.

The SNPs were genotyped using either restriction fragment length polymorphism (RFLP) of the PCR fragments or bidirectional PCR amplification of specific alleles (Bi-PASA) (Liu *et al*, 1997) when no suitable restriction site was present (Table 1). Seven of the eight SNPs that we used contained a restriction enzyme recognition sequence and so could easily be genotyped by RFLP of the DNA fragments amplified from individuals of the two backcross families. However, the SNP in *GH*2 had to be genotyped with Bi-PASA. For the Bi-PASA, we designed two new primers from our Arctic charr DNA sequences to selectively amplify the SNP allele with the G or the SNP allele with the T (Table 2).

We genotyped all the individual progeny from families one and two for each SNP marker for which their sire was heterozygous and their dam was homozygous

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(Table 1). Our crosses were designed to take advantage of chromosomes from each of the two strains segregating in the hybrid males. Consequently, if the sire was homozygous and the dam was heterozygous for a particular SNP marker or both were heterozygous or both were homozygous, then we did not bother to genotype their progeny for that particular SNP.

Statistical analysis

We tested for associations between the marker genotype at each of the five SNP loci and our three phenotypic variables: growth rate, condition index and gonadosomatic index by fitting a general linear model (GLM) to the data from the two backcross full sib families (SAS Institute, 2002). The GLM procedure used by SAS version 8.2 for UNIX is suitable for unbalanced designs (SAS Institute, 2002). Wet fish weight was transformed with a cube root transform before statistical analysis as this made its distribution closer to that of a normal distribution. Transformed growth rate (W) and condition index C were fitted to the linear model separately for measurement time 1 (W1 and C1, respectively), for measurement time 2 (W2 and C2, respectively), and for measurement time 3 (W3 and C3, respectively). However, GSI3 was only fitted for measurement time 3.

When both families 1 and 2 had a sire heterozygous for the SNP marker and a dam homozygous for the SNP marker, we used the nested linear model

$$Y_{ijk} = \mu + \alpha_i + \beta_{(i)j} + \delta_{(i)k} + \varepsilon_{ijk} \tag{1}$$

where Y_{ijk} is the phenotypic trait (eg, W1 or C1), α_i is the family, $\beta_{(i)j}$ is the tank in which the fish were raised (nested in family), $\delta_{(i)k}$ is the genotype at the SNP marker (nested in family) and ε_{ijk} is the random residual. Genotype was nested in family in case the marker allele that was associated with higher phenotypic values of the trait was different in different families.

When only family 1 had a sire heterozygous for the SNP marker and a dam homozygous for the SNP marker, we used the linear model

$$Y_{jkl} = \mu + \beta_j + \delta_k + \varepsilon_{jkl} \tag{2}$$

where Y_{ijk} is the phenotypic trait, β_j is the tank, δ_k is the genotype at the SNP marker and ε_{jkl} is the random residual. The proportion of the phenotypic variation explained by each SNP marker was calculated as its $r^2 = SS$ effect/SS total.

We also reanalysed the six growth rate and condition factor variables for the *GHRH/PACAP*2 locus with a similar linear model that included gender as a factor:

$$Y_{jkl} = \mu + \beta_j + \delta_k + \phi_l + \varepsilon_{jkl} \tag{3}$$

where Y_{ijk} is the phenotypic trait, β_j is the tank, δ_k is the genotype at the SNP marker, ϕ_l is gender and ε_{jkl} is the random residual.

We only ran this GLM with gender included for *GHRH/PACAP*2 because it was the most significant locus without gender. There was a substantial disadvantage in including gender as a factor in GLM for traits measured at time 1 or time 2. Many fish died before reaching sexual maturity, which meant that our sample size for the first measurement period was nearly three times as large if we included fish that died before we could determine their gender. To determine whether there was an association between SNP marker genotype and survival

to measurement period 3, we used Fisher's exact test on 2×2 contingency tables. We analysed both families separately for all five SNP marker loci.

We used a data permutation method to adjust the level of $\alpha = 0.05$ to correct for performing 36 multiple tests of association for different SNP marker–trait combinations (Churchill and Doerge, 1994) for the six growth rate and condition factor variables: W1, W2, W3, C1, C2, C3. To do this we (1) randomly assigned the observed marker genotypes to individual fish, (2) ran the appropriate linear model (eg, equation 1 for GH1C) for each of the 36 phenotypic traits (eg, W1), and 3) saved the smallest P value for the 36 F-tests of the factor 'marker genotype (family)'. We repeated this data permutation and statistical analysis 1000 times and then ranked the 1000 saved minimum P values from largest to smallest. We then used the 50th smallest of these 1000 saved P values as our 'experimentwise' significance level (see Churchill and Doerge, 1994), hereafter '$\alpha_{permutation}$'. Note that we use P values instead of F values because the degrees of freedom differed for each of the 36 linear models we used for testing marker–trait associations (G.A. Churchill, personal communication).

The analysis for GSI3 had to be done separately for each sex, which made the sample size too small to include tank as a factor. Therefore, we used the linear model

$$Y_k = \mu + \delta_k + \varepsilon_k \tag{4}$$

where Y_{ijk} is GSI3, the phenotypic trait, δ_k is the genotype at the SNP marker and ε_k is the random residual. We did not attempt to use a permutation test to determine a significance level for the GSI3 analysis because the small sample size for each sex made the analysis preliminary.

We also used Fisher's exact test on two locus genotype counts arranged in a 2×2 contingency table to determine whether the five SNP marker loci were in linkage disequilibrium.

Results

After being shifted from a water temperature of 4°C to one of 11°C, the fish grew very quickly. However, they showed considerable variation in size (CV length = 16–20%, CV weight = 40–50%; Table 3), which made it necessary to hand feed them and to feed a mixture of sizes of food pellets. After 7 months, Arctic charr had reached an average length of 17.1 cm (range 7.9–22.8 cm) and an average weight of 61.2 g (range 4.68–136.5 g). The variation was even more dramatic after 13 months, when the average length was 29.0 cm (range 16.7–38.6 cm) and the average weight 428.8 g (range 64.3–863.4 cm). Female fish grew significantly faster than male fish from the very first measuring period and this difference increased over time (Table 3).

The seven phenotypic traits—cube root of wet weight (W1, W2, W3), condition factor (C1, C2, C3), and GSI3—were not significantly correlated with each other except for a weak correlation between C1 and W1 ($r = 0.223$) and C1 and W2 ($r = 0.208$). However, W1 was significantly correlated with W2 ($r = 0.887$) and with W3 ($r = 0.740$). Similarly, W2 was significantly correlated with W3 ($r = 0.919$). Finally, there was a significant correlation between C1 and C2 ($r = 0.584$), a weak correlation between C2 and C3 ($r = 0.230$), but no correlation

Table 3 Means (standard deviations) for weight, fork length, condition factor and gonad weights

Months at 11°C	Sex	N	Length (cm)	Weight (g)	Condition factor ($g^{0.333}\,cm^{-1}$)	Gonad weight (g) (N=80)
7	All	321	16.9 (3.24)	61.2 (30.5)	0.225 (0.007)	—
7	M	81	17.1 (2.97)	63.2 (30.3)	0.226 (0.007)	—
7	F	70	18.7 (2.17)	78.3 (25.3)	0.226 (0.007)	—
11	All	184	25.0 (4.16)	202.6 (86.3)	0.229 (0.008)	—
11	M	81	24.2 (4.52)	189.1 (93.5)	0.230 (0.007)	—
11	F	69	26.8 (2.12)	234.4 (60.2)	0.228 (0.007)	—
13	All	138	31.0 (5.08)	428.8 (173.7)	0.239 (0.017)	1.44 (6.36)
13	M	68	29.0 (6.19)	368.1 (201.1)	0.237 (0.009)	0.843 (0.868)
13	F	62	33.1 (2.14)	497.5 (109.9)	0.242 (0.024)	2.240 (9.49)

Table 4 Analyses without gender for those candidate loci where both families 1 and 2 had a heterozygous sire and a homozygous dam

Traits[a]	Locus								
	GH2B			GH1C			IGF		
	F	P	N	F	P	N	F	P	N
W1	3.54	0.030	(312)	1.90	0.15	(294)	0.40	0.67	(287)
C1	0.12	0.89	(312)	0.49	0.61	(294)	1.91	0.15	(287)
W2	2.85	0.606	(178)	0.41	0.66	(166)	0.06	0.94	(165)
C2	0.09	0.91	(178)	1.17	0.31	(166)	2.22	0.11	(165)
W3	2.27	0.11	(134)	1.80	0.17	(129)	0.15	0.86	(124)
C3	0.15	0.86	(134)	0.69	0.51	(129)	0.71	0.49	(124)

The F values and probabilities for the factor 'marker genotype' for a particular trait are shown from ANOVAs done using the procedure GLM of the statistical package SAS (see equation (1) in text for the linear model).
[a]The six different phenotypic variables are: W1: cube root of first weight measurement; C1: W1/L1 (first length measurement); W2: cube root of second weight measurement; C2: W2/L2 (second length measurement); W3: cube root of third weight measurement; C3: W3/L3.

between C1 and C3 ($r = 0.096$). Generally, the phenotypic correlation between two traits was similar whether we analyzed all fish together or separately by sex, family or tank. The only exception to this was a weak correlation between C2 and W3 in females ($r = 0.452$) but not in males ($r = -0.086$) and a weak correlation between C2 and C3 in males ($r = -0.626$) but not in females ($r = 0.079$), which reflect changes in shape with sexual maturity.

To find segregating SNP markers in the proposed candidate genes, 13 gene fragments, covering almost 10 000 bp, were amplified with PCR and sequenced (Table 1). SNPs were found in all 10 gene regions investigated and totalled more than 30 transitions, transversions, insertions or deletions between or within the Nauyuk and the Labrador populations. However, the SNPs that had been detected in *Pit2, CRH, GHRH/PACAP*1, *IGFR*-α and *IGFR*-β were not segregating in the progeny of either of our backcross families (Table 1). In the end we used only eight SNP markers that were heterozygous in the male hybrid parent and homozygous in the female Nauyuk parent in at least one of the families.

We found four usable SNPs in *GH*1. One polymorphic *Nla*IV restriction site in the promoter region of the *GH*1 is an *Nla*IV restriction site ($G \rightarrow C$), one is an exonic *Blp*I transition (*GH*1C, $A \rightarrow G$) in the fifth exon, and the other two are intronic *Nla*IV and *Mnl*I restriction sites ($T \rightarrow C$) in the first and second introns, respectively (Table 1). All four SNPs are closely linked (*GH*1 gene length $\cong 4.3$ kb, Tao and Boulding, unpublished data) and the composite

SNP genotypes for *GH*1 represented only two haplotypes. Consequently, we present the statistical analysis only for one of these four SNPs, that in the *GH*1C fragment.

An SNP (a G-to-T transversion) detectable by RFLP (*Nla* IV) was found in the *IGF*-1 promoter region. Another SNP (an A-to-G transition) detectable by RFLP (*Bam*HI) was found in the intron A of *PIT*-1. Sequence analysis of the 785-bp *GHRH-PACAP*2 fragment revealed one G-to-A transition in the 18th base pair of the intron between the *GHRH*-like exon and the *PACAP* exon, which gave rise to two alleles detected by RFLP: allele *A* was cut by *Tsp*509I and allele G was not. A SNP polymorphic for *G* or *T* located in *GH*2 intron D (designed as *GH*2 *G/T*) was successfully genotyped with Bi-PASA.

The factor 'marker genotype' was significant at $\alpha = 0.05$ in seven out of the 36 linear models we used to test for significant associations between the phenotypic traits (W and C) and the genetic marker genotypes (Tables 4–6). However, permutation of data showed that the correct value for the experimentwise α adjusted for multiple tests was considerably smaller ('$\alpha_{permutation}$' = 0.0023). Our value of '$\alpha_{permutation}$' was somewhat larger than the experimentwise $\alpha = 0.05$ adjusted for 36 tests from the overly conservative Bonferroni method of $\alpha = 0.05/36 = $'$\alpha_{Bonferroni}$' = 0.0014.

Only one marker – trait association was significant at '$\alpha_{permutation}$' = 0.0023, that for the *GHRH/PACAP*2 marker genotype and weight at the first measurement period

Table 5 Analyses without gender for those loci where only family 1 had a heterozygous sire and a homozygous dam

Traits[a]	Locus					
	PIT-1			GHRH/PACAP2		
	F	P	N	F	P	N
W1	0.24	0.63	(211)	**21.76**	**0.00001**	**(217)**
C1	0.13	0.72	(211)	4.53	0.035	(217)
W2	1.70	0.19	(136)	3.63	0.059	(139)
C2	1.66	0.20	(136)	2.13	0.15	(139)
W3	0.98	0.32	(106)	1.52	0.22	(108)
C3	0.09	0.77	(106)	0.04	0.84	(108)

The F values and probabilities for the factor 'marker genotype' for a particular trait are shown from ANOVAs done in the statistical package SAS with the procedure GLM (see equation (2) in text for linear model).
[a]The six different phenotypic variables were: W1: cube root of first weight measurement; C1: W1/L1 (first length measurement); W2: cube root of second weight measurement; C2: W2/L2; W3: cube root of third weight measurement; C3: W3/L3.

Table 6 Analyses with gender for those loci where only family 1 had a heterozygous sire and a homozygous dam

Traits[b]	Locus[a]		
	GHRH/PACAP2		
	F	P	N
W1	6.83	0.0102	(117)
C1	0.00	0.97	(117)
W2	5.71	0.0186	(116)
C2	2.95	0.089	(116)
W3	6.34	0.0134	(103)
C3	0.03	0.86	(103)
GSI3 (m)	5.69	0.0207	(54)
GSI3 (f)	1.03	0.30	(49)

The F values and probabilities for the factor 'marker genotype' for a particular trait are shown from ANOVAs done with the procedure GLM of the statistical package SAS (see equations (3) and (4) in text for the linear models). The F values for the factor 'gender' are not shown but were always significant at a probability less than 0.001 for all traits except condition index.
[a]Note that these sample sizes are smaller than the total number of fish measured because they include only fish that survived until the last sampling period when their gender could be determined (Table 1).
[b]The seven different phenotypic variables were: W1: cube root of first weight measurement; C1: W1/L1 (first length measurement); W2: cube root of second weight measurement; C2: W2/L2, W3: cube root of third weight measurement; C3: W3/L3, GSI3–gonad wt/body wt at the third measurement period. Note that GSI3 was tested separately for males and females.

which could only be tested in family one. Indeed, the association between the SNP genotype and growth rate at the first measurement period was highly significant (W1, $P = 0.00001$ ($N = 217$)) when a linear model without gender was used (Table 5) with the SNP genotype GHRH/PACAP2 A/G having a faster early growth rate than the genotype GHRH/PACAP2 A/A (Figure 1a). The GHRH/PACAP2 SNP marker detected a major QTL for
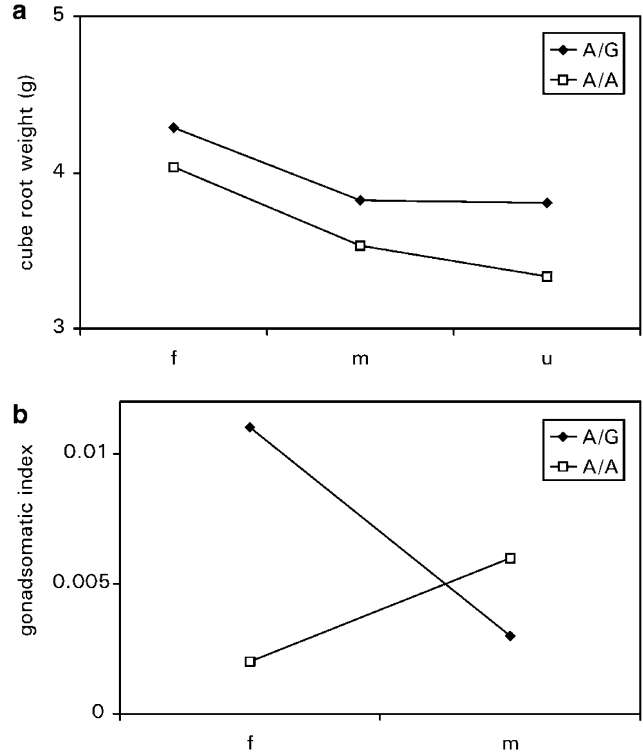
Figure 1 (a) PACAP2 marker genotypes and W1, (b) PACAP2 marker genotypes and GSI3. Gonad wt/body wt at the third measurement period. Note that GSI3 was tested separately for males and females and that males (CV = 1.40) have a smaller coefficient of variation than females (CV = 4.59).

growth rate, which accounted for 9.4% of the phenotypic variance in W1.

However, when the linear model with gender was used, the association between the GHRH/PACAP2 marker genotype and W1 was not quite significant ($P = 0.0102$ ($N = 117$)) at '$\alpha_{\text{permutation}}$' = 0.0023, likely because the sample size was only half as large (Tables 5 and 6). If we had been able to use a molecular method to sex all the fish at measurement period 1, it seems likely that the addition of gender would have made the association between W1 and the SNP genotype GHRH/PACAP2 A/G even more significant. This conclusion is supported by the observation that the addition of gender to the linear model for the second measurement period and for the third measurement period explained additional variance in the data while only slightly reducing sample sizes, and therefore resulted in smaller P values than in the analysis without gender. For example, the association of the GHRH/PACAP2 A/G genotype with W2 was weaker in the analysis without gender ($P = 0.059$ ($N = 139$), Table 5) than in the analysis with gender ($P = 0.0186$ ($N = 116$), Table 6). Similarly, the association of the GHRH/PACAP2 A/G genotype with W3 was weaker in the analysis without gender ($P = 0.22$ ($N = 108$), Table 5) than in the analysis with gender ($P = 0.0134$ ($N = 103$), Table 6). However, the associations between the GHRH/PACAP2 marker genotype and W2 and between the GHRH/PACAP2 marker genotype and W3 were not significant at '$\alpha_{\text{permutation}}$' = 0.0023 even when gender was included.

There was a trend for individuals with the GH2 G/T genotype to have a higher early growth rate (W1) than

the GH2 G/G genotype ($P = 0.03$) in both families, but it was not significant at '$\alpha_{permutation}$' $= 0.0023$ (Table 4). These two GH2 genotypes occurred in both males and females, demonstrating that this gene is not sex-linked in Arctic charr. However, for the 114 fish that could be sexed in family 1, there was a weak trend for the GH2 G/T genotype to be more common in females and the GH2 G/G genotype to be more common in males (Fisher's exact test, $P = 0.023$).

No significant associations were found between SNP genotypes of GH1C, IGF-1, and PIT-1 and growth rate (W1, W2 and W3) or condition index (C1, C2 and C3) (Tables 4 and 5).

There was a trend for early investment in gonads (ie, large GSI3) by males to be associated with the SNP genotype GHRH/PACAP2 A/A despite a small sample size ($P = 0.018$ ($N = 54$), Table 6; Figure 1b). However, there was no such trend for females ($P = 0.30$ ($N = 49$), Table 6; Figure 1b) or for any of our other SNP markers (Tao and Boulding, unpublished data).

There was no association between SNP marker genotype and survival to measurement period 3 for any of the five marker loci ($P > 0.05$, Fisher's exact test). Thus, there was no evidence that QTLs affecting growth rate were also affecting survival.

Our 2×2 contingency table analysis of two SNP marker locus genotypes (Tao and Boulding, unpublished data) revealed that GH1 and GH2 were in linkage disequilibrium ($P < 0.0117$ and $P < 0.0155$ for families 1 and 2, respectively, Fisher's exact test). All other two-locus genotypes for the five loci we genotyped (GH1, GH2, GHRH/PACAP2, IGF-1 and PIT-1) were in linkage equilibrium.

Discussion

Our results are of general interest for two reasons. First, they show that one of our SNP markers in our candidate genes (GHRH/PACAP2) accounted for a substantial amount (9.4%) of the phenotypic variation in early growth rate (W1). This is remarkable because we only tested five markers rather than the 200–400 markers that would be initially tested in a genome scan of a model organism (Lynch and Walsh, 1997). We used hybrid males that have low recombination rates proximal to the centromere (Sakamoto et al, 2000) and this, coupled with independent inheritance of the five markers, means that we have likely tracked the inheritance of QTL on five chromosome arms. Thus, our study shows that using comparative genomics information to develop SNP markers in candidate genes is very efficient in nonmodel organisms with large genome sizes like Arctic charr.

The linkage disequilibrium we observed between the two-locus genotypes for GH1 and GH2 likely results from a phenomenon known as 'pseudolinkage', which can occur in male salmonids (R Danzmann, personal communication). GH1 and GH2 were created by the duplication of a single GH gene in the tetraploid ancestor of modern salmonids, so would have initially been on homeologous chromosomes (Allendorf and Thorgaard, 1984). Homeologous chromosomes may form quadrivalents during meosis in male salmonids, which results in nonrandom segregation of their alleles (Sakamoto et al, 2000). All of the other two-locus genotypes were in

linkage equilibrium. This suggests that in Arctic charr these loci are located either on different chromosomes or far apart on the same chromosome.

Our decision to use candidate genes from the GH axis is timely because of recent work on transgenic Pacific salmonid fishes. A DNA construct injected into coho salmon eggs resulted in transgenic juveniles with 10 times the average growth rate of wild-type fish (Devlin et al, 1995). However, it was difficult to control the number of copies of the construct inserted into the germ line, and fish with many copies grew the fastest but were so deformed that they died before maturity (Devlin et al, 1995). In addition, there are potential environmental and ethical problems associated with the accidental release of transgenic salmon reared in ocean net pens into the wild (Devlin and Donaldson, 1992). Consequently, it is important to explore whether marker-assisted selection can speed up the development of a fast-growing 'domestic' fish strain from the existing strains of what are essentially wild populations of Arctic charr.

Allelic variations of the GH genes have previously been reported in several salmonid species (Park et al, 1995; McKay et al, 1998; Spruell et al, 1999). We did not find a significant association between growth rate or condition and the genotype at our SNP marker in the GH1 gene and only a weak trend at our SNP marker in the GH2 gene. We did not expect a direct effect of these particular SNP markers on our phenotypic traits because none of them represents a polymorphism at the amino-acid level or a mutation at a known regulatory element of the GH genes (Yang et al, 1997). Nevertheless, our SNP markers should have shown linkage disequilibrium with any nearby QTLs with a major effect on our phenotypic traits, so we conclude that there are none segregating in these populations.

Regulation of the GH genes in wild salmonid fishes is much more complex than regulation of the DNA construct in transgenic Pacific salmon. The DNA construct was composed of DNA sequences from GH1 of chinook salmon controlled by an antifreeze protein promoter sequence from an ocean pout, which resulted in abnormally high levels of GH secretion (Devlin et al, 1995). In wild-type fish, GH secretion is mediated in part by the insulin-like growth factor I (IGF1); the transcription factor Pit1/GHF1 belongs to the POU class of homeodomain proteins and is involved in the transcriptional regulation of the growth hormone, prolactin, and thyroid stimulating hormone beta genes and its own gene (Tuggle and Trenkle, 1996; Luskey, 1988). However, we found no significant associations between our SNP markers in Pit-1 and in IGF1 and our growth or condition traits.

In mammals, both growth hormone-releasing hormone (GHRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) belong to the same family of regulatory peptides, which regulate the release of GH. In fish, GHRH can cause a modest increase or have no effect on GH release while PACAP has a pronounced and prolonged stimulatory effect. This has been observed in rainbow trout (Oncorhynchus mykiss, Luo et al, 1990; Blaise et al, 1995), salmon (Oncorhynchus nerka, Parker et al, 1997), carp (Cyprinus carpio, Vaughan et al, 1992) and in a tilapia hybrid (Oreochromis niloticus × Oreochromis aureus, Melamed et al, 1995). These observations strongly suggest that, in fish, PACAP rather than GHRH plays a

major physiological role in the control of GH secretion (Montero *et al*, 2000).

Sequence variation in the *PACAP* gene could affect growth hormone secretion, thus indirectly affecting growth and condition traits. PACAP and GHRH are encoded in separate genes in mammals (Vaughan *et al*, 1992), but in the same gene precursor in fish (Parker *et al*, 1997; Fradinger and Sherwood, 2000). Salmon *GHRH-like/PACAP* gene has five exons and at least two copies (Parker *et al*, 1993; Fradinger and Sherwood, 2000). GHRH peptide is encoded on exon 4 and PACAP on exon 5. Our SNP marker is on the conjunction (or fourth) intron between exons 4 and 5 of *GHRH/PACAP*2. Alternative splicing of the messenger RNA transcribed from the *GHRH*-like/*PACAP* gene may explain the presence of two different mRNAs in the brains and intestines of salmonid fishes: a long mRNA and a short mRNA that is similar in sequence but is missing most of the exon that encodes GHRH (Parker *et al*, 1997).

We have three alternative hypotheses to explain the strong association of the SNP genotype *GHRH/PACAP*2 *A/G* with early growth (W1): (H1) The association has nothing directly to do with GHRH/PACAP2, but indicates an associated QTL somewhere on the same chromosome arm as the GHRH/PACAP2 gene. (H2) The introns of the *GHRH*-like/*PACAP* gene contain the consensus GT/AG splice donor/acceptor sites, a pyrimidine-rich region just upstream of the 3′-splice site and a branch point recognition sequence, all associated with intron splice recognition. If the *G* allele at our SNP marker biased the splicing of mRNA to favor the short mRNA, it could cause a greater expression of PACAP (Parker *et al*, 1997; Breathnach and Chambon, 1981). (H3) The association of our SNP marker on early growth rate could result from linkage disequilibrium with another mutation in the 5′ or 3′ region of the *GHRH/PACAP*2 gene that contains *trans*-acting regulatory sequences on the same chromosome. Known regulatory elements in the 5′-flanking region or the 3′ region of this gene, including TATA motifs, consensus sites for activating protein-2 (AP-2), CAAT, a half-cAMP response element (CRE), polyadenylation signals and (U) TG-rich elements (TTTTTCT, TGTTTT, and TTTGTA), are reported to be involved in polyadenylation signal recognition and 3′-cleavage (Parker *et al*, 1997).

Under aquaculture conditions, early sexual maturation is an undesirable process that reduces growth, increases mortality and results in inferior flesh quality (Tabachek and de March, 1990). The trend for early gonadal development in males to be associated with the SNP genotype *GHRH/PACAP*2 *A/A* ($P = 0.018$) may help predict which male fish will mature early and therefore should not be used as broodstock. What is most interesting about this trend is that this allele is the opposite allele to the allele *GHRH/PACAP*2 *A/G* that showed a highly significant association with high early growth rates.

SNP markers in candidate genes can be treated similarly to any other molecular markers used in mapping studies such as microsatellites or AFLPs. The advantage that SNP markers have is that their location in DNA regions that code for proteins in the somatotrophic axis means they are more likely to be near QTLs that affect growth. The SNP for *GHRH/PACAP*2 *A/G* seems especially promising for use in marker-selection pro-

grams to develop faster-growing strains of Arctic charr. The immediate use for this SNP marker would be to aid in identification of potential broodstock who have the genetic potential to grow quickly. First, we would need evidence that this association shows population-wide linkage disequilibrium in the Arctic charr strains used in the Canadian Aquaculture Industry. We already know that the G allele at this SNP marker is present in both strains of Arctic charr (Tao and Boulding, unpublished data). Marker-assisted selection is potentially important in fish aquaculture because of the large environmental component to the realized growth rate of a genetically superior fish. If such a fish is placed in a tank where social conditions (de March, 1997) or the physical environment are poor, the technicians may fail to recognize its genetic superiority, and the response of the strain to selection for increased growth rate will be slow.

Acknowledgements

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