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A single major chromosomal region controls natural killer cell-like activity in rainbow trout

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Abstract We report the identification of a single major chromosomal region controlling natural killer (NK) cell-like activity in rainbow trout (*Oncorhynchus mykiss*). A genetic map based on 484 AFLP and 39 microsatellite genotypes from 106 doubled haploid fish was constructed. These fish were produced by androgenesis from a hybrid of two clonal lines divergent in NK-like activity. NK-like activities for 75 of the doubled haploids were quantified by an in vitro chromium release assay utilizing ^{51}Cr-labeled YAC-1 target cells. Composite interval mapping revealed a single major quantitative trait locus (QTL) associated with NK-like activity in this rainbow trout model. Genetic mapping revealed this QTL to also be unlinked to: fragmented MHC class I and MHC class II regions, the leukocyte receptor cluster, the natural killer cell enhancement factor (*NKEF*) gene, the *RAG-1* gene, and two QTL associated with resistance to infectious pancreatic necrosis virus in rainbow trout. Collectively, these results extend the utility of rainbow trout as an immunological model and are consistent with the idea that a single chromosomal region homologous to the natural killer cell complex (NKC) located on syntenic portions of mouse chromosome (Chr) 6, human Chr 12, and rat Chr 4 may exist in a lower vertebrate model.

Keywords Natural killer cells · Fish · Quantitative trait locus

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Introduction

It is becoming increasingly evident that the innate immune system plays a major role in the overall health of both higher and lower vertebrates (Jones 2001). In mammals, natural killer (NK) cells are considered main effectors of the innate immune response. NK cells survey host tissues, eliminating foreign, virally infected, cancerous or otherwise compromised cells by cell-mediated lysis. Because suitable markers for NK cells have yet to be identified in lower vertebrates, it is unknown whether lower vertebrates contain homologues of mammalian NK cells. NK-like cells have been identified in amphibians (Ghoneum et al. 1990; Watson and Horton 1996) and fishes (Evans et al. 1984; Cleland and Sonstegard 1987; Faisal et al. 1989). Within fish, NK-like cells are often referred to as nonspecific cytotoxic cells (NCC) and are presumed to protect against bacteria, protozoan parasites, viruses and tumor growth (Jaso-Friedmann et al. 2001). The presence of functionally equivalent immune cells in fishes and higher vertebrates provides an interesting framework in which the evolution of innate immune responses can be studied.

It is plausible that extant differences in fish NK-like cells and mammalian NK cells may have paralleled the evolution of these organisms. In this regard, NK-like and NK cells may have diverged during the Silurian epoch some 400 million years ago to provide the same function in osteichythans and tetrapods, respectively. Because B and T cells appear conserved between mammals and fishes (Berstein et al. 1997), genetic differences in NK and NK-like cells may represent a major divergence in the evolution of the vertebrate immune system. Discerning

the genetics controlling NK-like cells in a lower vertebrate model may therefore provide information useful for divulging the phylogenetics and evolution of innate immune responses in vertebrates.

In mammals, NK cells eliminate bacteria, parasites, virally infected cells and tumor cells through a complex repertoire of inhibitory and activating receptors present on the surface of NK cells (Diefenbach and Raulet 2001; Lanier 2001; Ryan et al. 2001; Sawicki et al. 2001). The existence of multiple NK receptors requiring simultaneous engagement ensures the accuracy of target cell recognition while protecting normal somatic cells from NK cell mediated lysis (Cerwenka and Lanier 2001). While activating receptors trigger a NK cell to release perforin or other proteases into a target cell (Brown et al. 2001; Moretta et al. 2002; Pinkoski and Green 2002) inhibitory receptors bind self-MHC to restrain the cytolytic capacities of the NK cell (Miller 2001; Warren et al. 2001). The presence of NK inhibitory receptors supports the "missing-self hypothesis" as NK cells survey the cells of the body for normal expression of self-MHC I (Ljunggren and Karre 1990). When confronted with aberrant MHC, NK cells are activated to initiate cell-mediated killing to confer protection against bacteria, virally infected cells and tumor growth (Kim et al. 2000).

To date, NK receptors are poorly understood in all animals below the level of mammals (Yoder et al. 2002a). Even within mammals, major differences in NK receptors have been found which necessitate further explanation. In mammals, three distinct types of inhibitory receptors have been identified: the Ly49 receptor family, the killer cell immunoglobulin-like receptors (KIR), and the CD94/NKG2 receptors (Boyington et al. 2001; Mandelboim and Porgador 2001). An extensive Ly49 receptor family exists in mice yet only one Ly49 receptor, Ly49L, has been found in humans (McQueen et al. 2002). Conversely, KIR receptors are present in humans and other primates yet appear to be absent in rodents (Karlhofer et al. 1992; Smith et al. 2001).

The existence of functionally equivalent receptors resembling lectins in mice (Ly49) and immunoglobulins in humans (KIR) appears to represent convergent evolutionary pathways. In contrast, CD94/NKG2 receptors appear to be conserved throughout several mammalian species (humans, chimpanzees, cattle, pigs, rats, mice), indicating the possibility of a shared evolutionary history (Borrego et al. 1998; Braud et al. 1998; Lee et al. 1998; Vance et al. 1998; Shum et al. 2002; Ravetch and Sun 2003). Most recently, CD94/NKG2 homologues have been identified in the urochorodate *Botryllus schlosseri* (Khalturin et al. 2003) and cichlid fish *Paralabidochromis chilotes* (Sato et al. 2003). The existence of NK receptors in primates, rodents, a bony fish and a urochordate suggests that NK receptors arose early in the evolutionary history of vertebrates.

Genetic mapping studies with traditional mammalian model systems (humans, mice and rats) have revealed NK cell activity to be largely regulated by a tight cluster of inhibitory and activating receptor genes located within a single chromosomal region called the natural killer cell complex (NKC). These NKC genes are located on syntenic portions of the mouse chromosome (Chr) 6 (Yokoyama et al. 1991), human Chr 12 (Renedo et al. 1997) and rat Chr 4 (Dissen et al. 1996). In comparison to the mammalian NKC, little is known concerning the genetic regulation of NK-like cells in fishes and other lower vertebrates (Ravtech and Lanier 2000; Litman et al. 2001; Fischer et al. 2003). To our knowledge, the *CD94/NKG2* sequence identified in the cichlid (Sato et al. 2003) represents the only gene currently identified in a lower vertebrate that shares homology with the mammalian NKC. A putative activating receptor, NCCRP-1, has been reported in catfish (Jaso-Friedmann et al. 2002) although this receptor does not share homology with any genes currently identified within the mammalian NK complex. As such, it remains unclear whether a complex of genes comparable to the NKC of the mouse, rat and human genomes exists in fishes or any other lower vertebrates.

In this paper we report that a single major chromosomal region controls NK-like activity in rainbow trout. We identified this quantitative trait locus (QTL) within a doubled haploid population produced by androgenesis from an F1 hybrid of two clonal lines divergent in NK-like activity. Utilizing genetic mapping, we also show this QTL to be unlinked to either the major histocompatibility complex (MHC) or leukocyte receptor complex (LRC) in rainbow trout. These results are consistent with the idea that a region analogous to the NKC identified on syntenic portions of mouse Chromosome 6, human Chromosome 12, and rat Chromosome 4 may exist in rainbow trout. Moreover, the clonal rainbow trout lines and genetic map described herein provide a unique framework in which the complexity underlying NK-like cells in a lower vertebrate model can be further characterized.

Materials and methods

OSU and HC clonal lines of rainbow trout

Two clonal lines of rainbow trout (OSU and HC) were used in this study. The OSU clonal line was derived from a single fish obtained from a domesticated Oregon State University hatchery strain (OSU) and the HC clonal line was propagated from a single fish obtained from a hatchery strain from Hot Creek, California (HC). To produce clonal lines, outbred eggs were irradiated with ^{60}Cobalt gamma radiation to destroy the maternal nuclear genome of the eggs. Irradiated eggs were then fertilized with sperm from either the founding HC or OSU fish to stimulate the development of haploid zygotes. Diploidy within these fertilized eggs was then achieved by applying a heat shock treatment to prevent the first cell cleavage. Resultant fish were either sexually XX or YY and homozygous at all loci. To produce lines that were genetically identical a second round of androgenesis or gynogenesis was then performed (Parsons and Thorgaard 1985; Scheerer et al. 1986). These clonal lines represent the basis of several ongoing projects in our laboratory aimed at discerning the genetic framework of complex phenotypes in rainbow trout (Robison et al. 2001; Nichols et al. 2003). Clonal lines of fish are considered analogous to the inbred mice used extensively in biomedical research (Ristow et al. 1998). The isogenicity of both the OSU and HC clonal lines has been confirmed by DNA fingerprint analysis (Young et al. 1996;

Robison et al. 1999) and acceptance of skin grafts (Ristow et al. 1996).

OSU×HC doubled haploid rainbow trout

Individuals from the OSU (all female XX) and HC (all male YY) clonal lines were crossed to generate an all male (XY) F1 generation. Sperm from OSU×HC F1 hybrids was then used to produce a population of OSU×HC doubled haploids by androgenesis (Young *et al*. 1998). Briefly, outbred eggs were irradiated and fertilized with normal sperm from an OSU×HC F1 hybrid to create haploid zygotes. A heat shock treatment was subsequently applied to block the first mitotic division and this treatment resulted in the production of doubled haploid individuals. Doubled haploid progeny are genetically different from one another due to recombination events occurring during the production of gametes by the OSU×HC F1 hybrid.

Rainbow trout mapping progeny

Two separate OSU×HC doubled haploid populations (n=20 and n=86) were generated to yield the 106 fish utilized as the basis for constructing an OSU×HC genetic map. Genomic DNA was isolated from fin clips using the PureGene Genomic DNA purification kit (Gentra Systems, Minneapolis). Individuals in the n=86 OSU×HC doubled haploid population were PIT-tagged (BioMark, Boise, Idaho) so they could be identified in replicate testing of NK-like activity.

Rainbow trout tested for NK-like activity

The 86 PIT-tagged OSU×HC doubled haploid fish were raised in a semi-closed recirculating freshwater system at the Washington State University Hatchery facility. The fish were initially maintained in a single circular tank and to avoid overcrowding were randomly separated into two circular tanks within the same recirculating system at approximately 2 years of age. At 2.5 years of age, fish were tested for NK-like activity. A total of 75 fish were tested in triplicate in periods spanning 3 weeks during the summer season.

Phenotypic testing of NK-like activity

Fish were anesthetized and blood was collected by cardiac puncture. The NK-like phenotype was measured as described in Ristow et al. (2000). Briefly, NK-like cells were isolated from whole blood by centrifugation on Histopaque and Percoll gradients. NK-like cells from the OSU×HC doubled haploids were plated in 96-well cell culture plates at 10^6 cells/100 μl media/well in triplicate for each of three bleeds. ^{51}Cr-labeled YAC-1 target cells were added to each cell set at a concentration of 10^4 cells/100 μl. In addition, six wells containing 10^4 target cells/100 μl/well and 100 μl of 2% sodium dodecyl sulfate (total release) or 100 μl of media (spontaneous release) were plated as controls. Following an 18 h incubation at 20°C, plates were centrifuged and 100 μl of supernatant of each well was measured for release of ^{51}Cr using a gamma counter. NK-like cytotoxicity was calculated as: % cytotoxicity = (observed release−spontaneous release)/(total release−spontaneous release)×100.

AFLP genetic markers

The 106 doubled haploid fish were genotyped at 484 polymorphic amplified fragment length polymorphic (AFLP) markers following the Perkin Elmer Applied Biosystems AFLP mapping protocol for genomes. AFLP reactions were carried out as described in Vos et al. (1995) and as modified by Robison et al. (2001). Briefly, genomic DNA was digested using the restriction enzymes *Eco*RI and *Mse*I prior to the ligation of specific adaptors. Selective amplification was carried out using standard AFLP *Eco*RI and *Mse*I adaptor primers containing +3 selective bases. A total of 31 +3 *Eco*RI and *Mse*I primer combinations were analyzed and AFLP fragments were visualized on polyacrylamide gels using Cy5-labeled *Eco*RI primers on a Molecular Dynamics Storm imaging system. AFLP loci were named in accordance to the selective nucleotides of the *Eco*RI primer followed by those of the *Mse*I primer, the order of appearance on the gel, the size in base pairs, and the parental source of the band (OSU or HC).

Microsatellites

Microsatellites previously developed for salmonids were utilized to establish synteny between our OSU×HC genetic map and previ-

Table 1 Microsatellite acronyms. The nomenclature of each microsatellite indicates the fish species and source laboratory in which it was derived

Species acronym	Scientific name, common name	Laboratory acronym		Reference(s)
Ogo	*Oncorhynchus gorbuscha*, pink salmon	UW	University of Washington School of Fisheries, Seattle, USA	Olsen et al. (1998)
Omm	*Oncorhynchus mykiss*, rainbow trout	OMM	National Center for Aquaculture USDA-ARS Leetown, USA	Rexroad et al. (2001), Rexroad et al. (2002a, 2002b) Y. Palti (pers comm)
Omy	*Oncorhynchus mykiss*, rainbow trout	TUF	Tokyo University of Fisheries, Japan	Khoo et al. (2000); T. Sakamoto (pers comm)
Omy	*Oncorhynchus mykiss*, rainbow trout	UW	University of Washington School of Fisheries, Seattle, USA	P. Bentzen (pers comm); T. Seamons (pers comm)
Omy	*Oncorhynchus mykiss*, rainbow trout	DU	Dalhousie University, Halifax, Canada	Morris et al. (1996)
Omy	*Oncorhynchus mykiss*, rainbow trout	UoG	University of Guelph, Canada	Jackson et al. (1998)
One	*Oncorhynchus nerka*, sockeye salmon	ASC	Alaska Science Center Anchorage, USA	Scribner et al. (1996)
Ots	*Oncorhynchus tshawytscha*, chinook salmon	SSBI	SeaStar Biotech Inc., Victoria, Canada	Small et al. (1998)
Ots	*Oncorhynchus tshawytscha*, chinook salmon	NWFSC	Northwest Fisheries Science Center NOAA-NMFS, Seattle, USA	Naish and Park (2002)
Ssa	*Salmo salar*, Atlantic salmon	DU	Dalhousie University, Halifax, Canada	O'Reilly et al. (1996)
Ssa	*Salmo salar*, Atlantic salmon	NVH	Norwegian College of Veterinary Medicine, Oslo, Norway	B. Hoyheim (pers comm)

ously published OSU×Arlee (Young et al. 1998; updated Nichols et al. 2003b) and outbred (Sakamoto et al. 2000; Ozaki et al. 2001) rainbow trout maps. Primer sequences used to amplify microsatellites were obtained from sources listed in Table 1. Microsatellites were amplified using 50 ng of genomic template DNA with PCR conditions optimized for each microsatellite marker. In situations where size differences between the microsatellites amplified within the OSU and HC controls were large, microsatellites amplified in the doubled haploid progeny were scored on 2% agarose gels. Otherwise, microsatellites were run on polyacrylamide gels using Hex or Fam fluorescent labeled forward primers on an ABI system and scored using Genotyper software.

Genetic map construction

An OSU×HC genetic map based on AFLP and microsatellite genotypes of the doubled haploid progeny was constructed. This map was generated using the PC versions of Mapmaker 2.0 and Mapmaker/EXP 3.0 software (Lander et al. 1987) and the Mapmaker II program designed for the Macintosh (Dr. Scott Tingey, Dupont Experimental Station, Wilmington, Delaware). Linkage groups were established at a minimum LOD score of 3.0 and a maximum theta of 0.40 using the Kosambi map function as described in Nichols et al. (2003b).

Mapping of NKEF and MHC loci

The natural killer cell enhancement factor gene (*NKEF*) was genotyped and mapped on the OSU×HC map using the ABI SnaPshot dideoxynucleotide terminating protocol (Applied Biosystems International, USA). Primer sequences utilized to amplify the *NKEF* locus were designed to detect single nucleotide polymorphisms between the OSU and HC *NKEF* sequences reported in Zhang et al. (2001). Two MHC I loci (*MHCIa* and *LMP2*) and two MHC II loci (*DAB* and *DAA*) were previously mapped on the OSU×HC map as detailed in Phillips et al. (2003).

Sex phenotype

The OSU×HC doubled haploids used in the mapping panel were killed at approximately 4 years of age, at which time all were considered sexually mature. Upon sacrifice, the abdomens of each fish were cut open to expose the internal viscera. The sex of each OSU×HC doubled haploid fish was determined by a visual observation of the gonads and the male or female phenotype was assigned based on the respective presence of testis or ovaries. Phenotypic sex was mapped as a binary trait on the OSU×HC genetic map.

QTL analysis

Composite interval mapping was used to scan the OSU×HC genetic map for evidence of QTL influencing NK-like cytotoxicity in rainbow trout. This analysis was performed using Model 6 of the Windows QTL Cartographer computer program (Copyright 2001, Program in Statistical Genetics North Carolina State University) with a window size of 5.0 cM and five other markers used as cofactors in the model. Logarithm of odds (LOD) scores for the main effects were computed and significance thresholds for NK-like cytotoxicity were determined by a series of permutation tests at the 5% level (Churchill and Doerge 1994) with 1,000 replicates. Genetic markers exhibiting LOD scores greater than the calculated threshold value were then identified. Such markers are statistically correlated with regions of the rainbow trout genome associated with controlling NK-like cytotoxicity at or above the $P<0.05$ level of significance.

Results

Phenotypic variation of NK-like activity

The histogram shown in Fig. 1 depicts the range of NK-like activities found within both the OSU×HC doubled haploid progeny and the OSU and HC clonal lines. The phenotypic values of the 75 doubled haploids segregated into two clusters using Ward's minimum variance cluster analysis in SAS, indicating the possibility of two discrete phenotypic categories for the NK-like trait. Average NK-like cytotoxicity values of nine OSU and HC control fish were used to produce a classification function using the DISCRIM procedure in SAS. This function was utilized to classify the 75 OSU×HC doubled haploids in two populations: 39 classified as having NK-like activity comparable to the OSU parental line while 36 classified as having NK-like activity comparable to the HC line. The mean values of fish classified as comparable to the OSU line ranged from –0.90 to 21.39% while the 36 fish classified to be comparable to the HC line ranged from 24.13 to 57.60%. Two distinct phenotypes, high and low NK-like cytotoxicity, in approximately equal proportions in the OSU and HC parental lines, and 36 high and 39 low classifications in the OSU×HC doubled haploid progeny was consistent with, but did not prove that, a single gene or genetic region controlling NK-like cytotoxicity exists in our rainbow trout model.

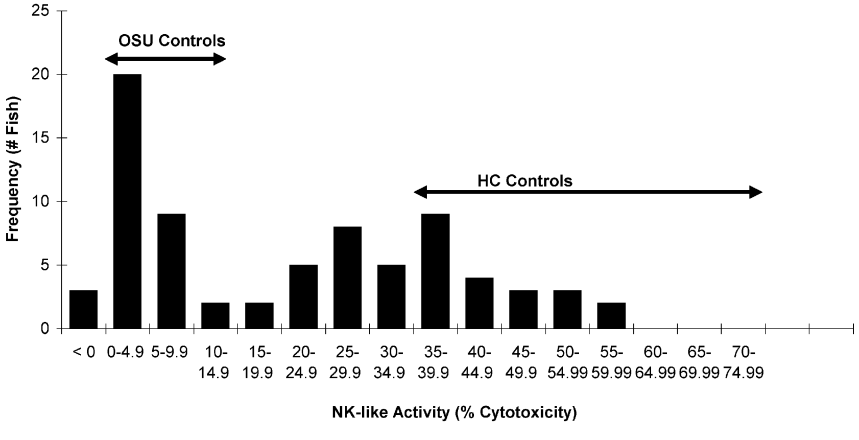

Fig. 1 Distribution of NK-like activity in OSU×HC doubled haploid rainbow trout. Values represent mean NK-like cytotoxicity for each of 75 fish tested in triplicate, with three replicates per testing. The range of OSU and HC control values are indicated by *arrows*

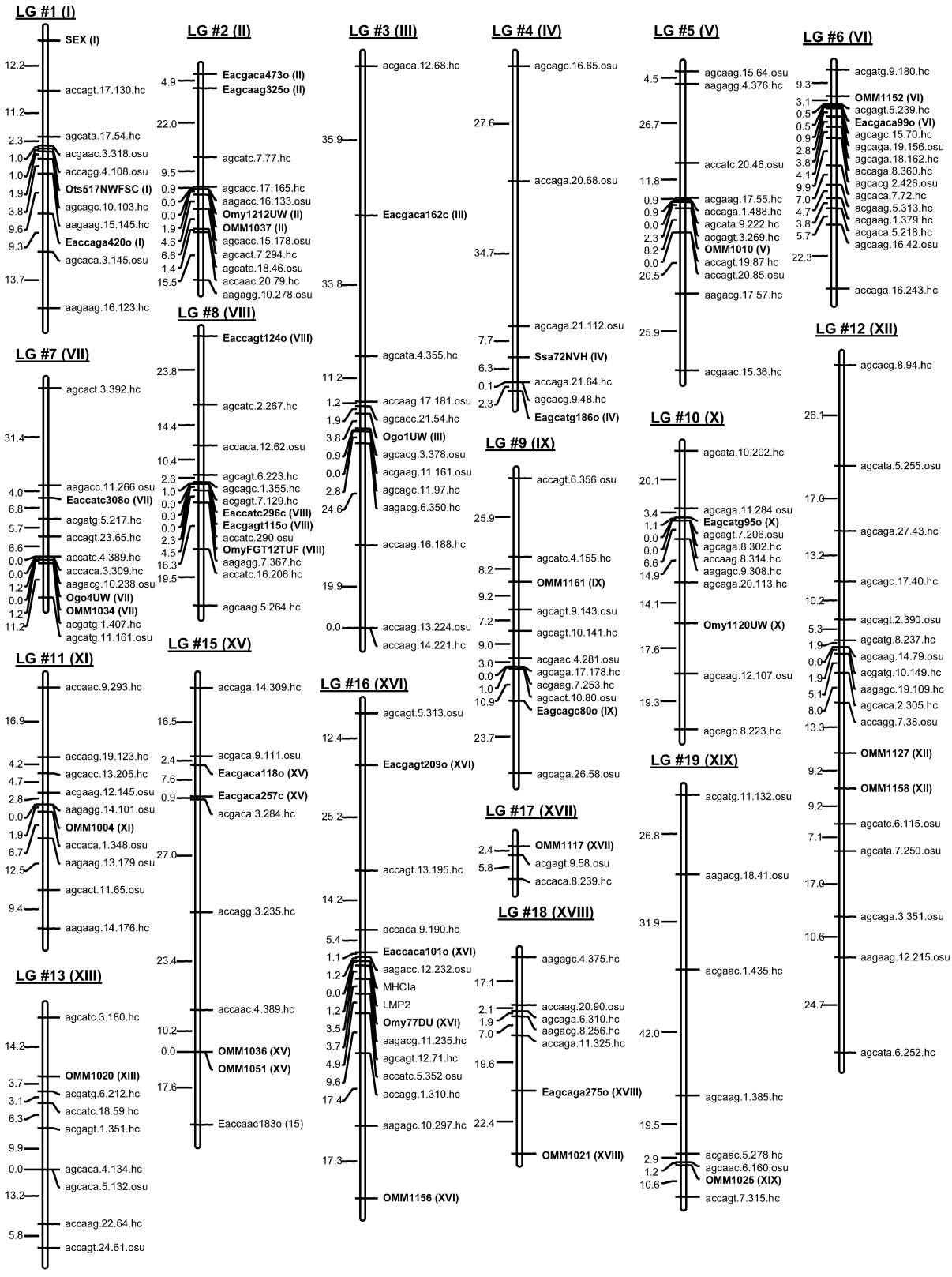

Fig. 2 OSU×HC Genetic map consisting of 34 linkage groups (*LG*). Labels indicate centimorgan distances and marker names. AFLP markers were named in accordance to the nucleotides of the *Eco*RI and *Mse*I primers, order of appearance on gels, base pair size and parental source of band. Syntenies with OSU×Arlee LG are indicated in *parentheses* with Roman numerals. Genetic markers encompassing QTL controlling NK-like activity are outlined on LG 31

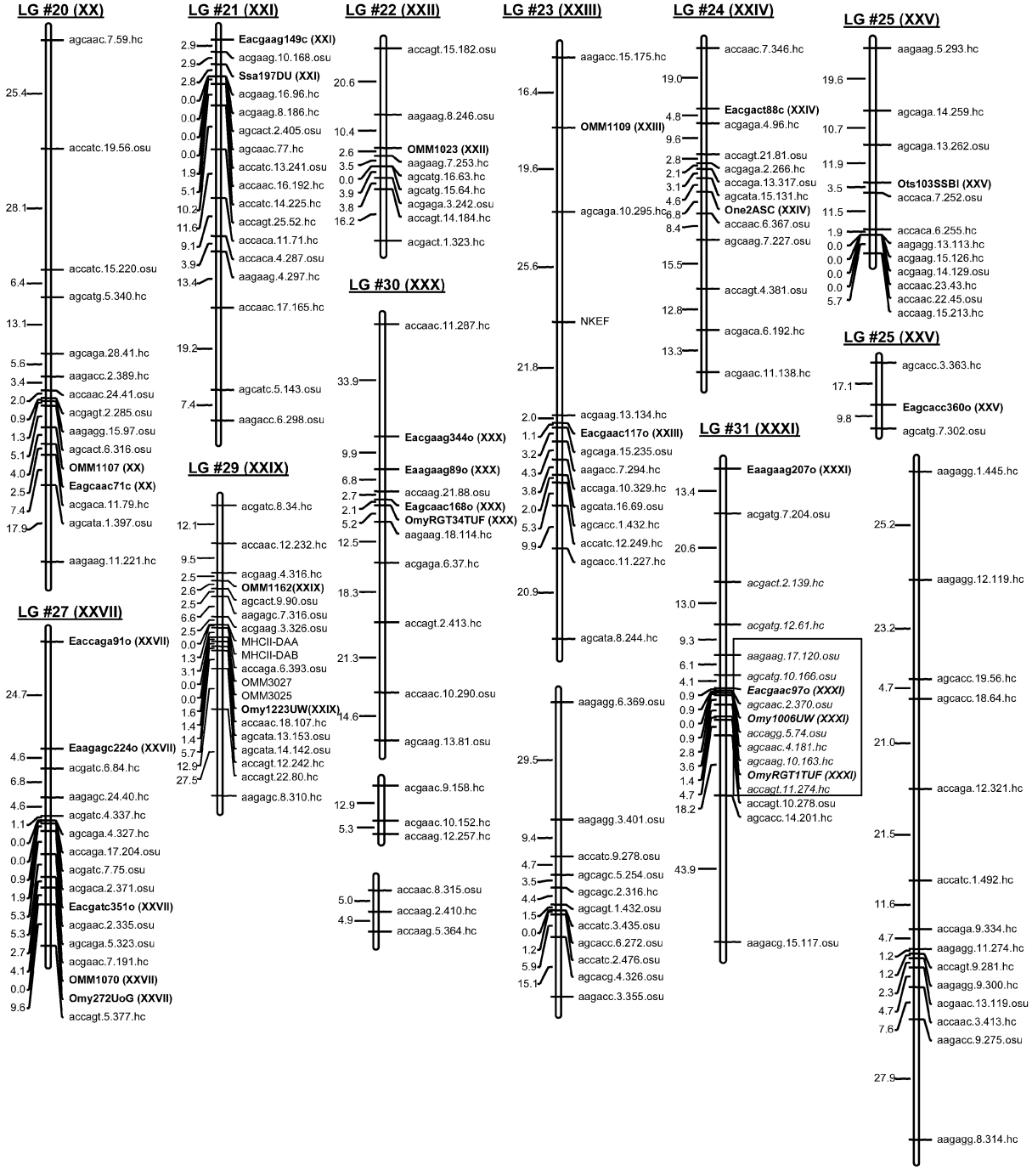

Fig. 2 (continued)

Genetic linkage map

A total of 523 genetic markers (484 AFLP and 39 microsatellites) were genotyped in order to construct the OSU×HC genetic map. Linkage analysis assigned 330 AFLP markers and 39 microsatellites to 29 major linkage groups and 5 smaller groups (Fig. 2). One hundred and fifty four of the AFLP markers genotyped remained unlinked at a LOD of 3.0 and a maximum θ of 0.40. Because fish within the OSU and HC clonal lines each have 60 chromosomes, 30 major linkage groups (corresponding to the haploid chromosome number) are expected within an OSU×HC population. In this respect, the constructed genetic map represents sound coverage of the OSU×HC genome. Furthermore, the 2,872.0 cM size of the OSU×HC map is consistent with the estimated 2,627.5 cM minimum size of the rainbow trout genome (Young et al. 1998).

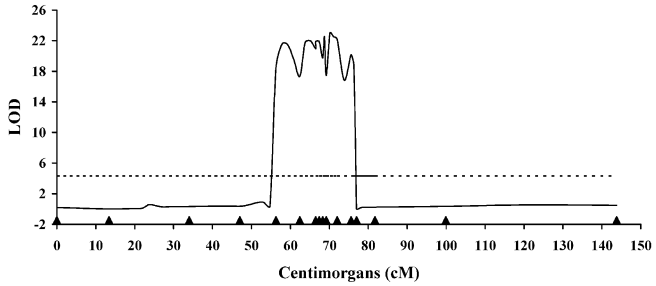

Fig. 3 Logarithm of odds (LOD) plot of the OSU×HC linkage group exhibiting NK-like QTL. Genetic distances are represented on the *x-axis* and the *y-axis* shows LOD scores. The *dashed line* indicates the 4.33 threshold value determined through a series of permutation tests at the P=0.05 level of significance. The *small triangles* represent positions of genetic markers on LG 31

Regions of the rainbow trout genome associated with NK-like cytotoxicity

A series of permutation tests at the 5% level with 1,000 replicates resulted in a LOD threshold value for NK-like cytotoxicity of 4.33. A total of 11 AFLP markers and two microsatellites had LOD scores exceeding this calculated threshold value (Fig. 3). Therefore, these 13 markers are statistically correlated with genetic regions associated with controlling NK-like cytotoxicity. Interestingly, these 13 markers are all located on linkage group 31 (Figs. 2, 3). This clustering of markers reveals a single major chromosomal region or QTL spanning genetic distance of 25.7 cM associated with NK-like cytotoxicity. The haploid genome of rainbow trout being 2.4×10^9 bp (Young et al. 1998) and the 2,872 cM size of the OSU×HC map gives a tentative estimate of approximately 836 kb per cM. This estimate is tentative as recombination rates are not consistent across all chromosomal regions (Sakamoto et al. 2000) and the OSU×HC genetic map may not span the entire genome. Nevertheless, the clustering of AFLP markers within the QTL suggests that a relatively large physical distance is associated with NK-like activity in rainbow trout. This QTL also explains 63.4% of the variation in the cytotoxicity phenotype observed within the doubled haploid progeny (Table 2) indicating this chromosomal region has a major effect on the NK-like cytotoxicity phenotype.

Synteny of the OSU×HC map with other published rainbow trout maps

Mapping of common microsatellites facilitated direct alignment of 28 linkage groups between the OSU×HC and published OSU×Arlee (Nichols et al. 2003b) genetic maps. Because 29 genes are mapped on the OSU×Arlee map, this alignment enabled the inference of the chromosomal locations of these genes on the OSU×HC map. Furthermore, as common microsatellites have been used to establish synteny between the OSU×Arlee (Nichols et al. 2003b) and outbred (Sakamoto et al. 2000; Ozaki et al. 2001) rainbow trout maps, we can also infer the synteny of linkage groups on the OSU×HC map with those of the outbred rainbow trout maps (Table 3). Synteny with other published maps is important for understanding the chromosomal associations of genes and QTLs identified in other rainbow trout crosses. For example, utilizing synteny we can demonstrate the QTL associated with NK-like activity is unlinked to the leukocyte receptor cluster

Table 2 Calculated effects of the QTL associated with NK-like activity in rainbow trout. R^2 is the proportion of variance in the NK-like phenotype explained by the QTL

QTL designation	Linkage group	LOD	R^2	Additive effect
NkOH31	31	22.80[a]	0.634	9.33

[a] Denotes a LOD above the significance threshold for the NK-like phenotype. The additive effect is the effect of substituting a maternal allele (OSU) with a paternal allele (HC)

Table 3 Synteny of OSU×HC linkage groups (LG) with published OSU×Arlee (Young et al. 1998; Nichols et al. 2003b) and outbred (Sakamoto et al. 2000; Ozaki et al. 2001) rainbow trout maps

Microsatellite	OSU×HC LG no.	OSU×Arlee LG no.	Outbred LG no.
Ots517NWFSC	1	I	18[a]
Omy1212UW	2	II	Oii[a]
OMM1037	2	II	Oii[a]
Ogo1UW	3	III	A
Ssa72NVH	4	IV	
OMM1010	5	V	W[a]
OMM1152	6	VI	S[a]
OMM1034	7	VII	R[a]
Ogo4UW	7	VII	R
OmyFGT12TUF	8	VIII	J
OMM1161	9	IX	Oi[a]
Omy1120UW	10	X	H[a]
OMM1004	11	XI	E[a], P[a]
OMM1127	12	XII	Fi[a]
OMM1158	12	XII	Fi[a]
OMM1020	13	XIII	N[a]
OMM1036	15	XV	8[a]
OMM1051	15	XV	8[a]
Omy77DU	16	XVI	Fii
OMM1156	16	XVI	Fii[a]
OMM1117	17	XVII	L[a]
OMM1021	18	XVIII	U[a]
OMM1025	19	XIX	I[a]
OMM1107	20	XX	T[a]
Ssa197DU	21	XXI	B
OMM1023	22	XXII	C[a]
OMM1109	23	XXIII	Q[a]
One2ASC	24	XXIV	A[a]
Ots103SSBI	25	XXV	K[a]
OMM1070	27	XXVII	15[a]
Omy272UoG	27	XXVII	15
OMM1162	29	XXIX	2[a]
Omy1223UW	29	XXIX	2[a]
OMM1124	29	XXIX	2[a]
OmyRGT34TUF	30	XXX	M
Omy1006UW	31	XXXI	S[a]
OmyRGT1TUF	31	XXXI	S[a]

[a] Denotes inferred synteny based on common microsatellites between OSUxArlee and Outbred maps.

(LRC) (Yoder et al. 2002a), MHC class I and II loci (Phillips et al. 2003), *RAG-1* gene (Nichols et al. 2003b) and two QTL associated with resistance to infectious pancreatic necrosis virus in rainbow trout (Ozaki et al. 2001).

Discussion

This is the first report concerning genetic mapping of NK-like activity in any lower vertebrate model. Utilizing cloned fish, doubled haploid progeny, in vitro immunological assays and PCR-based genetic mapping, we identified a single major QTL associated with NK-like cytotoxicity in rainbow trout. These results are consistent with the idea that a single major chromosomal region analogous to the NKC on syntenic portions of human Chromosome 12, mouse Chromosome 6 and rat Chromosome 4 may exist in a lower vertebrate model.

Our lower vertebrate model, rainbow trout (*Oncorhynchus mykiss*), constitutes an important species to both aquaculture and sport-fishing industries worldwide. Rainbow trout are members of the taxonomic family Salmonidae and, as salmonids, belong to one of the most primitive groups of bony fish existing today. Salmonids evolved as tetraploids by duplicating the genome of a diploid ancestor and their present genomes are in the process of rediploidization (Allendorf and Thorgaard 1984). This ancestral duplication of chromosome number and genetic fluctuation allow salmonids to tolerate a variety of chromosome set manipulation procedures which are currently not feasible or possible with traditional mammalian disease model systems.

For example, chromosome manipulations using androgenesis (Parsons and Thorgaard 1985) are used to create homozygous clonal lines of rainbow trout in only two generations. In comparison, typical inbreeding programs with mice often take up to 20 generations to produce offspring that are considered to be genetically identical. In a related approach, haploid gametes from an F1 fish can be subjected to heat shock treatments to double the chromosome number, which instantly results in progeny that are homozygous at all loci. The ability to genetically manipulate gametes to produce doubled haploid individuals is a technique currently restricted to certain plants and fishes (Lynch and Walsh 1998). This approach is amenable for genetic mapping experiments because the power of detecting QTL in experimental crosses is increased using doubled haploid designs (Carbonell et al. 1993; Martinez et al. 2002).

The combination of divergent NK-like phenotypes within the OSU and HC clonal lines and the ability to create OSU×HC doubled haploid fish provided us with a unique animal model to examine the genetic framework underlying NK-like cytotoxicity. Utilizing this model, we combined genetic mapping with phenotypic analysis to identify a single major chromosomal region controlling NK-like activity in rainbow trout. Our evidence for the existence of this genetic region is strong as this QTL accounts for 63.4% of the total variation in NK-like phenotypes observed within the doubled haploid progeny (Table 2). The additive effects of this QTL were also positive indicating that the allele(s) causing an increase in NK-like activity were inherited from the HC parent (Table 2). This finding is consistent with the increased NK-like cytotoxicity observed among individuals within the HC clonal line (Fig. 1).

Detection of a single QTL associated with NK-like cytotoxicity suggests several immediate hypotheses for NK-like cytotoxicity in this lower vertebrate model. One hypothesis is that within rainbow trout a single gene exhibiting a Mendelian inheritance pattern may control the phenotypic divergence of NK-like cytotoxicity between the OSU and HC lines. This result is consistent with suggestions that NK-like activation in channel catfish is dependent on a single receptor referred to as NCCRP-1 (Jaso-Friedmann et al. 2002). A gene carrying a mutation for NCCRP-1 in the OSU line might explain the apparent loss of function in NK-like cytotoxicity associated with the homozygous OSU genotype. To date, our attempts to amplify NCCRP-1 from cDNA derived from NK-like cells have proven unsuccessful in rainbow trout. Similarly, we have been unable to amplify a *CD94/NKG2* gene in the rainbow trout using degenerate primers based on human, cichlid and urochordate sequences.

It is also possible that the OSU line might exhibit a mutant allele for another NK-like receptor, co-receptor or molecule important in either the adhesion or the signaling cascade associated with cell-mediated lysis. Because the F1 OSU×HC generation exhibits NK-like cytotoxicity comparable with the HC line (Evenhuis and Ristow, unpublished results), it appears that a loss-of-function allele could be attributed to an activating receptor in the OSU line or an inhibitory receptor in the HC line. However, because many OSU×HC doubled haploid individuals exhibited NK-like cytotoxicity (Fig. 1) at levels intermediate to the OSU and HC controls, it appears unlikely that a single gene could fully account for this variation.

It is also plausible that NK-like cytotoxicity in rainbow trout is controlled by a series of genes clustered in a single chromosomal region on linkage group 31. Such a cluster of genes would tend to be inherited together, and this scenario would also explain the high level of NK-like cytotoxicity present in the F1 OSU×HC generation and the variation of NK-like cytotoxicity observed within the OSU×HC doubled haploid individuals. Given the existence of NK gene complexes within the genomes of more extensively studied mammalian models, it appears reasonable to suggest that lower vertebrates might also possess such a genetic complex consisting of several closely linked genes.

The genetic mapping of four MHC genes in the OSU×HC cross (Phillips et al. 2003) revealed that MHC I and MHC II complexes on linkage groups 16 and 29 are unlinked to the QTL associated with NK-like cytotoxicity on linkage group 31. Furthermore, utilizing the synteny established between our OSU×HC and other maps we can

demonstrate that the identified QTL is unlinked to the leukocyte receptor cluster (LRC) mapped to an OSU× Arlee linkage group (Yoder et al. 2002a) syntenic to OSU×HC linkage group 21 (Table 3). Similarly, through inferred synteny with an outbred rainbow trout map, the QTL controlling NK-like cytotoxicity is unlinked with two QTLs (inferred to be on OSU×HC linkage groups 3 and 24) that have been associated with resistance to infectious pancreatic necrosis virus in rainbow trout (Ozaki et al. 2001). In the present study, we also mapped the *NKEF* gene to linkage group 23 revealing that this gene is unlinked to the QTL controlling NK-like cytotoxicity. Collectively, these results establish a chromosomal framework of several important immune-related loci in rainbow trout. Moreover, these results and syntenies demonstrate that NK-like cytotoxicity, similar to the NK complex of mammals, is unlinked to both the MHC and LRC complexes.

Identification of the actual genes controlling the differences in NK-like cytotoxicity in rainbow trout is complicated by several factors including the large genetic size (in cM) of the identified QTL, possible allelic polymorphism for unrelated genes in this region, and the paucity of NK candidate genes identified in fishes. These factors exclude a direct sequencing approach for identifying the actual genes underlying the identified QTL. In efforts to overcome these obstacles, we have recently produced a new population of fish using an OSU× (OSU×HC) backcross design. These fish and subsequent backcrosses will enable genetic and phenotypic monitoring to occur at each generation for the retention of genetic markers associated with increased NK-like cytotoxicity. These populations should also enable us to conduct finer mapping on the genetic region controlling NK-like cytotoxicity in rainbow trout.

In summary, we report the identification of a single chromosomal region controlling NK-like activity differences in a rainbow trout model. This QTL was identified through the use of cloned fish, doubled haploid progeny, in vitro immunological assays and genetic mapping techniques. The OSU×HC genetic map and identified QTL presented within this paper provide a genetic framework that should facilitate further characterization of NK-like activity in rainbow trout. Given the phylogenetic distance between rainbow trout and the more traditionally studied mammalian disease models, we believe our lower vertebrate model may also prove valuable for identifying NK-related genes that may not be present in higher vertebrates. Collectively, the results presented herein are consistent with the idea that a genetic region analogous to the NKC located on syntenic portions of human Chromosome 12, mouse Chromosome 6 and the rat Chromosome 4 may also exist in lower vertebrates.

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