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ORIGINAL PAPER

# Cytogenetic mapping of immunoglobulin heavy chain genes in Antarctic fish

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**Abstract** The chromosomal location of the IgH locus has been analyzed in several bony fish of the Antarctic perciform group Notothenioidei. Two IgH probes were prepared from the species *Trematomus bernacchii* (family Nototheniidae, tribe Trematominae) and mapped onto the chromosomes of ten species belonging to the same genus (*Trematomus*) and in two outgroups, through one-color and two-color FISH. A single location of the IgH locus was found in the majority of the species examined, including the outgroups, whereas in four of them the IgH genes splited to two chromosomal loci. RT-PCR experiments revealed the presence of three allelic sequences in *T. newnesi*, a species in which the IgH genes were organized in two chromosomal loci. Possible pathways leading to IgH genes duplication during the diversification of trematomine fishes were inferred from the analysis of the FISH patterns in a phylogenetic context. The present work provides the first comprehensive picture of IgH genes organization at chromosomal level in a bony fish group.

**Keywords** Antarctic fish · Chromosomes · FISH · Cytogenetic mapping · IgH locus · *Trematomus*

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## Introduction

The body of information on the organization of the multigene immunoglobulin complex has grown over the past few years, as has its implication for understanding diversification during vertebrate evolution (e.g. Litman et al. 1999; Savan et al. 2005). In mammals there are five Ig classes (isotypes) that possess distinct effector functions, namely IgA, IgD, IgE, IgG and IgM. Until 2005, IgM and IgD were the only antibody isotypes found in bony fishes (Warr 1995; Wilson et al. 1997), but the recent finding of new Ig molecules (IgZ, IgT) in zebrafish (Danilova et al. 2005), rainbow trout (Hansen et al. 2005) and fugu (Savan et al. 2005) indicates that the Ig system in teleosts is more complex than was previously thought. The genomic organization of the Ig loci has been investigated in various fishes, and has focused mainly on the structure and complexity of the heavy chain (IgH) locus. Similar to the organization found in mammals, in the majority of the fish species the IgH locus consists of a series of variable gene segments (*VH*) upstream of the constant region genes (*CH*) coding for $\mu$ and $\delta$ chain isotypes (Bengtén et al. 2006; Solem and Stenvik 2006). An unusual pattern of genomic organization has been described in those species showing the IgZ, IgT isotypes (Danilova et al. 2005; Hansen et al. 2005; Savan et al. 2005).

In some fish species the molecular structure of the IgH genes displays a certain degree of specific variability and has suggested the possibility that duplication events could have led to their present organization. In the salmonids, one of the most extensively studied fish group, the IgH genes appear to be organized either in single or in double locus

(Hordvik et al. 1992; Hordvik et al. 2002; Hansen et al. 2005). A second cluster of IgH genes, possibly originating from a massive DNA duplication event, has been described for the channel catfish, *Ictalurus punctatus* (Gaffari & Lobb 1999; Bentgén et al. 2006). It has also been hypothesized that duplication events have given rise to the multiple IgH genes in *Cyprinus carpio* (Nakao et al. 1998).

Up to now, the complexity of the IgH genes architecture has been investigated mainly by employing molecular biology approaches. Little attention has been devoted to the study of these genes through in situ techniques at the chromosomal level, although the relevance of structural information obtained by molecular cytogenetics has largely been recognized. For example, the evidence of two different chromosomal locations recently detected by fluorescence in situ hybridization (FISH), confirmed the hypothesis that rainbow trout possess two IgH loci (Hansen et al. 2005).

To our knowledge, the only other application of in situ cytogenetic techniques for the study of the organization of Ig genes in bony fishes was made by Zhang et al. (1997) who used the in situ polymerase chain reaction and FISH to map the gene encoding the IgH on the chromosomes of the channel catfish, *I. punctatus*.

In the present work, the organization of the IgH genes has been analyzed at chromosomal level through the cytogenetic mapping of the IgH locus by means of FISH. The study has focused on a bony fish group, the tribe Trematominae (suborder Notothenioidei, family Nototheniidae), which inhabits the Antarctic continental shelf (DeWitt et al. 1990; Eastman 1993, 2005).

Trematomine species have been widely studied. Conventional karyotyping and molecular cytogenetics detected a high degree of karyotypic variability within the tribe, with diploid numbers ranging from 24 to 58 (Morescalchi et al. 1992; Ozouf-Costaz et al. 1999; Pisano and Ozouf-Costaz 2003). The phylogenetic relationships among these species have been matter of debate for many years and have been recently clarified (Sanchez et al. 2006). Immunological analyses have been performed on one of the species in the tribe, *Trematomus bernacchii*.

In *T. bernacchii*, IgM molecules have been extensively studied (Pucci et al. 2003; Abelli et al. 2005 among others) and both *VH* and *CH* genes have been investigated (Coscia et al. 2000; Oreste and Coscia 2002; Coscia and Oreste 2003).

The Trematominae is the most speciose taxon within the Nototheniidae, an important Antarctic fish family intensively studied as a model of adaptive radiation to cold environment (Eastman 2005; Near et al. 2004). At present, the Trematominae includes two genera (*Trematomus*, *Pagothenia*) and 13 recognized species (DeWitt et al. 1990) which radiated rapidly in the late Miocene and mid-Pliocene (2.5–10 million years ago), according to the estimate by Near et al. (2004).

The main aim of the present work was to obtain structural information on the organization of the IgH genes in the chromosomes of an appropriate number of closely related fish species. Therefore two IgH probes were prepared from one species (*T. bernacchii*) and mapped onto the chromosomes of ten species of the same genus through one- and two-color FISH. In order to infer the evolution of the IgH locus from FISH data, a comparative analysis of IgH chromosomal mapping was performed in a phylogenetic context, by including the species *Notothenia coriiceps* (family Nototheniidae, tribe Nototheninae) as an intra-family outgroup. The species *Eleginops maclovinus*, which belongs to the basal notothenioid family Eleginopidae, was included in the analysis as extra-family outgroup.

In the broader context of comparative genomics, the present work provides both a contribution to the knowledge of the arrangement of the chromosomal IgH genes and its evolution in fish, and more generally in vertebrates.

## Materials and methods

### Studied species and sampling

In this paper, we follow the taxonomic nototheniid nomenclature from DeWitt et al. (1990), except for the taxon *borchgrevinki* which has recently been assigned to the genus *Trematomus* according to phylogenetic evidence (Sanchez et al. 2006). Ten *Trematomus* species were studied, namely *T. bernacchii*, *T. borchgrevinki*, *T. eulepidotus*, *T. hansoni*, *T. lepidorhinus*, *T. loennbergii*, *T. newnesi*, *T. nicolai*, *T. pennellii* and *T. scotti.* The species *Notothenia coriiceps* (family Nototheniidae, tribe Nototheninae), and *Eleginops maclovinus* (Eleginopidae) were included in the analysis as outgroups.

The *Trematomus* and *Notothenia* species were collected in the seas off Victoria Land and Adélie Land, during French and Italian Antarctic Expeditions carried out between 2000 and 2004. *Eleginops maclovinus* was collected at Falkland Islands during the ICEFISH 2004 cruise (International Collaborative Expedition to collect & study Fish Indigenous to Sub-Antarctic Habitats) supported by NSF.

The specimens were caught by gill nets, traps or hand lines and were kept in aquaria with running, aerated sea water prior to tissue sampling and in vivo treatment with colchicine.

Blood cells, spleen and testis were fixed in liquid Nitrogen and preserved at –80°C for further molecular analyses.

Chromosome preparations were obtained from head kidney according to Doussau de Bazignan and Ozouf-Costaz (1985). Fixed cell suspensions and/or slide preparations were preserved at –20°C before FISH experiments.

## Probes for FISH

### *IgVH probe*

As a *VH* probe for FISH, a genomic clone isolated from a *T. bernacchii* genomic library was used. The library was constructed using genomic DNA isolated from the blood cells of a *T. bernacchii* specimen. The purified DNA was partially digested with *Eco*RI; fragments of 9–21 kb were ligated into *Eco*RI-digested Lambda DASH II vector arms (Stratagene) and in vitro packaged. The library was screened using a 508 bp *Eco*RI-*Pst*I fragment, encoding the *T. bernacchii* Ig heavy chain variable domain, from the previously isolated cDNA clone P40 (GenBank accession no. AF303569) (Coscia and Oreste 2003). The Random Primed DNA Labelling kit (Roche Diagnostics) was used to obtain the 32P-labelled probe. One of four hybridizing recombinant clones was further analyzed: phage DNA was digested with *Not*I and *Hin*dIII, and subcloned into pBluescript II KS vector (Stratagene). The positive clones were confirmed by DNA hybridization performed overnight at 65°C in 3 × SSC, 0.2% SDS, 1 × Denhardt's buffer; and then washed at 65°C two times with 3 × SSC, 0.2% SDS and four times, with 2 × SSC, 0.1% SDS. After a final wash with 2 × SSC, membranes were exposed to X-ray film (Kodak). Two positive clones were identified, one containing a 2.3 kb insert and a second, designated NH6 (GenBank accession no. AY775455), containing an insert of about 6.5 kb. The latter was used as *VH* probe for FISH after nick translation labelling with biotin-16dUTP (Roche Diagnostics) according to standard procedures.

### *IgCH probe*

As the *CH* probe for FISH, we used a cDNA fragment of about 1 kb from clone 2O4 (GenBank accession no. AY775454), encoding the constant domains of *T. bernacchii* IgH chain. Clone 2O4 was isolated from a spleen cDNA expression library immunoscreened with rabbit antibodies specific for *T. bernacchii* IgH chain, as described in Coscia et al. (2000). The fragments were excised out by digestion with *Eco*RI, subcloned into pBS SK vector, and the recombinant plasmids were transformed into *E. coli* HB101 cells for propagation. Plasmid DNA purification was performed by use of the Wizard *Plus* Maxipreps DNA Purification System (Promega).

The probe was labelled with digoxigenin-11dUTP (Roche Diagnostics) according standard procedures.

### *Fluorescence in situ hybridization (FISH)*

The labelled probes were purified by ethanol precipitation and dissolved in the hybridization buffer (65% formamide/2 × SSC, 40 mM $KH_2PO_4$, 10% Dextran Sulphate Sodium Salt) to yield a final concentration of 20 ng/μl.

The probes were denatured at 75°C for 5 min and then chilled on ice to prevent from re-annealing.

One-color FISH (IgVH probe) and two-color FISH (IgVH and IgCH probes) were performed as described previously (Mazzei et al. 2004). Briefly, the chromosome preparations were denatured by heating at 70°C for 1 min in 70% (v/v) formamide/2 × SSC (pH 7.0), dehydrated in a cold ethanol series and air-dried.

Hybridization was carried on overnight in a moist chamber at 37°C, followed by high-stringency washings: 5 min in 2 × SSC at 72°C and 2 min in 1 × PBD (MP Biomedicals) at room temperature. Detection was performed with streptavidin-FITC and anti-digoxigenin-Texas red (MP Biomedicals) following the manufacturer's instructions. Preparations were counterstained in DAPI (4,6-diamidino-2-phenylindole)/ 2 × SSC (0.3 μg/ml) and mounted in a standard antifade solution (Vector).

The chromosomal spreads were examined using a Zeiss Axiophot epifluorescence microscope. Digital micrographs were recorded by use of a cooled CCD camera (Sensys Photometrics) and the images were processed with Genus software for animal chromosomes (Applied Imaging).

### *Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA was isolated from the spleen of a *T. newnesi* specimen using the SV total RNA isolation System (Promega), following the manufacturer's instructions, and then subjected to reverse transcription using M-MLVReverse Transcriptase (Ambion). To accomplish PCR amplification of the double-stranded cDNA the

following two primers were used: forward (CH1: 5′-GCAGGGTGACTTCAAAAA GC-3′), designed on the sequence of the *T. bernacchii* Ig CH1 domain; reverse (anti-CH4: 5′-GCCCCACAAACACATAC-3′), specific for the *T. bernacchii* Ig CH4 domain.

The amplification was performed as follows: 94°C for 2 min, 30 cycles of 94°C (1 min) 57°C (1 min), and 72° C (1 min) with a final extension at 72°C for 7 min. The PCR products were further amplified by a second PCR using the same primers and following the same conditions. The PCR products (about 800 bp) were analyzed on 1% agarose gel, purified by QIA quick PCR purification kit (Qiagen) and cloned into the pGEM-T Easy vector arms (Promega). The sequence of the clones was determined with an ABI PRISM 3100 automated sequencer at PRIMM (Naples, Italy).

## Results

### FISH chromosomal patterns

IgH gene sequences were localized by one- and two-color FISH in the chromosomes of ten trematomine species and in the two outgroups (*N. coriiceps* and *E. maclovinus*). *T. bernacchii* was chosen as the main target species, having available species-specific probes (both *VH* and *CH* probes were obtained from its genome).

In *T. bernacchii* ($2n = 48$) a single chromosomal locus for the IgH genes was identified. The hybridization signals, obtained either by one-color FISH with IgVH probe (Fig. 1a, b) or two-color FISH with IgVH and IgCH probes (Fig. 1c–e), were localized in an interstitial-distal region in an acrocentric chromosome pair (Fig. 1a, frame).

This chromosomal region corresponds to a large DAPI-positive band, as shown in Fig. 1b (frame). Two-color FISH indicated the co-localization of the two probes in the same chromosomal band thus confirming the presence of a single chromosomal IgH locus in this species (Fig. 1c–e).

Due to the morphological similarity among the acrocentric chromosomes in the metaphases of *T. bernacchii* (Pisano and Ozouf-Costaz 2003), the assignment of the IgH locus to a specific chromosome pair was not possible.

One-color FISH and two-color FISH detected a single location of the IgH locus in six of the ten species of the genus *Trematomus*, and in the outgroup species *N. coriiceps* and *E. maclovinus* (Table 1).

In those species showing a single IgH locus (see examples in Fig. 2a–d), the hybridization signal was located at interstitial/distal position on a pair of acrocentric chromosomes (as in *T. hansoni*: Fig. 2a) or at a similar position along one of the two arms of metacentric chromosomes (as in *T. eulepidotus*: Fig. 2b) and in the intrafamiliar outgroup *N. coriiceps* (Fig. 2c). In the extrafamiliar outgroup, *E. maclovinus*, the IgH locus was detected at the peri-telomeric position of an acrocentric chromosome (Fig. 2d).

A different hybridization pattern was observed in *T. scotti*, *T. newnesi*, *T. pennellii* and *T. lepidorhinus.* In these species, one- and two-color FISH produced

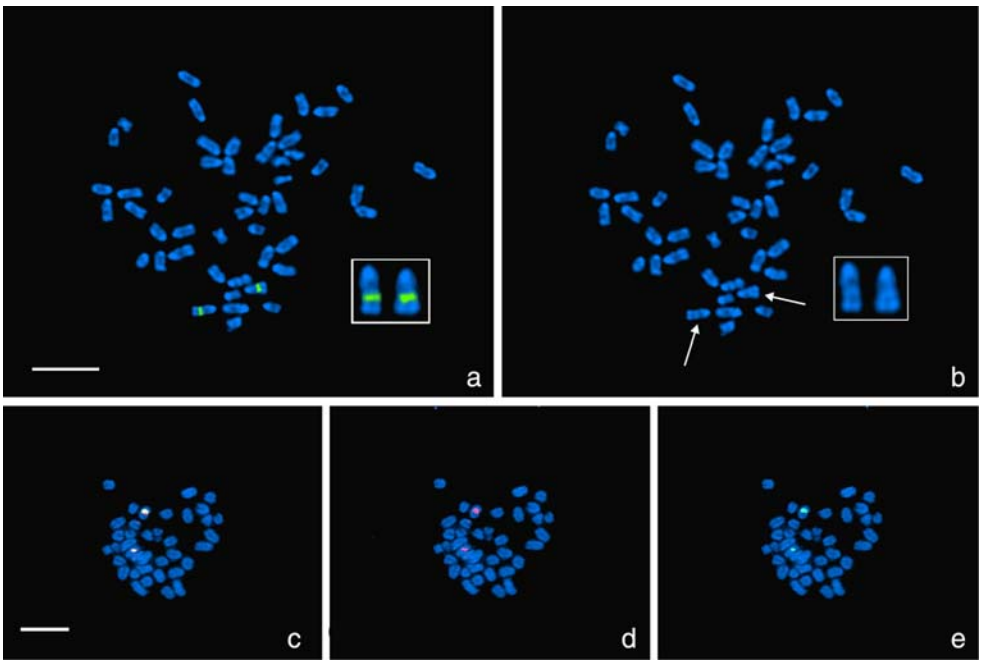


**Fig. 1** Cytogenetic mapping of IgH locus in *Trematomus bernacchii*. Metaphase chromosomes after FISH with the *VH* probe showing a single IgH locus in a pair of acrocentric chromosomes. The two chromosomes bearing IgH genes are enlarged to show details of the position of the FISH signal (frame) (**a**); the same metaphase after DAPI staining with details of the chromosomal region to which the locus has been assigned (frame) (**b**). Co-localized hybridization signals after two-color FISH using the probes *CH* and *VH* (**c**); signal of the *VH* probe (red) (**d**) and of the *CH* probe (green) (**e**) on the same hybridized metaphase. Bars = 10 μm

**Table 1** Summary of the results of the IgH genes cytogenetic mapping in the investigated species

| | Single IgH locus | Double IgH locus |
|---|---|---|
| Trematominae | | |
| *Trematomus bernacchii* | • | |
| *Trematomus borchgrevinki* | • | |
| *Trematomus eulepidotus* | • | |
| *Trematomus hansoni* | • | |
| *Trematomus lepidorhinus* | | • |
| *Trematomus loennbergii* | • | |
| *Trematomus newnesi* | | • |
| *Trematomus nicolai* | • | |
| *Trematomus pennellii* | | • |
| *Trematomus scotti* | | • |
| Outgroup species | | |
| *Notothenia coriiceps* | • | |
| *Eleginops maclovinus* | • | |

signals in four chromosomes suggesting the presence of two IgH loci (Table 1). As examples of such a double chromosomal IgH sequence location, probed metaphases from *T. newnesi* and *T. scotti* are included in Fig. 2 (e and f respectively).

It is worth noting that, irrespective of the single or double chromosomal location of IgH locus, the hybridization signals were strong and unambiguous in all the studied species.

## Analysis of *T. newnesi* cDNA clones

In order to acquire further data to verify whether multiple sequences are transcripted, RT-PCR experiments were performed in one of the species (*T. newnesi*) showing four FISH signals. Three PCR products

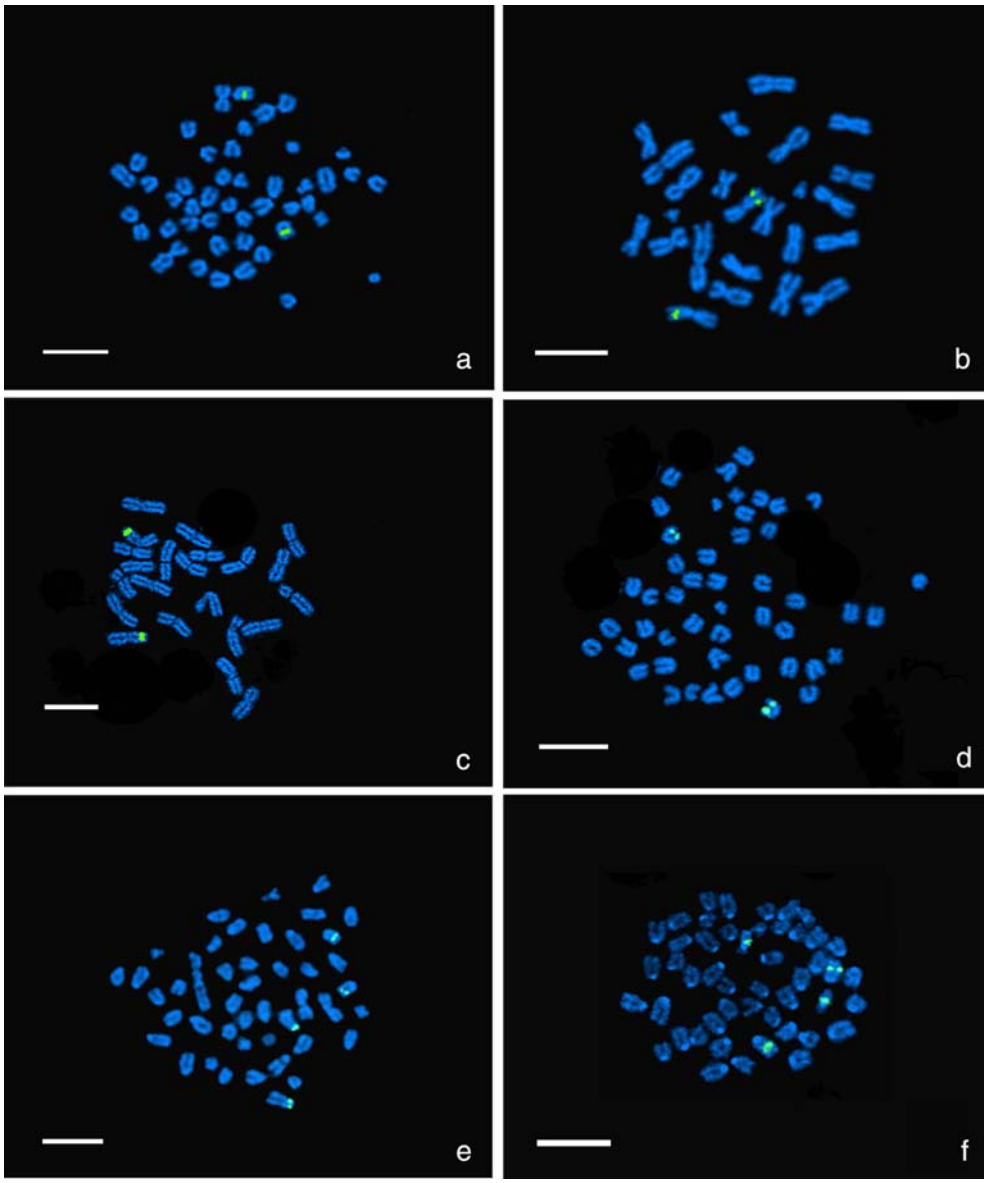


**Fig. 2** Examples of single IgH locus chromosome location in the trematomine species *T. hansoni* (**a**), *T. eulepidotus* (**b**) and in the outgroups *N. coriiceps* (**c**) and *E. maclovinus* (**d**). The hybridized chromosomes of *T. newnesi* (**e**) and *T. scotti* (**f**) are shown as examples of double IgH locus pattern. Bars = 10 μm

of about 800 bp, corresponding to three different allelic IgH isoforms, were obtained. The sequenced clones are named N42 (GenBank accession no. DQ248890), N43 (GenBank accession no. DQ248891) and N47 (GenBank accession no. DQ248892).

The allelic sequences showed a high nucleotide identity, with nucleotide differences at six positions (Table 2). Among these positions, two (61 and 178) are located in the CH2 domain, whereas the others are in the CH3 domain. Five of the six nucleotide differences result in non-conservative substitutions.

## Discussion

The data on Ig genes in bony fishes at the functional and structural levels have been growing over the past few years (e.g. Litman et al. 1999; Danilova et al. 2005; Hansen et al. 2005; Savan et al. 2005; Bentgén et al. 2006). A certain degree of plasticity has been detected for the Ig genomic region, and it is becoming evident that the IgH locus in teleosts is not just a simplified version of the mammalian translocon-type locus (Danilova et al. 2005; Savan et al. 2005). The complexity of the IgH locus architecture in several species supports the hypothesis that duplication events could have occurred leading to the present organization. For instance, in the catfish *I. punctatus* molecular data indicated that the IgH locus includes three regions (IgH1, IgH2, and IgH3) in a 725 kb stretch of chromosome; it has been suggested that these regions could be derived from a massive duplication of an original locus, followed by a second duplication of a smaller portion (Gaffari and Lobb 1999; Bentgén et al. 2006). Duplication events are hypothesized to be at the origin of the complex IgH gene organization in *Cyprinus carpio* (Nakao et al. 1998; Savan et al. 2005).

In salmonids, the IgH genes have been extensively studied, but the tetraploid origin of this fish group makes it more complicated to explain the different organization found in the various species. *Salmo salar* has two distinct, but highly homologous IgH genes, resulting from two IgH loci (IgHA and IgHB) present in haploid embryos (Hordvik et al. 1992). In *Oncorhynchus mykiss*, the hypothesis that at least one of IgH locus has been duplicated, has been formally confirmed by means of cytogenetic mapping, which demonstrated the occurrence of two IgH loci located onto two different pairs of chromosomes (Hansen et al. 2005).

In actual fact, although molecular cytogenetics (especially the fluorescence in situ hybridization) has made important contributions to genomic studies of fishes (Jaillon et al. 2004; Phillips 2006), very little information on the IgH chromosomal organization, through an in situ approach, was available before the present study. Here we compare the IgH locus cytogenetic mapping in twelve fish, and show the occurrence of one or two IgH chromosomal loci in closely related species. The results provide the first comprehensive picture of IgH genes organization at chromosomal level in a bony fish group.

We obtained unambiguous FISH signals in all the twelve species investigated, thus providing evidence for a single IgH locus in the majority of them, and two chromosomal loci in four *Trematomus* species. In *T. bernacchii* the hybridization signals were assigned to a large DAPI positive region, although a precise identification of the chromosome pair bearing the IgH sequences was not possible, due to the absence of distinctive morphological features. Indeed a detailed chromosome characterization in fish karyotypes remains a very difficult task, as Zhang et al. (1997) have remarked, in their attempt to study the IgH genes on the chromosomes of the catfish *I. punctatus*.

When considering the structural aspects, it is worth noting that, regardless of the single or double chromosomal location of IgH locus, the hybridization signals were equally strong. This suggests a similar gene segment multiplicity. Moreover, the fact that two-color FISH performed with the two IgVH and IgCH probes resulted in co-localized signals, is structurally relevant, since it indicates that each chromosomal locus contains both IgVH and IgCH gene sequences. This in turn suggests that the identified loci should be complete and possibly functional.

The results of RT-PCR experiments, although carried out in only one species, are consistent with a multiplicity of the IgH sequences and with the FISH patterns, thereby revealing the presence of three allelic sequences in *T. newnesi*, a species in which the IgH genes are organized in two chromosomal loci. Although a detailed and comparative study of the functional aspects of IgH sequences was outside the scope of the present study, the occurrence, in *T. newnesi*, of at least three different transcripts with a high

**Table 2** Differences among the cDNA clones obtained by RT-PCR in *T. newnesi*

| Clone | Position # | | | | | |
|---|---|---|---|---|---|---|
| | 61 | 178 | 581 | 589 | 605 | 606 |
| N42 | T (ser) | C (his) | A (asn) | G (gly) | A (val) | G (val) |
| N43 | C (pro) | C (his) | A (asn) | A (ser) | A (val) | G (val) |
| N47 | T (ser) | T (tyr) | C (thr) | G (gly) | T (val) | A (ile) |

Base positions and relative codon changes are indicated

percentage of nucleotide identity (94%) and the prevalence of non-synonymous substitutions, suggest that these sequences could have been selected to produce molecules of comparable functionality. Similar indications arise from the analysis of base substitutions occurring in *S. salar* IgH genes, which share a 98.2% nucleotide identity (Hordvik et al. 1992).

Two chromosomal IgH loci have been detected by FISH in only one other fish species, *O. mykiss*. This finding was interpreted as a remnant of the tetraploid event that is believed to have occurred in the salmonid lineage (Hansen et al. 2005). Since there is no evidence of a tetraploid ancestry in notothenioid fishes (Eastman 1993; Pisano and Ozouf-Costaz 2003), the only way to interpret the significance of intrafamiliar chromosomal IgH locus variation and to infer the evolution of IgH locus from the FISH data, was to analyze them in a phylogenetic context.

In spite of numerous investigations based on both morphological and molecular characters, the phylogeny of the Trematominae remained unresolved for a long time (Ritchie et al. 1996; Lecointre et al. 1997; Bargelloni and Lecointre 1998; Bargelloni et al. 2000; Near et al. 2004). However very recently Sanchez et al. (2006) obtained a consensus phylogenetic tree for the family Nototheniidae based on MLL (Mixed lineage leukemia like gene), Rhodopsin and mitochondrial d-loop and cytochrome b sequencing. This included a better resolution of the interrelationships among the Trematominae, which now appear to be monophyletic. Therefore the chromosomal patterns of IgH locus were mapped onto the topology of the above-mentioned, most-recent phylogenetic tree (Fig. 3). The tree includes a stem group (*T. scotti*, *T. newnesi* and *T. eulepidotus*) and a clade for the remaining *Trematomus* species, itself divided into two sister-groups: clade A (*pennellii*/*loennbergii-lepidorhinus*) and clade B (*bernacchii-hansoni*/*borchgrevinki-nicolai*). Since in both the outgroup species *E. maclovinus* and *N. coriiceps*, IgH genes have been found in a single chromosome pair, and assuming that the chromosomal region bearing the IgH- locus is syntenic, this ''single locus'' pattern can be considered as the plesiomorphic condition for the IgH chromosomal position (Fig. 3a).

Among the Trematominae, the IgH locus can be single or duplicated in a second chromosome pair. This duplication is observed in the basal species, *T. scotti* and *T. newnesi*, and also in *T. pennellii* and *T. lepidorhinus*, while the other Trematominae display a single chromosomal IgH locus. This would suggest that an event of duplication (Fig. 3b) occurred at the base of the clade Trematominae, followed by separate events of gene loss, leading back to the single IgH locus situation in *T. eulepidotus* (Fig. 3, c1), *T. loennbergii* (Fig. 3, c2) and in the whole clade B *bernacchii-hansoni*/*borchgrevinki-nicolai* (Fig. 3, c3). However

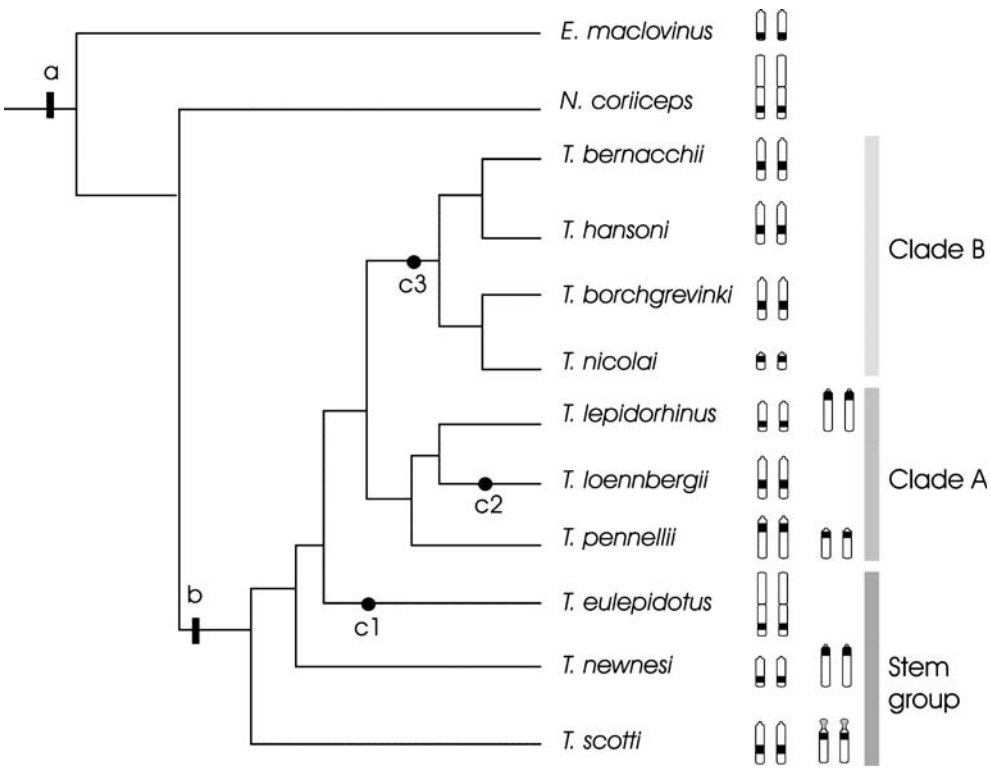


**Fig. 3** Comparative representation of the chromosome pairs bearing IgH genes in ten trematomine species and in two outgroups. The ideograms are drawn to scale. The black bands on ideograms indicate the position of the IgH loci. The topology of the phylogenetic tree is from Sanchez et al. (2006)

the present data cannot exclude the alternative hypothesis, also with four steps, that the extra IgH locus may have arisen independently in *T. lepidorhinus*, *T. pennellii*, *T. newnesi* and *T. scotti.*

Due to the absence of detailed structural information, we cannot assess with certainty, the interspecific homeology among the chromosome pairs bearing IgH sequences in the various species. However it seems that the possible plesiomorphic chromosomal IgH location, at interstitial–distal region, has been retained in most of the species and in the species showing two chromosomal IgH loci (*T. lepidorhinus*, *pennellii*, *newnesi* and *scotti*); the additional locus seems located in an similar pericentromeric position.

In the clade B, *T. bernacchii*, *T. hansoni* and *T. borchgrevinki* display the hypothesized IgH plesiomorphic pattern in a single acrocentric chromosome pair, which is probably syntenic among the three species. However the location of Ig sequences in some species suggests that further chromosomal rearrangements may have occurred leading to the present diversification in the IgH patterns. For instance *T. pennellii* has two loci at pericentromeric position (Fig. 3, clade A). In *T. nicolai* the IgH bearing chromosomes appear much smaller than those in the other species of the clade. This is not surprising, since among Trematominae this species possesses a very peculiar karyotype ($2n = 58$) made of an high number of small elements, probably originating from multiple fission events (Morescalchi et al. 1992).

In summary, our analysis provides the first structural evidence that duplication events of the IgH locus occurred during the diversification of the trematomine fishes. Comparative molecular data will be necessary to determine homologies among the IgH sequences on the duplicated loci in the various trematomine species and get insights into the evolutionary processes involved.

Although in some Trematominae the chromosomal pattern of IgH loci seems close to the one described in the rainbow trout by Hansen et al. (2005), we can exclude that genomic tetraploidization would explain the duplication of IgH locus in trematomines, given the absence of a tetraploid ancestry in notothenioid fishes (Eastman 1993; Pisano and Ozouf-Costaz 2003). On the other end, due to the highly diversified karyotypic features of Trematominae (e.g. Ozouf-Costaz et al. 1999), we cannot exclude the fact that specific karyotypic dynamics could have contributed to the present chromosomal IgH locus patterns in these fishes.

The lack of sufficient comparative information prevents us from suggesting any definitive hypothesis on chromosomal organization of IgH genes in the amazingly diverse groups of bony fish. Additional analyses on both closely- and distantly-related fish species are required to draw a comprehensive picture of the IgH locus organization and evolution. However, based on the results provided in the present study, it is likely that in bony fishes the occurrence of diversified IgH patterns could be expected. These could arise from duplication events combined with chromosomal rearrangements, in accordance with the peculiar genetic and cytogenetic histories of the various lineages.

In approaching any further analyses, the informative value of direct in situ information, acquired by molecular cytogenetics, should be taken into account in order to integrate molecular information into the broader context of the genome structure at chromosomal level (Jaillon et al. 2004; Volff 2005; Phillips 2006).

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