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Karyotypes of basal lineages in notothenioid fishes: the genus *Bovichtus*

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Abstract Using comparative cytogenetic techniques, we characterized the chromosomes of fishes from the family Bovichtidae, the basal lineage of the largely Antarctic suborder Notothenioidei. We focused on three Sub-Antarctic species of the genus *Bovichtus* that differ greatly in their circumpolar distributions: *B. diacanthus* (Tristan da Cunha Island Group), *B. variegatus* (New Zealand) and *B. angustifrons* (Tasmania). Chromosomes were analyzed both by conventional karyotyping and by cytogenetic mapping of ribosomal genes using fluorescence in situ hybridization. The three species displayed a strongly conserved karyotype consisting entirely of telocentric chromosomes (diploid number = 48; Fundamental Number = 48), in agreement with our previously published hypothesis that the bovichtid karyotype is the basal state for notothenioid fishes. The chromosomal distribution of ribosomal genes differed from those of most notothenioid species studied to date, with the 45S and 5S genes separated on two different chromosome pairs. Separation of two classes of ribosomal genes is the most widespread condition in teleosts, including the bovichtids. Most notothenioid lineages on the other hand exhibit a derived consolidation of these genes.

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Introduction

Among the eight families of the perciform suborder Notothenioidei, the Bovichtidae represent the most important non-Antarctic taxon in terms of species diversity. After the separation of the genus *Pseudaphritis* proposed by Balushkin (1992), also supported by molecular phylogenetic analyses (Lecointre et al. 1997; Ritchie et al. 1997) and with the inclusion of the new taxon *Halaphritis platycephala* Last, Balushkin, Hutchins 2002 (Last et al. 2002), the Bovichtidae are presently composed of three genera, the monospecific *Cottoperca*, species *C. gobio* (Günther 1861) endemic to South America, the monospecific *Halaphritis*, species *H. platycephala* (Southeastern Australia), and the polyspecific *Bovichtus* Cuvier 1831. Of the nine recognized bovichtid species, *B. angustifrons*, *B. argentinus*, *B. chilensis*, *B. diacanthus*, *B. elongatus*, *B. oculus*, *B. psychrolutes*, *B. variegatus*, *B. veneris* (Hardy 1988; Balushkin 2000), eight are non-Antarctic and widespread, inhabiting the shelf waters of Australia, New Zealand, South America, and several Sub-Antarctic islands of the Indian and Atlantic sectors of the Southern Ocean. *B. elongatus* is found south of the Antarctic Convergence (Gon and Heemstra 1990) and probably immigrated to Antarctic waters from South America or the Scotia Arc after the formation of the Antarctic Circumpolar Current (Eastman 1993).

The phylogenetic position of the Bovichtidae with respect to the Notothenioidei has been controversial for some time (Lecointre et al. 1997), but there is now substantial evidence, based on various molecular data sets, that this family is the most primitive lineage of notothenioids (Chen et al. 2003; Near et al. 2004). Furthermore a recent study of anatomical and histological features of systematic significance in *C. gobio* and *B. diacanthus* reinforced the phyletically basal position of bovichtids among Notothenioidei (Eastman 2006). Cladistic analysis of skeletal development also places the Bovichtidae as the sister lineage of all other notothenioids and supports a proposal to increase the taxonomic

status of the Bovichtidae to that of a superfamily (Voskoboinikova 2004).

Despite the crucial phylogenetic position of bovichtids, these temperate notothenioids have received much less attention than their Antarctic relatives, due in large part to the difficulty of collecting them from their remote, isolated habitats in the Southern Hemisphere. Thus, a major objective of the ICEFISH Cruise (International Collaborative Expedition to collect and study Fish Indigenous to Sub-Antarctic Habitats) of 2004 was to capture specimens of the Bovichtidae and other poorly sampled notothenioids. Perhaps the least studied member of the family is the Tristan klipfish, *Bovichtus diacanthus* (Carmichael 1818), a temperate species that is endemic to the Tristan da Cunha Island Group and lives at temperatures between 10 and 27°C (Andrew et al. 1995).

Here we report a comparative cytogenetic analysis of *B. diacanthus* and two congeners, *B. angustifrons* Regan 1913 and *B. variegatus* Richardson 1846, that live in the cool-temperate waters of Tasmania and Southern New Zealand, respectively. We find that the three species share a conserved karyotype consisting entirely of telocentric chromosomes (diploid number = 48; Fundamental Number = 48), which is the basal condition for notothenioid fishes (reviewed in Pisano and Ozouf-Costaz 2003). Furthermore, we show that the 45S and 5S ribosomal genes are present on two different chromosomes, as is true for most teleost species. The consolidated ribosomal gene locus of other notothenioid lineages is likely a derived phenotype.

Prior the present work cytogenetic data were available on *C. gobio* (Prirodina 1986; Pisano et al. 1995) whose systematic position among bovichtids has been questioned (Nakamura et al. 1986). Preliminary reports of the cytogenetic features of *Bovichtus* have been published (Pisano et al. 1998; Pisano and Ozouf-Costaz 2003).

Materials and methods

Fish collection

Specimens of *B. diacanthus* (three males, four females, one juvenile) were collected from tidepools near Edinburgh, Tristan da Cunha. They were transported to the *RVIB Nathaniel B. Palmer* for experimentation. *B. angustifrons* (ten juveniles) was sampled by diving at Eaglehawk Neck (southeastern coast of Tasmania) in February 1996 and processed at the laboratories of the Australian Antarctic Division, Kingston, Tasmania. *B. variegatus* (two females, two males, three juveniles) was obtained from tidepools at Otago Peninsula in March 1998 and studied at Portobello Marine Laboratory, Dunedin, New Zealand.

Reference specimens of the three species were deposited at the National Antarctic Museum of the University of Genova (*B. variegatus* and *B. angustifrons*) and in the University of Tennessee Research Collection of Fishes (*B. diacanthus*).

Chromosome preparation

Fishes were maintained in tanks supplied with fresh, aerated seawater at local ambient temperature. Specimens were treated in vivo with colchicine to induce metaphase arrest, suspensions of mitotic cells were obtained from head kidney and spleen, and chromosome spreads were prepared by the methods of Doussau de Bazignan and Ozouf-Costaz (1985) with slight modifications. Fixed cells were stored at −20°C for later analysis.

Karyotyping

Chromosome spreads on microscope slides were prepared for conventional karyotyping and chromosome banding as modified for notothenioid fishes (Ozouf-Costaz et al. 1997). Characterization of chromosomal morphology followed Levan et al. (1964). The karyotype of each fish was established by examination of multiple DAPI- and Giemsa-stained metaphases with an Olympus BX61 equipped with a Sensys (Photometrics) CCD camera for digital imaging. Micrographs were processed either by the use of Genus Software (Applied Imaging) or by application of Adobe Photoshop and Corel Photopaint image analysis software.

Ribosomal DNA probes and fluorescence in situ hybridization (FISH)

Nucleolar 45S ribosomal genes were detected by hybridization to a fragment of 28S rDNA of ~400 bp (Mazzei et al. 2004). Extranucleolar 5S rDNA was visualized by hybridization to a conserved coding fragment of 120 nucleotides obtained from the notothenioid species *Trematomus bernacchii*. The gene-specific probes, inserted into the plasmid vector pDrive Cloning Vector (Qiagen) were labeled with biotin (45S) or with digoxigenin (5S) by use of a nick translation kit (Boehringer). Labeled DNAs were purified by ethanol precipitation and dissolved in the hybridization buffer (65% formamide/2X SSC, 40 mM KH_2PO_4, 10% dextran sulfate).

One-color FISH (28S rDNA probe) and two-color FISH (28S and 5S rDNA probes) were performed as described previously (Mazzei et al. 2004). Briefly, the chromosomes of freshly prepared spreads were denatured by heating at 70°C for 1 min in 70% (v/v) formamide/2×SSC (pH 7), dehydrated in a cold ethanol series, and then air-dried. Ribosomal gene probes were dissolved individually or together in hybridization buffer to yield final concentrations of 10 ng/μl (28S rDNA) and 20 ng/μl (5S rDNA), denatured by heat treatment at 75°C for 10 min, and then chilled on ice to prevent reannealing. The probes were applied to chromosomal

spreads (20 µl per slide) and incubated overnight in a moist chamber at 37°C. High-stringency post-hybridization washing was performed in 2×SSC at 72°C (5 min) followed by 2 min in 1×PBD (MP Biomedicals) at room temperature. Bound 28S probe was detected by incubation of chromosomal spreads with Rhodamine-conjugated anti-digoxigenin antibody (Roche), and 28S/5S sequences were visualized by co-application of the Rhodamine-anti-digoxigenin antibody and streptavidin-FITC (MP Biomedicals). Chromosomal spreads were counterstained in 0.3 µg/ml DAPI/2×SSC and mounted in anti-fade solution (Vector).

Stained chromosomal spreads were examined using a Olympus BX61 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).

Results

The karyotypes

Examination of metaphase plates showed that each of the three bovichtids possessed 48 telocentric (mono-armed) chromosomes (2n = 48; FN = 48). The specific karyotypes (Fig. 1a–c) were established by arranging DAPI- or Giemsa-stained chromosomes in 24 pairs according to size (large to small) and to morphology. Due to the very similar features of most of the chromosomes, it was impossible to infer unambiguous homology (correspondence between the chromosomes within a karyotype) or homeology (correspondence between chromosomes of the three specific karyotypes). However, one chromosome pair, number 23, could be identified consistently in each species because the chromosomes contained a Giemsa- and DAPI- achromatic region of variable extent at a pericentromeric position. This segment of chromosome 23 was positive to Ag-NOR banding (data not shown) used to detect the nucleolar organizing regions (NORs), and appeared heteromorphic in length between the two homologues.

Mapping of the ribosomal genes

To locate ribosomal gene complexes, FISH was performed on chromosome spreads of *B. variegatus* (Fig. 2a, b) and of *B. diacanthus* (Fig. 2c, d, e). The nucleolar ribosomal genes (Fig. 2a, c, e), detected with the 28S rDNA probe, were found at an interstitial position in a pair of small chromosomes. The position of the hybridization signal corresponded to the achromatic band of the NOR-bearing chromosomes of the pair number 23. The same site was Chromomycin A3-positive (data not

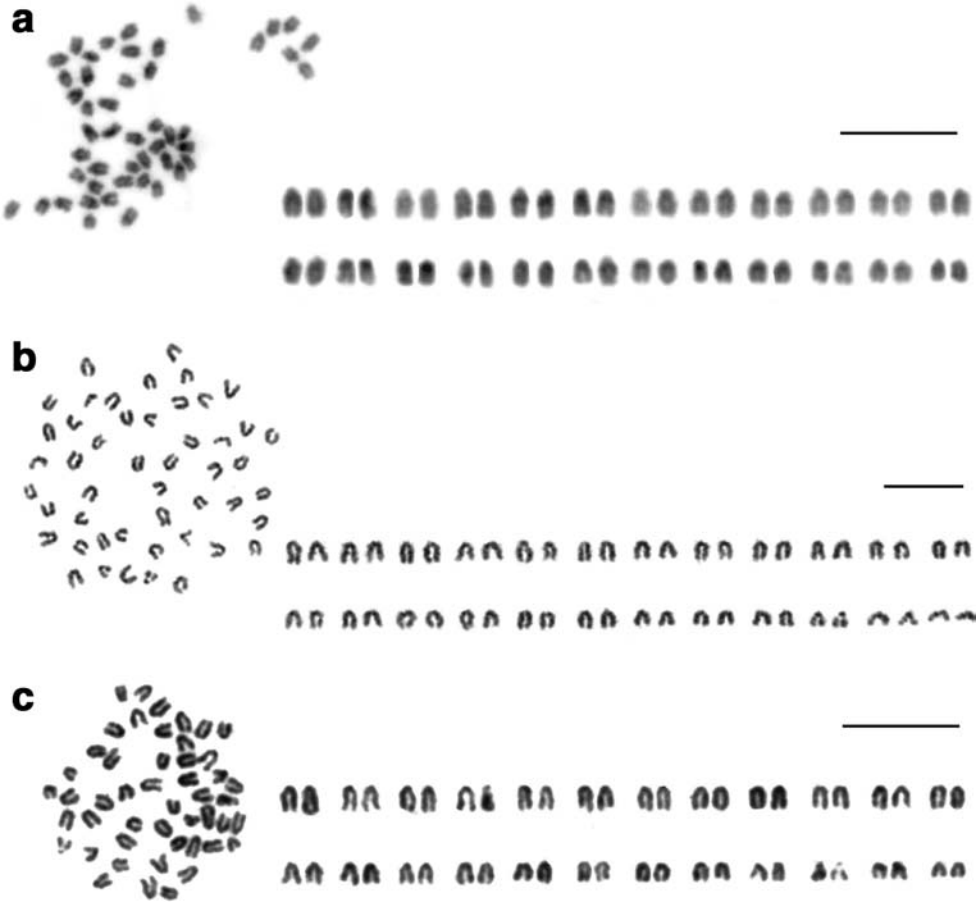

Fig. 1 The karyotypes of three *Bovichtus* congeners: *Bovichtus diacanthus* (**a**), *Bovichtus variegatus* (**b**), *Bovichtus angustifrons* (**c**). Karyotypes were assembled from DAPI- (**a**, **b**) or Giemsa-stained (**c**) metaphase chromosomes. Bars = 10 µm

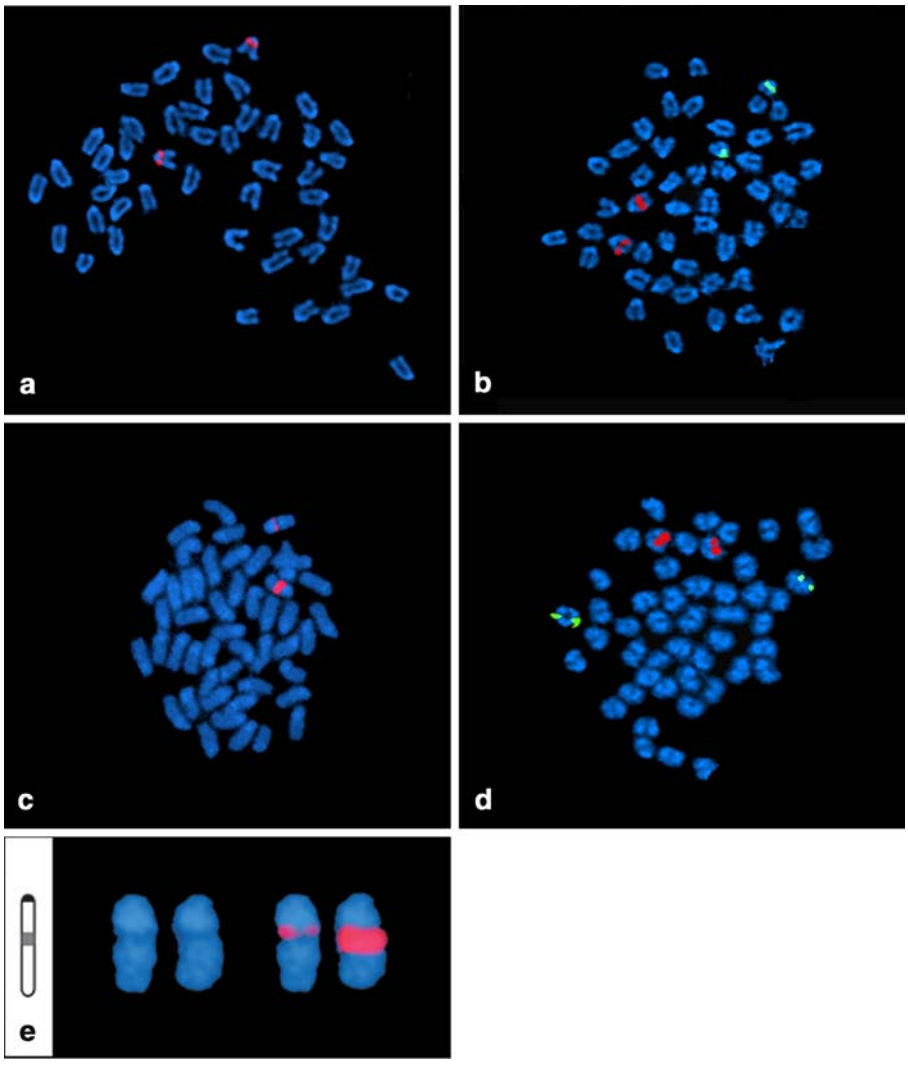

Fig. 2 Mapping of the ribosomal genes of *B. variegatus* and *B. diacanthus*. One-color FISH shows that nucleolar rDNA is found in an interstitial, DAPI-negative region of a single pair of chromosomes in *B. variegatus* (**a**) and *B. diacanthus* (**c**). Magnification of the pair of chromosomes that bear nucleolar rDNA in *B. diacanthus* (**e**) illustrates the length heteromorphism of this region between the two homologous chromosomes. Two-color FISH shows that the nucleolar (*green*) and extranucleolar (*red*) ribosomal genes map on two different chromosome pairs in *B. variegatus* (**b**) and *B. diacanthus* (**d**)

shown), which indicated that the nucleolar rDNA maps to a region that is rich in guanine and cytosine.

In specimens that showed heteromorphic NORs the FISH signals differed in intensity (Fig. 2e), which indicated that the ribosomal gene locus on the two homologues of the pair differed in gene copy number.

The extranucleolar 5S ribosomal DNA genes of *B. variegatus* and of *B. diacanthus* were found at an interstitial position in a chromosome pair of medium size. The 5S genes could not be assigned to a unique chromosome pair due to the absence of distinctive morphological features or banding patterns. However, two-color FISH revealed unambiguously that the nucleolar and extranucleolar ribosomal gene loci were located on different chromosome pairs in each of the species (Fig. 2b, d).

Discussion

The karyotype of the family Bovichtidae

Our results demonstrate that the karyotypes of three Sub-Antarctic species of the genus *Bovichtus* are simple and remarkably similar. Each contains 48 telocentric chromosomes with morphologies lacking distinctive, species-specific characters. Furthermore, the karyotype of *Cottoperca gobio* (Prirodina 1986; Pisano et al. 1995) is virtually identical to those of the *Bovichtus* congeners described here. Thus, the bovichtid karyotype appears to be highly conserved, although we cannot exclude the occurrence of small rearrangements during the diversification of the family that are undetectable with our methods. Given the phylogenetically basal position of the bovichtids among notothenioids, our results provide compelling support for our hypothesis that a karyotype of 48 telocentric chromosomes is the primitive condition for fishes of the notothenioid suborder. The final test of this hypothesis must await definition of the karyotype of the sister group of the notothenioids, whose identity remains uncertain (Dettaï and Lecointre 2004).

Having defined the bovichtid karyotype, we can now propose refined hypotheses regarding the directional polarity of the chromosomal changes that occurred during notothenioid diversification. These fishes display a range of karyotypes, with diploid numbers ranging from 20 (*Prionodraco evansi*, Bathydraconidae) to 58

(*Trematomus nicolai*, Nototheniidae). Of the 57 phylogenetically derived notothenioids whose karyotypes have been characterized cytogenetically (Pisano and Ozouf-Costaz 2003), only *Psilodraco breviceps* is known to possess solely uniarmed chromosomes (48) (Prirodina 1994). However the lack of detailed cytogenetic information on *P. breviceps* does not allow assessment of whether its karyotype is the expression of a very conservative karyotypic feature within the highly derived dragonfish family (Bathydraconidae) or whether it is a secondary condition that resulted from chromosomal rearrangements of a derived karyotype.

It is noteworthy that all three bovichtids, despite different distributions in the Southern Ocean, have maintained a stable gross karyotype (chromosome number and formula) and they seem a very cytogenetically conservative lineage.

However, considering a possible long specific divergence time, we cannot exclude the possibility that minor chromosome changes have also occurred during their diversification, and these are not recognizable by the cytogenetic techniques used in the present comparative analysis.

In the context of the notothenioid radiation, the highly conserved gross karyotypic features of bovichtids are of particular interest when compared to the high degree of karyotype diversification observed in some Antarctic notothenioid relatives, such as the genus *Trematomus* (e.g., Ozouf- Costaz et al. 1999) whose speciation has occurred rapidly, according to recent estimates (Near 2004).

Organization of ribosomal genes

Our data show clearly that the nucleolar 45S/28S ribosomal gene complex of the bovichtids is located at an interstitial, pericentromeric position corresponding to the NORs on a pair of small acrocentric chromosomes. In the karyotypes of most Antarctic species, however, these genes are found either in the heteromorphic arms of a pair of submetacentric chromosomes or in a large interstitial region of a pair of metacentric chromosomes. The length heteromorphism of the NOR-bearing chromosome pair of the bovichtids is a feature common to species of the suborder (reviewed in Pisano and Ozouf-Costaz 2003).

Two-color hybridization revealed unexpectedly that the 5S ribosomal genes of the bovichtids are separated from the 45S genes on different pairs of chromosomes. Separate chromosomal locations have been also observed in the karyotype of *Eleginops maclovinus* (Ozouf-Costaz et al., unpublished data), whereas the two ribosomal genes classes are present in a linked configuration and co-localized on the same chromosome in all the Antarctic notothenioid taxa studied to date. The organization of ribosomal genes on notothenioid chromosomes therefore constitutes a molecular phylogenetic trait of considerable importance.

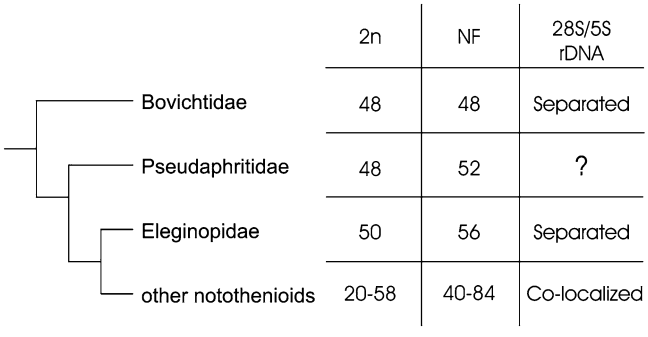

Fig. 3 Relevant cytogenetic features of basal notothenioid taxa in a phylogenetic context. The original cladogram of relationships among notothenioid lineages results from maximum parsimony analysis of molecular data (Near et al. 2004). Diploid chromosome number (2n), fundamental number (FN), and 28S and 5S rDNAs organization (linked or separate chromosomal locations) are reported

Cytogenetic comparison of Sub-Antarctic and Antarctic notothenioids

Figure 3 presents a summary of the main cytogenetic features of non-Antarctic (Bovichtidae, Pseudaphritidae, Eleginopidae) and Antarctic notothenioids, in which the lineages have been arranged according to their presently recognized phylogenetic positions (Near et al. 2004). Karyotypes of the Bovichtidae show the least derived features among the basal taxa (2n = 48 and FN = 48), whereas those of Pseudaphritidae and Eleginopidae are characterized by a higher fundamental number due to the presence of biarmed (i.e., more derived) chromosomes. Although the status of ribosomal gene organization in *Cottoperca gobio* and in Pseudaphritidae is unknown, our results for *Bovichtus* and Eleginopidae suggest strongly that separation of the two classes of ribosomal genes is a basal feature in notothenioids. Martins (2006) has reported that separate chromosomal locations for these two genes classes is the most widespread condition in teleosts, including the candidate notothenioid sister groups (Ozouf-Costaz et al. in preparation). Therefore, separation of the ribosomal genes is the primitive notothenioid condition, and the consolidated loci observed in most other notothenioids is a derived feature that arose in the sister lineage of the Eleginopidae.

Summary

Our results show that chromosomal characters, such as ''ribosomal gene location'', have substantial discriminatory power in phylogenetic studies of the notothenioid fishes. We are currently developing a set of chromosomal markers to be used in more detailed analyses of Antarctic and non-Antarctic notothenioids and in candidate outgroup teleost taxa. Thus, the karyotypes of the Sub-Antarctic notothenioids collected during the ICEFISH 2004 Cruise will provide a crucial link in determining

the phylogenetic position of the notothenioid suborder with respect to other teleost fishes.

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