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BIOLOGY OF THE ROSS SEA

Cytogenetic diversity of notothenioid fish from the Ross sea: historical overview and updates

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Abstract Cytogenetics provides a unique platform to study in situ structural, functional, and evolutionary aspects of the genome. As such it holds powerful promise in decoding mechanisms and processes of genome architectural changes and their role in organism's diversification and evolution. Since the early 80s, such an approach has been applied to the study of the Antarctic notothenioid fishes. In almost three decades, the cytogenetic information has expanded to cover half of the known species inhabiting the high Antarctic waters. Although started 10 years later, cytogenetic studies of species from the Ross sea region have provided valuable contributions to this bulk of knowledge. Here, we synthesize the currently available cytogenetic information on Antarctic notothenioid fishes from the Ross Sea Region, inclusive of both conventional karyotyping and gene mapping. In addition, new karyotypic data on four species (*Lepidonotothen squamifrons*, *Trematomus scotti*, *T. loennbergii,* and *T. lepidorhinus*) are provided. In discussing these data, specific focus is made on the patterns and subtleties of cytogenetic diversity at inter- and intraspecific levels aiming at contributing to the refinement of the knowledge of fish diversity in a region, the Ross Sea area, whose primary ecological value is widely recognized.

Keywords Antarctic fish · Chromosomes · gene mapping · Karyotype

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Introduction

According to the most recent census by Duhamel et al. (2014), the modern fish fauna of the Southern Ocean

(SO) includes 374 species in 47 families. Among them, the family Nototheniidae (suborder Notothenioidei) predominates, accounting for 115 species or 30.75% of all SO species. The diversification and dominance of notothenioid fishes in the Southern Ocean is the result of a unique evolutionary history influenced by the tectonic, climatic, and oceanographic events that led to the isolation of Antarctica and the establishment of the modern cold marine environment (Eastman, 2005).

Recent estimates of notothenioid divergence dates (Matschiner et al., 2011; Near et al., 2012) through analyses of molecular data principally supported the systematics-based phylogeographic scenario previously proposed by Balushkin (2000). From a presumed benthic notothenioid ancestor, living on South Australian continental shelves in the late Cretaceous, three lineages (Pseudaphritidae, Bovichtidae, and Eleginopsidae) diverged early and diversified slowly during the fragmentation of shelf areas between Australia and New Zealand, and detachment of Australia from Antarctica. These three families (totaling 11 species) presently represent a small minority of notothenioid species, distributed in cool-temperate non-Antarctic waters of the southern landmasses, except for one bovichtid species known to also occur in low-latitude West Antarctic Peninsula (Hureau & Tomo, 1977).

The Antarctic notothenioid lineages are hypothesized to have originated from an Eocene ancestor related to the only known notothenioid fossil species *Proeleginops grandeastmanorum* (Balushkin, 1994). Subsequent abrupt global cooling in early Oligocene precipitated a steep greenhouse to icehouse climatic transition, and ice sheets grew over most of Antarctica (Coxall et al., 2005). Ice sheet scouring of coastal shelves and sharp decline of seawater temperatures disrupted habitats and altered the trophodynamics in the marine ecosystem (Clarke & Johnston, 1996), so that the rich Eocene Antarctic ichthyofauna suffered a near-complete extinction (Eastman, 2005). Under these strong environmental selection pressures, the notothenioid ancestor evolved antifreeze glycoprotein (AFGP) (Chen et al., 1997), which protected against freezing to death (DeVries, 1971; DeVries & Cheng 2005). In the absence of significant niche competition post-Eocene extinction (Matschiner et al., 2011) and presumably also at subsequent episodes of species collapse associated with later climatic transitions (Near et al., 2012), the antifreeze-protected notothenioids were able to invade newly developing ice-associated niches vacated by extinct fish groups, diversify and undergo adaptive radiation in the changing Antarctic marine environment (Eastman, 2005).

Whether antifreeze glycoprotein (AFGP) served as the main trigger of the notothenioid adaptive radiation was a subject of debate (Matschiner et al., 2011; Near et al., 2012), AFGP evolution is nonetheless widely acknowledged as a key innovation, enabling the survival of the Antarctic notothenioid in a cooling and icy Antarctic marine environment where freezing avoidance is a matter of life or death (Cheng & Detrich, 2007). Living in perennial cold requires fundamental system-wide adaptations or adjustments for adequate functioning at subzero temperatures beside freeze avoidance by the action of AFGPs. The Antarctic notothenioids show a wide range of evolutionary adaptive changes and modifications. These include cold-able microtubule assembly systems (Detrich et al., 2000), high membrane lipid unsaturation for homeoviscous adaptation (Logue et al., 2000), cold-stable lens crystallin proteins to maintain lens transparency at low temperatures (Kiss et al., 2004), and expansion of gene families of recognized importance in mitigating stresses at freezing temperatures (Chen et al., 2008), among others. Profound trait alterations also accompanied notothenioid evolution in chronic cold. Unique among vertebrates, all the species of the Antarctic notothenioid lineage Channichthyidae live in complete absence of hemoglobin and red-blood cells, the presumed indispensable oxygen transport system, relying on simple diffusion in the cold and oxygen rich Antarctic waters for oxygen supply (Cocca et al., 1995; Cheng & Detrich, 2007). Another example is evolution of secondary pelagicism in multiple lineages from their plesiomorphic swim-bladder-less condition, achieving partial or full neutral buoyancy through lipid deposition and reduced ossification, which enabled expansion into semi-pelagic, pelagic, and cryopelagic habitats. The monophyly, speciosity, high level of endemism, morphological and biological diversity, and dominance in biomass lead to the recognition of the Antarctic notothenioids as a species flock (Eastman & McCune, 2000), and stimulated wide interests in understanding their evolutionary histories and mechanisms of diversification (Rutschmann et al., 2011; Lecointre et al., 2013). Indeed, notothenioid species flock has been used as a model to

test the hypothesis that the Antarctic shelf could act as a species flock generator, and as a starting point to investigate processes leading to flock-like patterning of biodiversity (Lecointre et al., 2013).

The molecular phylogeny of Notothenioidei has recently been intensively investigated with a variety of markers and greater and greater taxon sampling (Rutschmann et al., 2011; Near et al., 2012; Dettai et al., 2012), from which alternate phylogenetic hypothesis has emerged (Dettai et al., 2012).

There is a uniform agreement on the phylogenetic position of the basal non-Antarctic families (Bovichtidae, Pseudaphritidae, and Eleginopsidae) that diverged before the isolation of Antarctica. The designation of the other five families comprised of species predominantly or exclusively endemic to the Antarctic as a High Antarctic clade (Near et al., 2004) has also been generally accepted until recently. The phylogenetic study by Dettai et al. (2012) found strong support for the paraphyly of the predominantly Antarctic family Nototheniidae, with some of the nototheniids recovered as sister group of the clade containing the families Channichthyidae, Harpagiferidae, Artedidraconidae, and Bathydraconidae. Notothenid monophyly would require inclusion of these four families into Nototheniidae and conversion into subfamilies (Dettai et al., 2012).

The alternate phylogenetic hypothesis of Dettai et al. (2012) was adopted by the authors of the comprehensive Antarctic ichthyofauna census described in the Biogeographic Atlas of the Southern Ocean (De Broyer et al., 2014). In this new classification, the nototheniid subfamily Trematominae includes six genera (*Cryothenia*, *Indonotothenia, Lepidonotothen, Pagothenia, Patagonotothen,* and *Trematomus)*, the subfamily Nototheniinae includes *Notothenia* and *Paranotothenia,* whereas the genus *Gobionotothen* is erected as subfamily Gobionototheninae, and the highly divergent genus *Pleuragramma* is placed in its own subfamily Pleuragramminae. The former Channichthyidae, Harpagiferidae, and Artedidraconidae are considered as subfamilies, and the former Bathydraconidae is split into three subfamilies Bathydraconinae, Gymnodraconinae, and Cygnodraconinae.

A complementary approach to understanding notothenioid diversification is investigations of whole genome blueprints at the chromosomal level. Antarctic notothenioid karyological and cytogenetics studies have been carried out for an increasingly large number of species in the past two decades. The goal of this review is to provide an updated overview on the cytogenetic features of Antarctic notothenioids distributed in the faunally rich Ross Sea. The review will follow the recent classification and nomenclature adopted by Duhamel et al. (2014).

The Ross Sea region lies between Victoria Land and Cape Colbeck and encompasses waters between longitudes 150°W and 160°E, and latitudes from 60°S to the Antarctic continental perimeter. As such it comprises the continental shelf with its banks and gulleys, the slope, a portion of the abyssal plain, and seamounts, some of which emerge as archipelagos such as the case of the Balleny Islands. This area corresponds to part of the FAO Major Fishing Area 88, and particularly Subareas 88.1 (Eastern Ross Sea) and part of the 88.2 (Western Ross Sea).

Ichthyofaunal studies in the Ross Sea have customarily been focused on a more restricted area, Ross Sea sensu stricto, encompassing only the continental shelf and slope down to a depth of 2000 m (northern limit is Cape Adare and Iselin Bank at about 71–72°S). This is the largest continental shelf ecosystem south of the Antarctic Polar Front, and one of the better known portions of south polar seas. Relatively isolated from human civilization, and protected under the Antarctic Treaty, it includes several Antarctic Specially Protected Areas (ASPAs). It is thus far the least anthropogenically affected stretch of ocean on Earth, and a compelling candidate for future marine protection initiatives (Ballard et al., 2012). This review will cover studied species from the Ross Sea sensu stricto and the greater Ross Sea.

Cytogenetics: historical digressions, advancements and potential for ichthyological research

As the only form of DNA within natural cellular context of an organism readily observable with light microscopy, chromosomes provide a unique platform to study in situ structural, functional, and evolutionary aspects of the genome. During mitosis and meiosis, diffused chromatin strands condense and organize into chromosomes of distinctive sizes and shapes, providing informative diagnostic characters. For many years, classical karyotyping studies utilized staining and banding methods to assess chromosomes number and morphology, determine the presence of sex-linked heterochromosomes, as well as detect gross structural features that would inform on chromosomal changes

such as re-arrangements or aberrations. With the advent of cytogenomics approach utilizing a combination of molecular biology, genetics, and cytogenetics, as well as sophisticated image visualization and capture technologies, studies of chromosomal details advanced from gross morphology to interrogating much finer molecular information carried within chromosomes (Speicher & Carter, 2005). Central to the cytogenomics approach is Fluorescence In Situ Hybridization (FISH) that can probe and reveal target DNA sequences on the chromosomes. Chromosomal FISH offers an important level of native genomic view that bridges whole genome nucleotide sequences from isolated DNA and gross chromosomal morphology. It can capture progressively finer grains of structural information of chromosomes, from selective visualization of entire chromosomes and/or chromosome arms, to the detection of specific chromosomal regions, to localizing individual genes. These levels of resolution greatly aid in discerning genetic and structural variations within and between species. Conventional karyotyping to reveal species-distinctive morphology of the chromosomal set as proxy of genome structure, and to track patterns of karyotype diversification that accompanied phyletic diversification, remains useful in correlating genomic change to speciation. When combined with chromosomal FISH mapping of genic and genomic markers and broadly applied across phylogeny, much finer scale of structural genomic changes would be uncovered that will enhance our ability to address the process of genome evolution. Finally, combined insights from cytogenomics, whole genome sequencing and bioinformatics hold powerful promise in decoding the mechanism and process of genome architectural evolution and its role in the diversification and evolution of organismal lineages, such as the remarkable Antarctic notothenioid radiation.

Cytogenetic analysis of notothenioid fish

The first karyological data on notothenioid fishes dated back to the early 80's. In that decade, notothenioid cytogenetic studies thrived as a new frontier and approach in understanding notothenioid phylogeny and evolution, and complementing the taxonomical synthesis from classical systematics, and emerging knowledge from biochemical and molecular analyses of these fishes. A flurry of research activities took place in multiple countries, and soon after, the first papers describing the basic chromosomal features and standard karyotypes of notothenioid species were published by Russian (Prirodina, 1984; Prirodina & Neyelov, 1984), French (Doussau de Bazignan & Ozouf-Costaz, 1985) and Brasilian/Japanese (Phan et al., 1986; 1987) polar fish biologists. Some of those pioneering studies investigated not only species with Antarctic distribution, but also circum-Antarctic species, laying the foundation for comparative karyological analyses in a broader species evolutionary context. However, while several different sectors of the Southern Ocean were investigated, none of the cytogenetically studied specimens was collected in the Ross Sea.

It was only after the Italian Antarctic station (Mario Zucchelli Station) was built at Terra Nova Bay (74°41′42″S, 164°07′23″E) that the first papers on the cytogenetics of fishes from the Western Ross Sea area was published (Morescalchi et al., 1992a, b). However, taxonomic representation was restricted to accessible sampling areas around the Zucchelli Station. This was considerably expanded by subsequent scientific collaborations between nations operating in the Ross Sea region, which enabled sampling in McMurdo Sound by Ross Island to the south (cooperation between USA and Italy), and the waters of the Balleny Islands to the north (cooperation between New Zealand and Italy, especially in the framework of the Victoria Land Transect Project and BioRoss Programme). At the same time, Italian and French biologists collaborated on intra-species comparisons of specimens from the Ross Sea and those collected in the Weddell Sea (international EPOS cruise Leg 3 on the R/V Polarstern, 1989) and along the coast of Adelie Land (ICOTA and REVOLTA programmes of the French polar Institute IPEV). Additional comparative cytogenetics studies between Antarctic and sub-Antarctic species finally were made possible in 2006 by sampling during French Expeditions to the Kerguelen Island region such as the POKER campaign (POissons de KERguelen, campagne d'évaluation de la biomass de poissons à Kerguelen). Scientific cooperation between Italy and Australia led to the inclusion of cytogenetics study of fish fauna in the Australian Antarctic and Subant-arctic Programme (Pisano et al., 2011), and sampling on the shelf around Heard Island was performed in during the THIRST (Third Heard Island Research Survey Trip, 1993) voyage. Chromosomal analyses were included in the

ICEFISH (International Collaborative Expedition to collect and study Fish Indigenous to Sub-antarctic Habitats, 2004) cruise, an international expedition supported by USA NSF Polar Programs aimed at comparing Antarctic notothenioid fishes and cool/temperate notothenioids living in the sub-Antarctic areas of the Atlantic Ocean.

The first standard protocol for chromosome preparations from notothenioid fishes was developed during the Terres australes et antarctiques françaises (TaaF) summer campaign icHTYo-GeneT (Doussau de Bazignan and Ozouf-costaz, 1985) to the Kergulen. With time, it was refined to optimize the quality of the preparations. Cell division is characteristically infrequent in adult Antarctic notothenioids in general, and particularly so in specimens inhabiting the extreme cold waters of high latitudes. Recently, a protocol of short-term cell culture from the cephalic kidney and spleen has been developed in field laboratory (Rey et al., 2015). The protocol, successfully tested on a wide spectrum of notothenioids (genera *Dissostichus*, *Notothenia*, *Trematomus*, *Gymnodraco*, *Pogonophryne*, *Chionodraco*, etc.), may aid in increasing the number of mitotic figures and to obtain chromosomes from specimens in bad conditions that quickly die after capture. Application of cytogenomics techniques, advancement in microscopy and improvements in the image capture system have led to significant steps forward in understanding chromosomal structures (Ozouf-Costaz et al., 2015). FISH mapping of marker gene sequences (such as telomeric sequences, ribosomal genes, globin genes, etc.) enabled more accurate description of karyotypes and inter- and intra-specific analyses, facilitating comparative and evolutionary genomic investigations in Antarctic fish (e.g., Pisano et al., 2003; Negrisolo et al., 2008; Nicodemus-Johnson et al., 2011).

This review provides a synthesis of the cytogenetics information for notothenioid fishes from the Ross Sea obtained through an extensive literature survey, and includes new karyotypic data on four species (*Lepidonotothen squamifrons*, *Trematomus scotti*, *T. loennbergii,* and *T. lepidorhinus*). This body of information is discussed in the broader context of the cytogenetic data currently available for Antarctic notothenioids. The review also integrates classical karyotyping information with recent chromosomal in situ gene mapping data to highlight patterns and subtleties of cytogenetic diversity at inter- and intra-specific levels. Such an integrated picture contributes to a robust and interdisciplinary characterization of the fish diversity in the Ross Sea region whose primary ecological value is widely recognized (Ballard et al., 2012).

Materials and methods

Fish sampling and chromosome preparation

The four notothenioid species used for the new karyotyping were collected during Italian Antarctic expeditions as well as through international collaborative station-based activities and cruises conducted in the Ross Sea area.

Trematomus scotti specimens were collected during the *RV Italica* cruise 2004 (Victoria Land Transect-VLT Project). The sampling area was located approximately between 71°10′S and 74°50′S across a latitudinal gradient of about 4° off Victoria Land. *T. loennbergii* specimens were collected in McMurdo Sound (77° 55′ S, 166° 40′ E) with traps through large holes drilled through sea ice during the US Antarctic expedition 2004/05. *Lepidonotothen squamifrons* (formerly *L. kempi*) and *Notothenia coriiceps* specimens were caught by bottom trawls during the Western Ross Sea Voyage 2004 aboard the New Zealand R/V *Tangaroa*. Specimens of *Trematomus lepidorhinus* were collected near the Italian Mario Zucchelli Station at Terra Nova Bay (74° 41′ S, 164° 7′ E) during multiple Italian Antarctic Expeditions. Table 1 summarizes this sampling information.

Mitotic chromosomes were obtained according to standard protocols for chromosome preparation (Ozouf-Costaz et al., 2015), slightly modified for species living in cold waters. Briefly, specimens were maintained in tanks supplied with flow through fresh, aerated seawater at local ambient temperature. Fish was injected intraperitoneally with colchicine (2 mg colchicine/100 g fish), and at an appropriate time later sacrificed with an anesthetic (MS222) overdose. Head kidney and spleen were harvested, and after tissue disaggregation and cell hypotonization, cell suspensions were fixed in 3/1 methanol/acetic acid (v/v) and stored at −20°C until further analyses.

Chromosome spreads in fixed cell suspension spread on microscope slide were DAPI (4,4′,6-diamidino-2-phenylindole) stained, and examined with an Olympus BX61 microscope equipped with a SenSys CCD camera (Photometrics). Micrographs

Table 1 Details on the samples used for karyotyping

Species	Sex	SA	SS#	Expedition
Lepidonotothen squamifrons	1f, 1u	WRS	836	TAN 2004[a]
	3f, 1 m	WRS	3,784	
	1f, 3 m	WRS	3,805	
Notothenia coriiceps	1f	WRS	584,	TAN 2004[a]
	1f	WRS	674	
	1f	WRS	681	
	1f, 2 m	WRS	961	
	1f, 3 m	WRS	3,869	
Trematomus lepidorhinus	2f, 1 m	WRS		PNRA 98/99[b]
	4f	WRS	544, 689, 783, 835	TAN 2004[a]
	1 m	WRS	A4	ITA 2004[c]
	4f	WRS	Hin5	
	1 m	WRS	Hout3	
Trematomus loennbergii	8f, 3 m	MCMS		USA 2004/05
Trematomus scotti	1f, 1u	WRS	Hout4	ITA 2004[c]
	2f	WRS	Hout3	
	1 m, 1u	WRS	BTNSMN	
	1f	WRS	BTNR2	
	1f	WRS	A2	
	1u	WRS	Hin5	

Number and sex of the specimens, sampling area and stations (when applicable), and expeditions are summarized. Further information (including the geographical coordinates of the sampling stations) can be found in the data report pertaining to each expedition (references a, b, c in the table)

SA sampling area, *SS#* sampling station number (when applicable), *f* female, *m* male, *u* undetermined, *WRS* Western Ross Sea, *MCMS* McMurdo Sound, *TAN 2004* RV Tangaroa cruise 2004, *PNRA 98/99* Italian Antarctic Expedition 1998/99, *ITA 2004* RV Italica cruise 2004, *USA 2004/05* US Antarctic Exp. 2004/05

[a] Mitchell & Clark (2004)

[b] Pugliatti & Ramorino (1999)

[c] Ramorino (2004)

were processed with CytoVision Genus software (Applied Imaging). Chromosomes were classified following Levan et al. (1964) according to the centromeric position and arm lengths ratio. Chromosomes were arranged in species-specific karyotypes (or the intra-species karyomorphs, when more than a single chromosomal set was found in a species) in decreasing order of size. FISH with a 28S rDNA probe was performed according to Ghigliotti et al. (2007).

Cytogenetic data inventory

In this review we refer to the list of notothenioid species by Eastman & Eakin (2014). The taxonomic classification and nomenclature follows Duhamel et al. (2014).

We therefore use the designation *Trematomus borchgrevinki* instead of the former *Pagothenia borchgrevinki, Trematomus amphitreta* instead of *Cryothenia amphitreta*, and *Trematomus peninsulae* instead of *Cryothenia peninsulae.* Also we used *Pleuragramma antarctica* the new valid scientific name of the Antarctic silverfish instead of the former *P. antarcticum.*

Notothenioid karyotypic data were derived from the database by Arai (2011) and updated data available in the literature. Information on the chromosomal localization of genes and sequences was obtained from the original scientific publications.

Results

New cytogenetic data for nototheniids from the Ross sea

Lepidonotothen squamifrons

Examination of multiple metaphases from each specimen consistently indicated a diploid number of 48 chromosomes and karyotype formula 4 m/sm + 44st/t (Fig. 1a). A dim DAPI-stained band along the arm of a pair of telocentric chromosomes (arrows) corresponds to the chromosomal locus of ribosomal genes clusters (Tomaszkiewicz et al., 2011). No sex-linked karyotype difference was observed between males and females. Similar results were reported for specimens from other sectors of the Southern Ocean, namely Bouvet island (Tomaszkiewicz et al., 2011), Heard Island and Chiuchia Bank (Ozouf-Costaz & Doussau de Bazignan, 1987), Heard island (Pisano et al., 2011 and unpublished data).

Trematomus loennbergii

A certain degree of variability was detected among individuals, with diploid numbers ranging between 26 and 33 (Fig. 1b–f). The differences in the number of elements in the complement accompanied with variations in the morphology of the chromosomes, as indicated by the karyotypic formulae (Table 2), were not linked to the sex of the specimens. Conversely, despite numeric and morphologic plasticity, the total number of chromosomal arms remains the same, as indicated by the consistency of the fundamental number (52).

Trematomus scotti

Examination of multiple metaphases from each specimen consistently indicated a diploid number of 48 and karyotype formula 4 m/sm + 44 st/t (Fig. 2a, a′). Among the two-armed elements, some degree of variability in the length of the p arms was sometimes detected between the two-armed chromosomes of pair 1. Those chromosomes were found homeomorphic or heteromorphic depending on the individual. The p arms of this chromosome pair were found to bear the major ribosomal gene locus (Fig. 2b). The smallest pair of two-armed homologues is consistently in the form of metacentric chromosomes. No differences in the karyotype were observed between males and females.

Trematomus lepidorhinus

A sex-related difference was detected in the diploid number with females having 48 chromosomes (karyotype formula 4 m/sm + 44 st/t), and males having 2n = 47 (karyotype formula 5 m/sm + 42 st/t). The additional metacentric chromosome in males is a large Y-chromosome, clearly recognizable in the metaphase plates (Fig. 3a, a'). The X1 and X2 chromosomes were recognized based on morphology and banding features: a dim DAPI-stained peri-centromeric region characterize X1 among telocentric chromosomes, whereas X2 is the only sub-telocentric chromosome of the complement. Size heteromorphism, due to the size variability of a dim DAPI-stained region extending along the p arm, was detected in the small sized sub-metacentric elements of the complement.

Notothenia coriiceps

The chromosomal set of the specimens in this study is congruent with the karyotype of specimens collected at more southern location in the Ross Sea (Morescalchi et al., 1992a) and in various other regions of the Southern Ocean (Prirodina & Neyelov, 1984; Phan et al., 1987; Ozouf-Costaz et al., 1999). The karyotype of *N. coriiceps* is made up of 22 two-armed chromosomes (2n = 22; Fig. 3b, b′).

The elements of pair 1 are submetacentrics, easily recognizable by DAPI staining. The centromeres are weak DAPI banded and flanked by strong DAPI-positive sub-centromeric regions. A large portion of the p arm is poorly stained by DAPI. This region is positive to silver staining (unpublished data) and bears the Nucleolar Organizing Regions (NORs). In addition, ribosomal genes have been found located at this chromosomal site through fluorescence in situ hybridization (Pisano et al., 2000). The chromosomes of the pair 1 are homomorphic or heteromorphic according to specimens due to variability in the length of the short arm between the homologues.

The submetacentric chromosomes of pairs 2 and 3 can be distinguished based on size and DAPI staining of the centromeric regions. A large centromeric area of pair 2 is weakly DAPI-stained; conversely, the

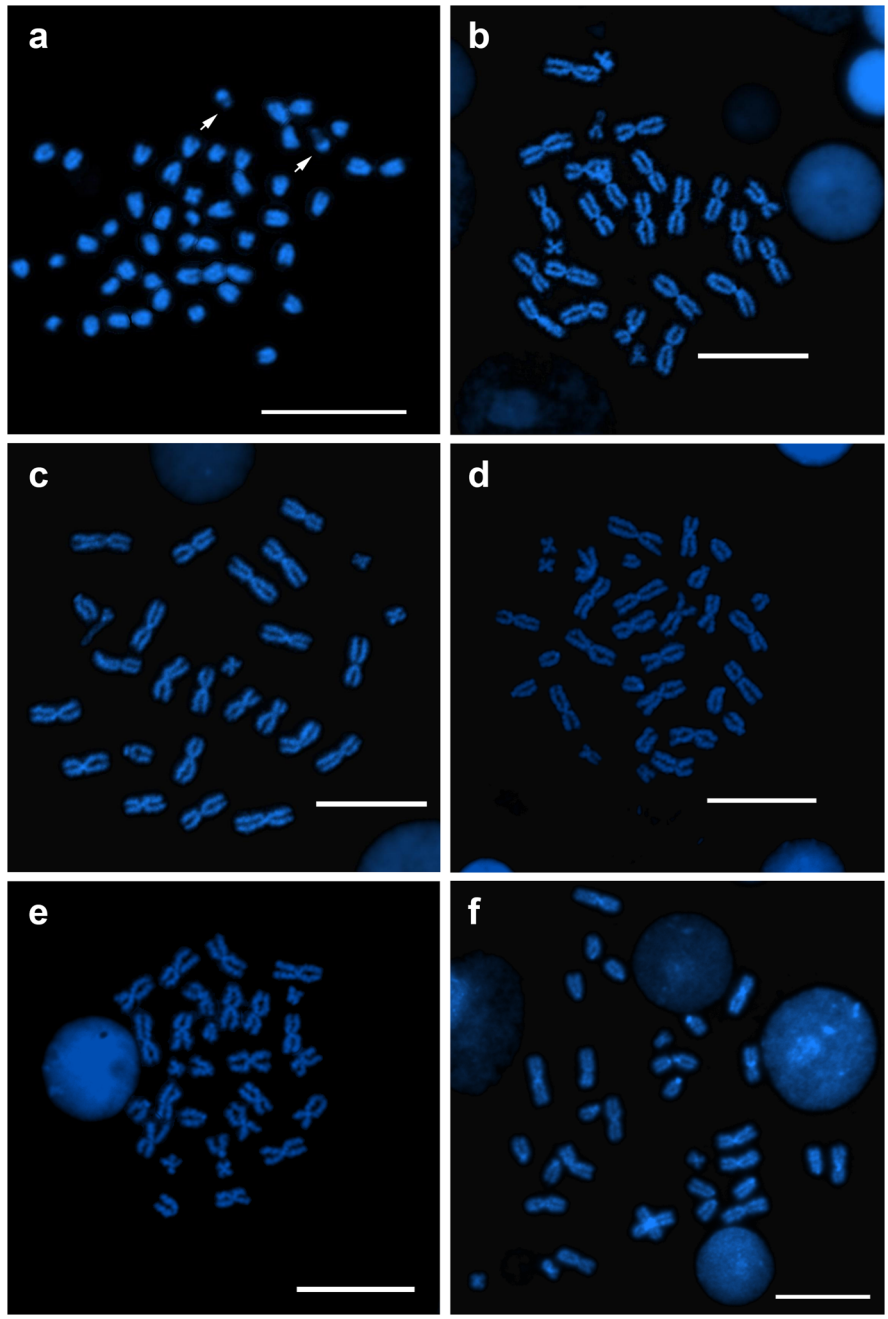

Fig. 1 DAPI-stained metaphase plates of *Lepidonotothen squamifrons* (**a**) and *Trematomus loennbergii* (**b**, **c**, **d**, **e**, **f**). Variants with 26 (**b**), 27 (**c**), 29 (**d**), 31 (**e**) and 33 (**f**) chromosomes are shown for the species *T. loennbergii*. *Scale bars* = 10 µm

medium-sized submetacentrics of pair 3 have DAPI-positive centromeres.

All other chromosomes are metacentric of comparable size. The specie-specific karyotype could be reconstructed by taking into consideration size, average arm ratio values, and DAPI-banding pattern. The homologues of pair 4 are the largest metacentrics having dim DAPI-stained centromeres and intensely stained peri-centromeric regions. Pair 5 and 6 are composed by chromosomes that fall into the category of metacentrics but that have arm ratio lower than 0.5. The elements of those two pairs are weak DAPI-stained in the centromeric region. The pair 7 is medium-sized metacentric characterized by large and

Table 2 Synthesis of the main chromosomal data on *T. loennbergii* from the Ross Sea area

2n	Karyotypic formula	FN	sex	Morphology and position of the unpaired chromosome
26	26 msm	52	1 m	Two-armed chromosome located between pair 5 and 6
27	25 msm + 2stt	52	1 f	
28	24 msm + 4stt	52	4 f, 1 m	
29	23msm + 6stt	52	1f, 1 m	Two-armed chromosome located between pair 9 and 10
30	22msm + 8stt	52		
31	21msm + 10stt	52	1f	Two-armed chromosome located between pair 8 and 9
33	19 msm + 14stt	52	1f	Two-armed chromosome located between pair 7 and 8

2n diploid number, *FN* fundamental number, *msm* meta/submetacentric chromosomes, *stt* subtelo/telocentric chromosomes

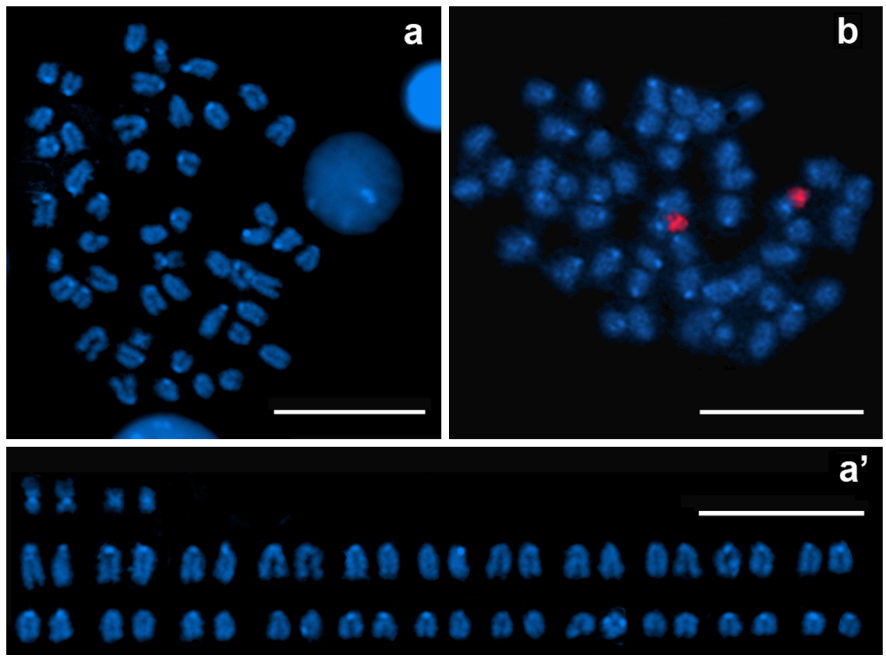

Fig. 2 *Trematomus scotti*, metaphase plate (**a**) and corresponding karyotype (**a**′) after DAPI-staining. Physical mapping of 28S rDNA (*red signals*) is shown in (**b**). *Scale bars* = 10 μm

typical DAPI-positive bands at the peri-centromeric regions. Pairs 8 and 9 are medium-sized metacentric chromosomes with DAPI-positive centromeres. The smallest elements of the karyotype are metacentrics with dim centromeric DAPI staining.

Synthesis on the cytogenetic information of species distributed in the Ross Sea

According to the available taxonomic and faunistic information (summarized in Table 3) 109 notothenioid species, in 11 subfamilies, have Antarctic distribution. Basic cytogenetic information is available for 59 of those species (54.13%), and 33 have been characterized through in situ cytogenetic mapping (Table 3; Fig. 4a).

For the Ross Sea Region alone, the number of occurring species is 68, in 9 subfamilies (Fig. 4b). Gobionototheninae and Harpagiferinae have never been recorded in this area where Artedidraconinae and Trematominae are the most speciose taxa. From the Ross Sea Region, basic cytogenetic analyses have been performed on 27 species (39.71%), and almost all of them (22) have been characterized in various details by cytogenetic mapping (Fig. 4b). The proportion between the number of species per subfamily and the number of cytogenetically studied species vary among the lineages (Fig. 4b). Eight of the species, comprising

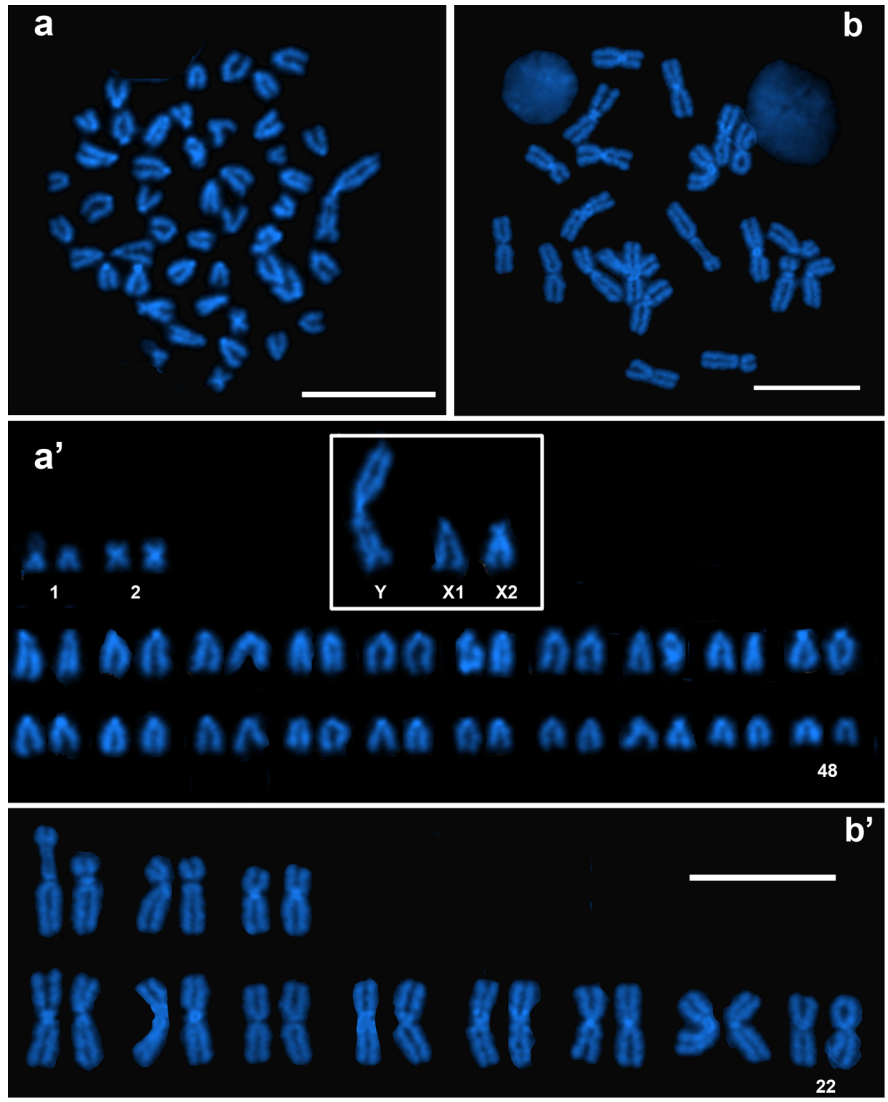

Fig. 3 Metaphase plates and corresponding karyotype of *Trematomus lepidorhinus* male (**a** and **a'**, respectively) and *Notothenia coriiceps* (**b** and **b'**, respectively) after DAPI staining. The sex chromosome system of *T. lepidorhinus* is shown in the *white rectangle*. *Scale bars* = 10 µm

three Artedidraconinae (*Artedidraco glareobarbatus*, *A. skottsbergi*, and *Histiodraco velifer*), four Trematominae (*Trematomus borchgrevinki*, *T. loennbergii*, *T. newnesi*, *T. nicolai*), and one Channichthyidae (*Cryodraco atkinsoni*) species, have been cytogenetically described only based on specimens collected in the Ross Sea Region.

The diploid numbers of notothenioid species studied from the Ross Sea Region range between 2n = 22 (*Notothenia coriiceps*) and 2n = 58 (*Trematomus nicolai*). The most common diploid number is 48 (Table 3, column 2n). In a minority of taxa (9), more than one diploid number has been found within the same species.

Discussion

Overview on 20 years of cytogenetic studies in the Ross Sea

The first cytogenetic studies on Antarctic Notothenioid fishes dated back to the early 80's (Prirodina, 1984; Prirodina & Neyelov, 1984; Doussau de Bazignan & Ozouf-Costaz, 1985; Phan et al., 1986; 1987). More than three decades later, cytogenetic information has expanded to cover half of the known species inhabiting the high Antarctic waters, and cytogenetic studies of species from the Ross sea region since the

Table 3 Synthesis of the cytogenetic information available for notothenioid fishes of the Ross Sea

Antarctic notothothenioid species	RS	RSR	CIRSR	CIORSR	2n	SLHC	ISMI
Dissostichinae							
Aethotaxis mitopteryx DeWitt 1962	Yes	Yes	No	No	–	–	–
Dissostichus eleginoides Smitt 1898	Yes	Yes	No	[1]	48	No	28S rDNA[2], 5S rDNA [2]
Dissostichus mawsoni Norman 1937	Yes	Yes	[2]	[3]	48	No	28S rDNA [2], 5S rDNA [2], Rex1 and Rex3 retrotransposons [4], AFGP sequences [5]
Gvozdarus svetovidovi Balushkin 1989	Yes	No	No	No	–	–	–
Gobionototheninae							
Gobionotothen acuta (Günther1880)	No	No	–	[6] [7]	48	–	28S rDNA [7], 5S rDNA [7]
Gobionotothen barsukovi Balushkin 1991	No	No	–	No	–	–	–
Gobionotothen gibberifrons (Lönnberg 1905)	No	No	–	[7] [8] [9]	46	No	28S rDNA [7], 5S rDNA [7]
Gobionotothen marionensis (Günther 1880)	No	No	–	[7]	50	No	28S rDNA [7], 5S rDNA [7]
Nototheninae							
Notothenia coriiceps Richardson 1844	Yes	Yes	[10]	[9] [11] [12]	22	No	Globin genes [13], telomeric sequences [14], 28S rDNA genes [14], IgH [15], Rex1 and Rex3 retrotransposons [4]
Notothenia rossii Richardson 1844	Yes	No	No	[1] [11]	24	No	–
Paranotothenia dewitti Balushkin 1990	Yes	No	No	No	–	–	–
Paranotothenia magellanica (Forster in Bloch and Schneider 1801)	Yes	Yes	No	[1]	26	–	–
Pleuragramminae							
Pleuragramma antartica Boulenger 1902	Yes	Yes	No	[3]	48	No	–
Trematominae							
Indonotothenia cyanobrancha Richardson 1844	No	No	–	[1]	48	–	–
Lepidonotothen larseni (Lönnberg 1905)	No	Yes	No	[7]	48	No	28S rDNA [7], 5S rDNA [7]
Lepidonotothen mizops (Günther 1880)	No	No	–	[6] [7]	48	No	28S rDNA [7], 5S rDNA [7]
Lepidonotothen nudifrons (Lönnberg 1905)	No	No	–	[7]	28	No	28S rDNA [7], 5S rDNA [7]

Table 3 continued

Antarctic notothothenioid species	RS	RSR	CIRSR	CIORSR	2n	SLHC	ISMI
Lepidonotothen squamifrons (Günther 1880)	Yes	Yes	p.p.	[6] [7]	48	No	28S rDNA [7], 5S rDNA [7]
Pagothenia brachysoma (Pappenheim 1912)	Yes	Yes	No	No	–	–	–
Patagonotothen guntheri (Norman 1937)	No	No	–	[7]	48	–	28S rDNA [7], 5S rDNA [7]
Trematomus amphitreta Cziko and Cheng 2006	Yes	No	No	No	–	–	–
Trematomus bernacchii Boulenger 1902	Yes	Yes	[10] [16]	[3] [8]	48	No	IgH genes [15]
Trematomus borchgrevinki (Boulenger 1902)	Yes	Yes	[10]	No	45/46	Yes	IgH genes [15], 5S rDNA [17], AFGP genes [17]
Trematomus eulepidotus Regan 1914	Yes	Yes	[10]	[3] [6]	24	No	IgH genes [15]
Trematomus hansoni Boulenger 1902	Yes	Yes	[10]	[3] [8]	45/46-48	Yes	globin genes [13], IgH genes [15], 5S rDNA [17], AFGP genes [17]
Trematomus lepidorhinus (Pappenheim 1911)	Yes	Yes	p.p	[3] [6]	47/48	Yes	IgH genes [15], 5S rDNA [17], AFGP genes [17]
Trematomus loennbergii Regan 1913	Yes	Yes	[10] p.p		26-30	No	IgH genes [15]
Trematomus newnesi Boulenger 1902	Yes	Yes	[10] [16]		45/46	Yes	IgH genes [15], 5S rDNA [17], AFGP genes [17], Rex1 and Rex3 retrotransposons [4]
Trematomus nicolai (Boulenger 1902)	Yes	Yes	[10]		57/58	Yes	IgH genes [15], 5S rDNA [17], AFGP genes [17], telomeric sequences [18]
Trematomus peninsulae Daniels 1981	No	No	–	No	–	–	–
Trematomus pennellii Regan 1914	Yes	Yes	[10]	[3]	32	No	globin genes [13], IgH genes [15], 5S rDNA [17], AFGP genes [17], telomeric sequences [18]
Trematomus scotti (Boulenger 1907)	Yes	Yes	p.p	[3]	48–50	No	IgH genes [15]
Trematomus tokarevi Andriashev 1978	Yes	Yes	No	No	–	–	–
Trematomus vicarius Lönnberg 1905	No	No	No	No	–	–	–
Harpagiferinae							
Harpagifer andriashevi Prirodina 2000	No	No	–	[19]	48	–	–
Harpagifer antarcticus Nybelin 1947	No	No	–	[19]	48		–
Harpagifer bispinis (Schneider in Bloch and Schneider 1801)	No	No	–		–	–	–
Harpagifer crozetensis Prirodina 2004	No	No	–	No	–	–	–

Table 3 continued

Antarctic notothothenioid species	RS	RSR	CIRSR	CIORSR	2n	SLHC	ISMI
Harpagifer georgianus Nybelin 1947	No	No	–	No	–	–	–
Harpagifer kerguelensis Nybelin 1947	No	No	–	No	–	–	–
Harpagifer macquariensis Prirodina 2000	No	No	–	No	–	–	–
Harpagifer nybelini Prirodina 2002	No	No	–	No	–	–	–
Harpagifer permitini Neyelov and Prirodina 2006	No	No	–	No	–	–	–
Harpagifer palliolatus Richardson 1845	No	No	–	No	–	–	–
Harpagifer spinosus Hureau, Louis, Tomo and Ozouf 1980	No	No	–	No	–	–	–
Artedidraconinae							
Artedidraco glareobarbatus Eastman and Eakin 1999	Yes	No	[20]	No	46	No	28S rDNA [20]
Artedidraco loennbergi Roule 1913	Yes	Yes	No	No	–	–	–
Artedidraco mirus Lönnberg 1905	No	No	–	[19]	46	–	–
Artedidraco orianae Regan 1914	Yes	Yes	[20]	[3]	46	No	28S rDNA [20]
Artedidraco shackletoni Waite 1911	Yes	No	[20]	[3]	46	No	28S rDNA [20]
Artedidraco skottsbergi Lönnberg 1905	Yes	No	[20]		45/46	Yes	28S rDNA [20], 5S rDNA [17], AFGP genes [17]
Dolloidraco longedorsalis Roule 1913	Yes	No	No	No	–	–	–
Histiodraco velifer (Regan 1914)	Yes	No	[21] [20]	–	46	No	28S rDNA [20], telomeric sequences [21]
Pogonophryne albipinna Eakin 1981	No	Yes	No	No	–	–	–
Pogonophryne barsukovi Andriashev 1967	Yes	No	No	[3]	46	No	–
Pogonophryne bellingshausenensis Eakin, Eastman and Matallanas 2008	No	No	–	No	–	–	–
Pogonophryne brevibarbata Balushkin, Petrov and Prutko 2010	Yes	No	No	No	–	–	–
Pogonophryne berebropogon Eakin and Eastman 1998	Yes	No	No	No	–	–	–

Table 3 continued

Antarctic notothothenioid species	RS	RSR	CIRSR	CIORSR	2n	SLHC	ISMI
Pogonophryne dewitti Eakin 1988	No	No	–	No	–	–	–
Pogonophryne eakini Balushkin 1999	No	No	–	No	–	–	–
Pogonophryne favosa Balushkin and Eakin 1998	No	No	–	No	–	–	–
Pogonophryne fusca Balushkin and Eakin 1998	No	No	–	No	–	–	–
Pogonophryne immaculata Eakin 1981	Yes	No	No	No	–	–	–
Pogonophryne lanceobarbata Eakin 1987	Yes	No	No	No	–	–	–
Pogonophryne macropogon Eakin 1981	Yes	No	No	No	–	–	–
Pogonophryne maculiventrata Spodareva and Balushkin 2014	No	No	–	No	–	–	–
Pogonophryne marmorata Norman 1938	Yes	No	No	[3]	46	–	–
Pogonophryne mentella Andriashev 1967	Yes	No	No	[3]	46	No	–
Pogonophryne neyelovi Shandikov and Eakin 2013	Yes	No	No	No	–	–	–
Pogonophryne orangiensis Eakin and Balushkin 1998	No	Yes	No	No	–	–	–
Pogonophryne permitini Andriashev 1967	Yes	No	No	No	–	–	–
Pogonophryne platypogon Eakin 1988	No	No	–	No	–	–	–
Pogonophryne scotti Regan 1914	Yes	No	[16]	[3]	46	No	–
Pogonophryne skorai Balushkin and Spodareva 2013	No	No	–	No	–	–	–
Pogonophryne stewarti Eakin, Eastman and Near 2009	No	No	–	No	–	–	–
Pogonophryne squamibarbata Eakin and Balushkin 2000	No	No	–	No	–	–	–
Pogonophryne tronio Shandivov, Eakin and Usachev 2013	Yes	No	No	No	–	–	–
Pogonophryne ventrimaculata Eakin, 1987	No	No	–	No	–	–	–
Gymnodraconinae							
Gymnodraco acuticeps Boulenger 1902	Yes	Yes	[22]	[23]	48	–	28S rDNA [22], Rex1 and Rex3 transposons [4]
Acanthodraco dewitti Skòra 1995	Yes	No	No	No	–	–	–

Table 3 continued

Antarctic notothothenioid species	RS	RSR	CIRSR	CIORSR	2n	SLHC	ISMI
Psilodraco breviceps Norman 1937	No	Yes	–	[23] [3]	48	–	–
Bathydraconinae							
Akarotaxis nudiceps (Waite 1916)	Yes	No	No	No	–	–	–
Bathydraco antarcticus Günther 1878	Yes	No	No	No	–	–	–
Bathydraco joannae DeWitt 1985	No	No	–	No	–	–	–
Bathydraco macrolepis Boulenger 1907	Yes	Yes	No	No	–	–	–
Bathydraco marri Norman 1938	Yes	No	No	[3]	39/38	Yes	–
Bathydraco scotiae Dollo 1906	Yes	No	No	No	–	–	–
Prionodraco evansii Regan 1914	Yes	No	No	[23] [3]	20	–	–
Racovitzia glacialis Dollo 1900	Yes	No	No	[3]	36	–	–
Vomeridens infuscipinnis (DeWitt 1964)	Yes	No	No	No	–	–	–
Cygnodraconinae							
Cygnodraco mawsoni Waite 1916	Yes	No	[16]	[3]	48	No	–
Parachaenichthys charcoti (Vaillant 1906)	No	No	–	No	–	–	–
Parachaenichthys georgianus (Fischer 1885)	No	No	–	[23] [3]	48	–	–
Gerlachea australis Dollo 1900	Yes	No	No	[3]	48	–	–
Channichthyinae							
Chaenocephalus aceratus (Lönnberg 1906)	No	No	–	[24]	48	–	–
Chaenodraco wilsoni Regan 1914	Yes	Yes	No	[3] [25]	47/48	Yes	–
Champsocephalus gunnari Lönnberg 1905	No	No	–	[1] [26]	48	No	28S rDNA [27], 5S rDNA [28]
Channichthys rhinoceratus Richardson 1844	No	No	–	[1]	48	No	28S rDNA [28], 5S rDNA [28]
Chionobathyscus dewitti Andriashev and Neelov 1978	Yes	Yes	No	[3]	47	Yes	–
Chionodraco hamatus (Lönnberg 1905)	Yes	Yes	[29]	[3]	47/48	Yes	Tc1-like transposon [30], 28S rDNA [28], 5S rDNA [28], telomeric sequences [17], Rex1 and Rex3 transposons [4], HeliNoto transposon sequence [31], AFGP sequences [17]

Table 3 continued

Antarctic notothothenioid species	RS	RSR	CIRSR	CIORSR	2n	SLHC	ISMI
Chionodraco myersi DeWitt and Tyler 1960	Yes	Yes	No	[3] [25]	47/48	Yes	–
Chionodraco rastrospinosus DeWitt and Hureau 1979	No	No	–	[24]	48	–	–
Cryodraco antarcticus Dollo1900	Yes	Yes	[29]	[3]	48	No	–
Cryodraco akinsoni Regan 1914	Yes	No	[28]	No	48	No	28S rDNA [28], 5S rDNA [28]
Dacodraco hunteri Waite 1916	Yes	No	No	No	–	–	–
Neopagetopsis ionah Nybelin 1947	Yes	Yes	No	[3]	48	No	28S rDNA [28], 5S rDNA [28]
Pagetopsis macropterus (Boulenger 1907)	Yes	Yes	[29]	[3]	47/48	Yes	28S rDNA [28], 5S rDNA [28], AFGP sequences [17]
Pagetopsis maculatus Barsukov and Permitin 1958	Yes	Yes	No	[3]	48	–	–
Pseudochaenichthys georgianus Norman 1937	No	No	–	[24]	48	–	–
Species total 109	64	35					

The information was organized in the form of a table with 8 columns as follows: (1) Column 1 (Antarctic notothenioid species) includes current scientific names of the studied taxa according to Eastman & Eakin (2014) and Eschmeyer (2014). Classification of higher taxonomical level than species followed Duhamel et al. (2014). (2) Column 2 (RS) and 3 (RSR) contain information on the occurrence of a species in the Ross sea sensu stricto (meaning the continental shelf and slope) or in the Ross Sea region, respectively. Data are from Hanchet et al. (2013) and Duhamel et al. (2014), and additional information for species of the genus *Pogonophryne* is from Balushkin & Spodareva (2013) and from Shandikov & Eakin (2013). (3) Column 4 (CIRSR) includes information on the availability of cytogenetic data from specimens collected in the Ross Sea area from previous publications (reference) or presented herein (p.p.). (4) Column 5 (CIORSR) includes information on the availability of cytogenetic data from specimens collected in other Antarctic areas. When data are available, the source reference is reported. (5) Column 6 (2n) contains diploid numbers. When sex-linked chromosomes were found in a species, the male/female-specific diploid numbers are reported. (6) Column 7 (SLHC) contains information on the occurrence (yes) or non-occurrence (no) of sex-linked heterochromosomes; (–) is included when a species was cytogenetically studied but sex-linked features were not investigated, or when no cytogenetic information is available. (7) Column 8 (ISMI) reports on the availability of in situ mapping information. Occurrence (yes) or nonoccurrence the list of localized sequences and genes, along with source references, is reported

[1] Doussau de Bazignan & Ozouf-Costaz (1985); [2] Ghigliotti et al. (2007); [3] Ozouf-Costaz et al. (1991); [4] Ozouf-Costaz et al. (2004); [5] Nicodemus-Johnson et al. (2011); [6] Ozouf-Costaz & Doussau de Bazignan (1987); [7] Tomaszkiewicz et al. (2011); [8] Phan et al. (1986); [9] Phan et al. (1987); [10] Morescalchi et al. (1992a); [11] Prirodina & Neyelov (1984); [12] Ozouf-Costaz et al. (1999); [13] Pisano et al. (2003); [14] Pisano et al. (2000); [15] Pisano et al. (2007); [16] Prirodina (1984); [16] Morescalchi et al. (1996); [17] Ghigliotti et al. (2013); [18] Caputo et al. (2002); [19] Prirodina & Ozouf-Costaz (1995); [20] Ghigliotti et al. (2010); [21] Caputo et al. (2003); [22] Pisano et al. (2001); [23] Prirodina (1990); [24] Prirodina (1989); [25] Ozouf-Costaz (1987); [26] Pisano et al. (1997); [27] Ozouf-Costaz et al. (1996); [28] Mazzei et al. (2004); [29] Morescalchi et al. (1992b); [30] Capriglione et al. (2002); [31] Cocca et al. (2011)

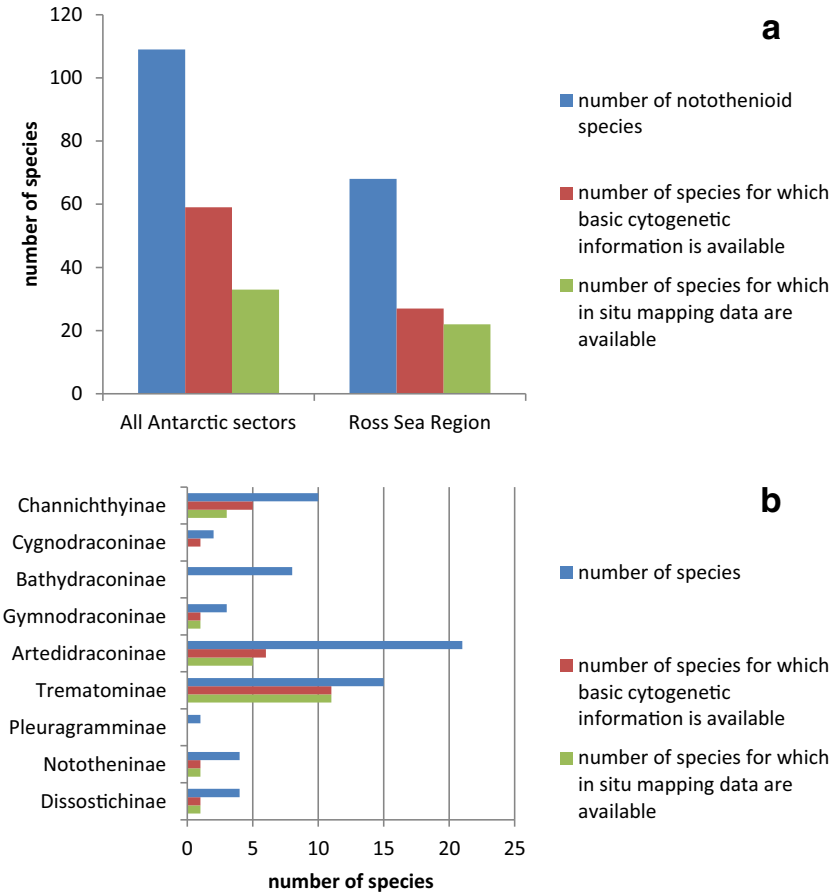

Fig. 4 The availability of basic or in situ mapping information in Antarctic Notothenioid species in the Antarctic waters and, specifically in the Ross Sea Region is illustrated. **a** All Antarctic sectors versus Ross Sea region. **b** Focus on the Ross Sea Region

early 90s have provided valuable contributions to this improvement.

The karyotypes of eight species could be described for the first time as a result of specimens caught from the Ross Sea. Besides the description of new karyotypes, more than 20 years of cytogenetic studies of Ross Sea Region specimens have generated data resources for comparative analyses useful in gaining insights into the diversity of Antarctic notothenioids at the chromosomal level. Starting from the basic cytogenetic parameter, that is chromosome number (2n), continuing with the chromosomes shape and arm size, summarized in the chromosomal formula and schematized in the karyotype, and eventually by the in situ the mapping of known sequences, the increasing collection of cytogenetic data year after year allowed in depth characterizations that help clarify and inform on the nature of chromosomal diversity within notothenioids.

The diploid numbers found in specimens from the Ross Sea Region do not deviate much from those described for Notothenioids in general. They range from $2n = 22$ in *Notothenia coriiceps* (Nototheninae), to $2n = 58$ in *Trematomus nicolai* (Trematominae). Diploid numbers are most conservative in Artedidraconinae, being 46 in all the species studied to-date

Similar to most notothenioids, diploid number found in species from the Ross Sea Region is most frequently 48. This is not surprising since previous cytogenetic studies in both non-Antarctic and

Antarctic notothenioids (e.g., Pisano et al., 2003; Mazzei et al., 2006; Ghigliotti et al., 2007) in this monophyletic suborder (Near et al., 2012) support the hypothesis that a karyotype of 48 one-armed chromosomes as the ancestral set for notothenioids (Pisano & Ozouf-Costaz, 2003).

However, despite the prevalence of 2n = 48, many other diploid numbers occur within notothenioids. Indeed, important chromosomal changes have accompanied the diversification of Antarctic notothenioids generating various levels of cytogenetic diversity appearing in the variety of karyotypes found even in closely related species (karyotypic divergence), as well as in the occurrence of karyotypic variants at intra-specific level (karyotype plasticity).

Major changes resulting in species-specific divergent karyotypes are chromosome rearrangements, already discussed in previous papers (Ozouf-Costaz et al., 1997; Pisano & Ozouf-Costaz, 2003; Tomaszkiewicz et al., 2011). Irrespective of the underlying mechanism, and the ongoing debate as to what extent the chromosomal changes could have influenced the processes of speciation and adaptation in notothenioids, chromosomal break points where recombination is suppressed most strongly, are currently acknowledged to permit both adaptive and non-adaptive divergence (Strasburg et al., 2009).

Besides chromosomal rearrangements, repetitive DNAs and transposable elements might have played a role in the diversification of the notothenioid karyotypes. In fishes, they have occasionally been found to accumulate in regions of the genome associated with possible events of chromosomal rearrangements (e.g., Schneider et al., 2013). In Antarctic notothenioid fishes, new LINE (Long Interspersed Nuclear Elements) gene families have emerged from extensive Antarctic-specific duplications (Chen et al., 2008), and other transposable elements have accumulated at chromosomal hot spots of recombination (Belkadi et al., 2014).

Spatial and local intra-specific cytogenetic diversity

Comparative analyses of specimens of the same species from different geographic areas sometimes reveal spatial distribution-related differences, showing there is a deeper level of cytogenetic diversity: the geographic-related intra-specific diversity.

T. hansoni is a good example of such within species diversity. In this circum-Antarctic notothenioid, specimens from the Atlantic sector have 48 chromosomes (Phan et al., 1986; Ozouf-Costaz et al., 1991), the population from the Ross Sea have 45/46 chromosomes and sex chromosomes (Morescalchi et al., 1992a), and the population from Adélie Land have 46 chromosomes, no sex-chromosomes and a peculiar pattern of heterochromatin (Ozouf- Costaz et al., 1999; Pisano & Ozouf- Costaz, 2000). Such intra-specific divergence may imply a reduction of genetic exchanges between populations, and/or the occurrence of intra-specific reproductive barriers. If this holds truth, spatial distribution-related intra-specific chromosomal diversity could be interpreted as the first cue of occurrence of sibling species, thus providing elements for further taxonomic and evolutionary investigations.

Sometimes, a degree of intra-specific diversity is detected in specimens within the same geographic area. In the Ross Sea Region, the analysis of multiple specimens of species that are abundant and widely distributed in the area has revealed the co-existence of individuals with different karyotypes both at inter- and intra-population levels.

Illustrative is the case of *T. loennbergii,* a single specific taxon cytogenetically represented by multiple karyotypic sympatric variants. Morescalchi and coll. (1992a) reported the presence of two karyotype variants with 2n = 28 and 2n = 30 in specimens caught in Terra Nova Bay. Here, we report five additional variants (2n = 26; 2n = 27; 2n = 29, 2n = 31, 2n = 33) (Fig. 1b–f) found in specimens collected in the same area (McMurdo Sound). The various diploid numbers correspond to different chromosomal morphologies and karyotypic formulas, but retain the same fundamental number (Table 2). The consistency in the number of chromosomal arms, suggests that the various karyotypic variants could be linked to each other though rearrangements of the Robertsonian type (White, 1978). However, differently from what described for mammals, Robertsonian fusions are not the most frequent mechanism of evolutionary karyotypic changes in fish (Galetti et al., 2006) therefore pericentric inversions and, possibly, also centromeric-drive (Molina et al., 2014) might also be taken into consideration to explain the intra-specific karyotypic polymorphism found in *T. loennbergii*.

Another level of intra-specific karyotype diversity is found between males and females when sex- linked heteromorphic chromosomes occur. In the Ross Sea Region, sex-linked chromosomes have been reported for *Trematomus borchgrevinki*, *T. hansoni*, *T. newnesi*, *T. nicolai* (Morescalchi et al., 1992a), *Chionodraco hamatus* and *Pagetopsis macropterus* (Morescalchi et al., 1992b), and *Artedidraco skottsbergi* (Ghigliotti et al., 2010) whose sex-chromosome system was described with specimens available from the Ross Sea. The occurrence of the sex-chromosome system of *T. lepidorhinus* is reported here for the first time (Fig. 3) since heteromorphic sex-linked chromosomes were not detected in previous studies on specimens from the Weddell Sea (Ozouf-Costaz et al., 1991). Thus, our new data of *T. lepidorhrinus* provide a second example where both sex-related and geographic-related karyotype diversity occur, besides *T. hansoni*.

Sex-related chromosomes have been reported for 26.67% of the cytogenetically studied notothenioid species (Ghigliotti et al., 2014), a much higher frequency compared to teleosts (4%) in general (Arai, 2011; The Tree of Sex Consortium, 2014). The finding of sex-linked heterochromosomes to occur only in cold-adapted species in a single family Nototheniidae (per Duhamel et al., 2014), but never in temperate species of the three basal non-Antarctic notothenioid families, has recently been hypothesized to be a possible evolutionary/adaptive trajectory toward genetic control of sex determination as a prevailing control in Antarctic notothenioids living in constantly frigid polar conditions, where temperature variations as a extrinsic control for sex determination are absent (Ghigliotti et al., 2014).

Insights into notothenioid chromosome structure by cytogenetic mapping

Due to a combination of historical and logistic reasons, cytogenetic studies of fish from the Ross Sea Region started later than in other Antarctic sectors. On the other hand, they started in a period of important methodological improvements that allowed integration of classical karyotypic approaches with molecular cytogenetics (Fig. 4b; Table 3, column ISMI), producing more refined and detailed structural analyses. Physical mapping of known DNA sequences in situ onto chromosomes has been conducted for various purposes. The possibility to label and recognize individual chromosomes for a correct pairing of the homologues has been extremely useful to gain robust karyotype assessment. For broader evolutionary questions, in situ localization of known sequences allowed visualization of genomic regions involved in structural changes that happened during Antarctic notothenioid evolution, such as the loss of the globin trait and the gain of the novel AFGP. Chromosomal positions of repetitive sequences (e.g., telomeric sequences, ribosomal genes clusters, transposable elements, etc.) have frequently been used as markers in comparative analyses across species to investigate gross karyotypic change. For instance, the occurrence of interstitial remnants of telomeric sequences, detected by FISH, in two-armed chromosomes of *Notothenia coriiceps* (Pisano & Ozouf-Costaz, 2003) has been considered as footprints of chromosomal fusion events that likely led to the formation of the 22 two-armed chromosomes in this taxon (Fig. 3b and b'), thus indicating the direction of karyotypic change in that lineage was from high to low diploid numbers.

Ribosomal sequences, which are among the most used markers in molecular cytogenetics of fish species (Gornung, 2013) have been extensively mapped in Antarctic notothenioids, including several species from the Ross Sea Region, where they mostly occur as a single locus (Pisano & Ghigliotti, 2009).

Interestingly comparative mapping of the multigenic ribosomal DNA units in *Dissostichus mawsoni* (Antarctic toothfish) and in the phylogenetically very close *Dissostichus eleginoides* (Patagonian toothfish) revealed an unexpected difference between these two giant congeneric species (Ghigliotti et al., 2007). A single locus is present in *D. eleginoides*, whereas *D. mawsoni* has a duplicated locus located on two pairs of chromosomes. It is unclear why these two morphologically similar sister toothfish species, both growing to very large sizes, would have differential abundance of rDNA genes. A potential explanation relates to their evolutionary history in distinct thermal environments. *D. eleginoides* occur in non-freezing subantarctic waters and has no (nor requires) antifreeze glycoproteins (AFGP). *D. mawsoni* is endemic to the icy freezing waters of Antarctica, possesses a large family of AFGP genes and produces high levels of circulatory AFGPs to avoid freezing (DeVries & Cheng, 2005). An extra set of ribosomal genes would support increased ribosomes production, which in turn would

support AFGPs synthesis, an effort not required by the Patagonian toothfish (Ghigliotti et al., 2007).

Molecular cytogenetics also complements molecular studies of cold adaptive and/or cold specialization changes in the genome. These include FISH mapping of the conserved chromosomal sites for alpha–beta-globin gene clusters in four red blooded fishes (Pisano et al., 2003) providing the reference chromosomal region where the primary deletion event resulting in the peculiar hemoglobin-less phenotype occurred in the white-blooded Antarctic icefishes (Cocca et al., 1995; Cheng & Detrich, 2007). In situ chromosomal mapping of AFGP genes, the key adaptive trait in Antarctic notothenioids, confirmed that they occupy a single genomic region, an important information that enabled appropriate sequence assembly of this large multigene family (Nicodemus-Johnson et al., 2011).

Multiple faces of diversity in a single taxon: the Trematomus case

Recent molecular phylogenetic analyses (Sanchez et al., 2007; Kuhn & Near, 2009; Lautrédou et al., 2012) synonymized the genera *Pagothenia* and *Cryothenia* with *Trematomus*, resulting in a total of 15 species in the genus, all endemic to high Antarctic waters and accounts for about 10% of all notothenioids (Eastman & Eakin, 2014). With the exception of *T. vicarius*, all trematomid species have been reported for the Ross Sea (Eastman & Hubold, 1999; Hanchet et al., 2013). Two trematomids, *T. eulepidotus,* and *T. lepidorhinus* are among the eight most abundant fish species in the Ross Sea area sensu stricto (Hanchet et al., 2013). This species richness is paired with a remarkable degree of ecological diversity. They are adapted to life in a wide range of niches including epibenthic, semi-pelagic, and cryo-pelagic habitats, forming an important component of the Antarctic coastal waters ichthyofauna in biomass (Ekau & Gutt, 1991; La Mesa et al., 2004; Causse et al., 2011).

Due to their recognized monophyly, species richness, high endemism, ecological diversity, and dominance of habitat, the genus *Trematomus* (formerly subfamily Trematominae) is considered as a full species flock, nested within the main flock of Antarctic notothenioids (Lecointre et al., 2013). With the exception of *T. scotti*, recognized as the sister group taxon of all other *Trematomus* (Sanchez et al., 2007), the species of the genus *Trematomus* comprise a burst of diversification in the recent past (10 Ma) (Near, 2004). The degree of diversification is even higher if one considers the intra-specific eco-phenotypic plasticity recorded in some species, such as the presence of two color morphs in *T. bernacchii* (Bernardi & Goswami, 1997), and two "mouth" morphs in *T. newnesi* (Piacentino & Barrera-Oro, 2009; Eastman & Barrera-Oro, 2010).

Their abundance in coastal regions made the trematomids one of the best cytogenetically studied notothenioid group from the Ross Sea. With diploid numbers ranging from $2n = 24$ to $2n = 58$ (Table 3), and the occurrence of very different karyotype morphologies, trematomids exhibit the highest karyotypic diversity among notothenioids, indicative of a high rate of chromosomal change that occurred during their adaptive radiation. The degree of diversification within the genus is even higher when intra-specific karyotype variability is examined. Karyotype variants have been observed in *T. loennbergii* and in *T. hansoni*, as described earlier. In addition, chromosomal plasticity typical of *Trematomus* is manifested in heteromorphic sex-linked chromosomes in many of the species, namely *T. hansoni, T. lepidorhinus, T. newnesi, T. nicolai,* and *T. borchgrevinki* (Ghigliotti et al., 2014).

In some cases, the cytogenetic differences between species served as useful tool to definitively solve taxonomic ambiguities, when the distinction of different species is hindered by indistinct morphological and molecular characters, as epitomized by *T. lepidorhinus* and *T. loennbergii* (De Witt et al., 1993; Lautrédou et al., 2010). The chromosome set of these two species are markedly distinct. *T. lepidorhinus* has a poorly rearranged chromosome set consistently made up of 47 and 48 chromosomes in males and females, respectively. In contrast, *T. loennbergii* is represented by multiple co-existing karyotypic variants with substantially lower diploid numbers (ranging between 26 and 33), prevalence of two-armed chromosomes (rearranged chromosomal set), and no sex-linked differences.

Concluding remarks

In summary: (a) In about 20 years, basic cytogenetic information on Antarctic notothenioid from the Ross Sea Region has been collected for 27 of the 68 species occurring in that area. In parallel in almost all of the studied species, various details of the chromosome

structure and organization have been characterized by cytogenetic mapping. (b) The cytogenetic features of the notothenioid species living in the Ross Sea area do not deviate from those of Antarctic notothenioids, with diploid numbers ranging between 2n = 22 (*Notothenia coriiceps*) and 2n = 58 (*Trematomus nicolai*), and the most common chromosomal complement made up of 48 elements. (c) Processes of karyotypic divergence leading to a variety of karyotypes in closely related species as well as karyotype plasticity, epitomized in the occurrence of karyotypic variants at intra-specific level, occur in these fishes. (d) Different levels of cytogenetic diversity are detectable within Antarctic notothenioids including geographic-related intra-specific diversity (e.g., *T. hansoni*), intra-population diversity (e.g., *T. loennbergii*) and sex-linked diversity (e.g., *T. lepidorhinus*). (e) The application of in situ mapping, besides producing more refined and detailed structural analyses, allowed to address broader evolutionary questions and to contribute to a better understanding of adaptive genome changes happened during the Antarctic notothenioid radiation.

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