Comparative cytogenetic studies of the Nototheniidae (Teleostei: Acanthomorpha) from the Indian (Kerguelen-Heard Plateau) and Atlantic (South Georgia, South Sandwich, Falkland/Malvinas, Bouvet Islands) sectors of the Southern Ocean

by

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ABSTRACT. - Comparative cytogenetics is a powerful tool that can be combined with molecular phylogenetics and morphological systematics to infer the evolutionary relationships among species. In this report we describe the karyotypes (including 5S and 28S ribosomal RNA gene positions) of ten species from three understudied genera of the family Nototheniidae (suborder Notothenioidei); five of the karyotypes (species underlined) are reported for the first time: *Gobionotothen marionensis* (Günther, 1880), *G. acuta* (Günther, 1880), *G. gibberifrons* (Lönnberg, 1905), *Lepidonotothen larseni* (Lönnberg, 1905), *L. mizops* (Günther, 1880), *L. nudifrons (*Lönnberg, 1905), *L. squamifrons* (Günther, 1880), *Patagonotothen guntheri* (Norman, 1937), *P. ramsayi* (Regan, 1913), and *P. tessellata* (Richardson, 1845). Furthermore, we place these species in the notothenid phylogenetic tree by extensive sequencing of the mitochondrial cytochrome oxidase I (COI) genes, including COIs from notothenid genera with well characterized karyotypes (Notothenia (Richardson, 1844)) and from species chosen as outgroups (the notothenioids *Bovichtus diacanthus* (Carmichael, 1819), *Eleginops maclovinus* (Cuvier, 1830), *Chionodraco hamatus* (Lönnberg, 1905), and the serraniform *Zanclorhynchus spinifer* (Günther, 1880)). The COI sequences clearly delineate all species analysed, and the resulting tree is congruent with phylogenies based on nuclear gene sequencing. We also show that many notothenid clades (e.g., *Gobionotothen*, *Notothenia*) are supported by common derived chromosomal rearrangements, whereas the karyotypic alterations of others (e.g., *Lepidonotothen*) are not easily explained. We hypothesize that notothenioid diversification has been driven in part by specific patterns of chromosomal rearrangement.

RÉSUMÉ. - Cytogénétique comparée des Nototheniidae (Téléostei : Acanthomorpha) de secteurs indien (Plateau Kerguelen-Heard) et atlantique (îles de Géorgie du Sud, Sandwich du Sud, Falkland-Malouines, Bouvet) de l'océan Austral.

La cytogénétique comparée est un outil puissant, qui peut être combiné avec les résultats des phylogénies fondées sur la morphologie ou les séquences d'ADN pour inférer les relations de parenté entre les espèces. Nous décrivons ici les caryotypes (y compris les positions des gènes ribosomiques 5S et 28S) de dix espèces appartenant à deux genres moins étudiés de la famille des Nototheniidae (sous-ordre des Notothenioïdes). Cinq de ces caryotypes (noms d'espèces soulignés) sont décrits ici pour la première fois : *Gobionotothen marionensis* (Günther, 1880), *G. acuta* (Günther, 1880), *G. gibberifrons* (Lönnberg, 1905), *Lepidonotothen larseni* (Lönnberg, 1905), *L. mizops* (Günther, 1880), *L. nudifrons (*Lönnberg, 1905), *L. squamifrons* (Günther, 1880), *Patagonotothen guntheri* (Norman, 1937), *P. ramsayi* (Regan, 1913), et *P. tessellata* (Richardson, 1845). Nous replaçons également ces espèces dans l'arbre phylogénétique des nototheniidés avec l'aide de nouvelles séquences du gène mitochondrial de la cytochrome oxydase I (COI), en incluant des séquences représentant des genres de nototheniidés dont le caryotype a été établi (*Notothenia* (Richardson, 1844)) ainsi que de plusieurs groupes externes (les notothenioides *Bovichtus diacanthus* (Carmichael, 1819), *Eleginops maclovinus* (Cuvier, 1830), et *Chionodraco hamatus* (Lönnberg, 1905) ; et le serraniforme *Zanclorhynchus spinifer* (Günther, 1880)). Le COI permet de regrouper clairement les espèces analysées lorsque plusieurs spécimens sont présents, et l'arbre qui en résulte est congruent avec des phylogénies moléculaires précédentes fondées sur des gènes nucléaires. Nous montrons également que de nombreux clades de nototheniidés (par ex. *Gobionotothen*, *Notothenia*) sont soutenus par des caractères chromosomiques dérivés par-

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tagés, tandis que les altérations des caryotypes de certains autres tel *Lepidonotothen* sont plus difficiles à expliquer. Nous faisons l'hypothèse que la diversification des notothenioides a été poussée en partie par certains types de réarrangements chromosomiques.

Key words. - Nototheniidae - Chromosomes - Indian and Atlantic Sub-Antarctic sectors.

Notothenioid fish, which are geographically restricted and highly endemic to the Southern Ocean, are the best described example of a marine species flock (Eastman and McCune, 2000). As their marine habitat cooled and most fish taxa became locally extinct, the notothenioids, which lack swim bladders, have undergone an adaptive radiation to occupy vacated niches in the water column by reduction of skeletal mineralization and enhancement of lipid deposition. Thus, the Antarctic notothenioids, a group containing 130 species, provide a rare and advantageous model system for understanding the evolutionary processes that enabled diversification in an extreme marine environment. We are using comparative cytogenetics and molecular phylogenetics to examine the role of chromosomal rearrangements in notothenioid evolution.

The karyotypes of Antarctic and Sub-Antarctic notothenioid fish are arguably the best characterized among marine teleosts. Since 1985, systematic surveys of notothenioid karyotypes have been conducted, mainly by French and Italian teams (Caputo *et al*., 2002; Pisano *et al*., 2003 ; Mazzei *et al*., 2004 ; 2006, 2008; Ghigliotti *et al*., 2007, 2010; Mazzei *et al*., 2008), on numerous ship-based campaigns to diverse localities and through laboratory work at various Antarctic stations**.** Of particular importance, a consistent protocol for chromosomal preparation in the field was developed at Kerguelen in 1984 during the Terres australes et antarctiques françaises (TAAF) summer campaign ICHTYO-GENET (Doussau de Bazignan and Ozouf-Costaz, 1985), and this method has been applied in most studies of notothenioid cytogenetics. Chromosome preparations collected during these different expeditions are stored deep-frozen at the MNHN (Museum national d'Histoire naturelle, MNHN) in Paris and in other national museums. For many preparations, the formalin-fixed specimen they come from has been kept as voucher, allowing parallel cytogenetic and morphological investigations.

Contrary to the karyotypic consistency typically observed in other marine teleost groups (Galetti *et al*., 2000), karyotypic heterogeneity is common in the notothenioid suborder. For example, species belonging to the Nototheniidae, the most speciose family of the suborder, exhibit significant chromosomal variability, with diploid numbers ranging from $2n = 22$ chromosomes in *Notothenia coriiceps* (Richardson, 1844) to $2n = 58$ in *Trematomus nicolaï* (Boulenger, 1902; Pisano and Ozouf-Costaz, 2003). Moreover, cryptic intraspecific chromosomal rearrangements occur in some notothenioid species, especially changes involving Robertsonian fusions and the number and position of heterochromatic segments and/or the nucleolar organizers (Morescalchi *et al*., 1992; Ozouf-Costaz *et al*., 1999; Pisano and Ghigliotti, 2009). Such rearrangements have very likely played an important role in notothenioid speciation, and chromosomal divergence between populations may therefore indicate ongoing processes of reproductive isolation and species divergence (Gauthier *et al*., 2010).

In the present study, we concentrate on the comparative cytogenetics and molecular phylogenetics of three understudied genera of Nototheniidae collected from Sub-Antarctic peri-insular plateaux in the Indian and Atlantic sectors of the Southern Ocean: *Gobionotothen*, *Lepidonotothen*, and *Patagonotothen*. Ten species (*G. marionensis* (Günther, 1880), *G. acuta* (Günther, 1880), *G. gibberifrons* (Lönnberg, 1905), *L. larseni* (Günther, 1880), *L. mizops* (Lönnberg, 1905), *L. nudifrons (*Lönnberg, 1905), *L. squamifrons* (Günther, 1880), *Patagonotothen guntheri* (Norman, 1937), *P. ramsayi* (Regan, 1913), and *P. tessellata* [Richardson, 1845)] were caught at diverse locations; chromosomal preparations were made, and mitochondrial DNA was isolated. We describe the karyotypes of five species for the first time (*G. marionensis*, *L. larseni*, *L. nudifrons*, *P. guntheri*, and *P. tessellata*) and map the 5S and 28S RNA ribosomal RNA genes on the karyotypes of all ten by fluorescence *in situ* hybridization (FISH). The phylogenetic relationships of the ten species were examined in the context of the notothenioid suborder by sequencing their mitochondrial COI genes and those of outgroup species (the notothenioids: *Bovichtus diacanthus* (Carmichael, 1819), *Eleginops maclovinus* (Cuvier, 1830), *Chionodraco hamatus* (Lönnberg, 1905), and the serraniform *Zanclorhynchus spinifer* (Günther, 1880), (Li *et al*., 2009).

Compared to previous studies, especially for Kerguelen plateau, we have considerably ameliorated the sampling for the genera under focus in the present paper, especially *Gobionotothen* and *Lepidonotothen*. Previously published karyotypes and COI sequences for several notothenioid outgroups have been incorporated into our analyses. Finally, we mapped chromosomal rearrangements and ribosomal gene patterns on to the branches of the COI molecular tree to determine whether the changes are clade-specific and whether their evolutionary sequence can be deduced.

MATERIALS AND METHODS

Collection and sampling of notothenioids

Fish were collected by bottom trawling during two winter campaigns in the Atlantic and Indian sectors of the Southern Ocean: ICEFISH (International Collaborative Expedition to collect and study Fish Indigenous to Sub-Antarctic Habitats, 2004) on board the RV *Nathaniel B. Palmer* and POKER (POissons de KERguelen, Campagne d'évaluation de la biomasse de poissons à Kerguelen, 2006) on board the trawler *Austral*.

Male and/or female specimens of *Gobionotothen acuta*, *G. gibberifrons*, *G. marionensis*, *Lepidonotothen larseni*, *L. mizops*, *L. nudifrons*, *L. squamifrons*, *Patagonotothen guntheri*, *P. ramsayi*, and *P. tessellata* were collected at six sampling sites around the Indian and Atlantic Sub-Antarctic islands (Tab. I). Animals were captured using bottom trawls during the ICEFISH (May-July 2004) and POKER (September-October 2006) campaigns. Reference specimens caught during the ICEFISH cruise are kept in the collections of either the MNHN or the South African Institute for Aquatic Biodiversity (SAIAB, Grahamstown), whereas those sampled during the POKER cruise are maintained in the collections of the MNHN. Each species/population analysed is represented by at least one voucher, although some specimens could not be preserved because they were used for other analyses.

Molecular phylogenetics

DNA extraction, PCR and sequencing

Muscle and skin tissues from species of the three genera examined were stored in 85% ethanol at +4°C. The samples were extracted according to the protocol of Winnepenninckx *et al.* (1993). The "Folmer region" of the COI coding gene was amplified with primers FishF1-5'TCAACCAACCACAAAGACATTGGCAC3' and FishR1-5'TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward *et al.*, 2005). PCR was performed using Biometra thermocyclers in 25 μl reactions consisting of 5% DMSO, 5 μg bovine serum albumin, 300 μM dNTPs, 0.3 μM *Taq* DNA polymerase (Qiagen), 2.5 μl of 10X PCR buffer, and 1.7 pM of each of the two primers. Denaturation was performed at 94°C for 2 min, and amplification was performed for 45 cycles (94°C for 20 s; 52°C for 25 s; 72°C for 45 s) with a terminal elongation of 3 min. The same primers were used for sequencing. Both purification of the PCR products and sequencing of DNAs (both directions) were performed by Genoscope (http://www.genoscope.cns.fr/). The sequences were manually checked using Sequencher (Gene Codes Corporation). Sequence alignment and pairwise sequence comparison was performed using Bioedit (Hall, 1999). Additional COI sequences from *Gobionotothen* and *Lepidonotothen* species were downloaded from GenBank (Accession Numbers starting with EU indicated in the tree).

To place the three genera in the phylogenetic context of the Notothenioidei, we extracted DNA from additional species and sequenced their COI genes by the methods described in the preceding paragraph. These species included at least one representative of each clade of notothenioids: *Bovichtus diacanthus*, *Eleginops maclovinus*, *Chionodraco hamatus*, *Notothenia angustata*, *N. rossi*, *N. coriiceps*, *Paranotothenia magellanica*, *Trematomus scotti*, *T. nicolai*, and *Patagonotothen wiltoni* were used to represent the four families (Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae) that form the "High Antarctic Clade" (Sanchez *et al.*, 2007; Near and Cheng 2008). The COI gene for several specimens of *Zanclorhynchus spinifer* was also sequenced.

Phylogenetic inference

Zanclorhynchus spinifer (Congiopodidae) was chosen as the first outgroup because it may be the sister group of the Notothenioidei (Smith and Wheeler, 2004; however, see also Li *et al.*, 2009). Based on the consistent pattern from previous phylogenetic analyses (Near and Cheng, 2008; Li *et al.*, 2009; Fig. 5), *B. diacanthus* was selected as a second outgroup for the phylogenetic tree, as it is the first group to diverge within the notothenioids. The sequences were analysed by Bayesian inference (BI: MrBayes 3.1, Huelsenbeck and Ronquist, 2001) and maximum parsimony (MP: PAUP*4.0b10; Swofford, 1999). For BI analysis, the GTR (General Time Reversible) + I (Invariant) + G (Gamma) model was selected, with default settings for the priors and the 1st, 2nd and 3rd codon positions as different partitions. Four distinct analyses were run with four chains each, 10 million generations, and sampling of every 100th tree. We discarded the first (20 000) trees after determining that the burn-in zone was included in this interval. After verifying that convergence had been reached, the trees and parameter samples were pooled and combined in a consensus. For MP, a heuristic search (TBR, tree bisection-reconnection search, 1 000 replicates starting from random trees) and 1 000 bootstrap replicates were performed on the dataset. Nodes with Bayesian posterior probabilities equal or higher to 0.95 were considered significantly supported.

Chromosome preparations and fluorescence *in situ* hybridization (FISH)

Chromosomes were prepared and fixed from kidney and spleen mitotic cells according to the method of Doussau de Bazignan and Ozouf-Costaz (1985). Fixed cell suspensions were stored at -20°C, then thawed immediately before spreading onto microscope slides for FISH procedures.

The 28S rDNA and 5S rDNA probes (1049 and 87 bp, respectively) were amplified by PCR using DNA from *Trematomus bernacchii* as template, their sizes were veri-

Table I. - Reference data on each specimen for the studied genera.

Genus	Species	Survey	Locality	Chromosome sample ref	DNA sample ref	BOLD reference	Sex	Voucher
Gobionotothen								
	G. acuta	POKER 2006	Kerguelen Islands	AUS13	AUS13	FCKI025-11	F	MNHN 2007-1774
				AUS14	AUS14	FKCI026-11	F	MNHN 2007-1775
				AUS21	AUS21	FKCI029-11	F	MNHN 2007-1807
				AUS31	AUS31	FKCI034-11	F	MNHN 2007-1832
	G. gibberifrons	ICEFISH 2004	South Georgia	GG2	1228GoGi	FKCI047-11	M	MNHN 2005-0099
				GG4	1229GoGi	FKCI048-11	M	no
				GG6	1230GoGi	FKCI049-11	F	no
				-	ICTI309	FKCI065-11	?	no
	G. marionensis	ICEFISH 2004	South Sandwich Isl., Candlemas Isl.	GM2	1225GoMa	FKCI044-11	M	MNHN 2005-0088
				GM4, GM5	1227GoMa	FKCI046-11	F	SAIAB 75080
			South Georgia	-	1226GoMa	FKCI045-11	?	no
Lepidonotothen								
	L. larseni	ICEFISH 2004	South Georgia	LL2	-		M	no
				LL3	-		F	no
				LL4	-		F	no
				-	1246LeLa	FKCI052-11	?	MNHN 2005-85
				-	1248LeLa	FKCI048-11	?	no
			Bouvet Island	LL7	-		F	no
				LL8	-		F	no
				LL9	1247LeLa	FKCI053-11	F	SAIAB 74974
				LL12	-		F	no
				LL13	-		F	no
	L. mizops	POKER 2006	Kerguelen Islands	AUS7	AUS7	FKCI040-11	F	MNHN 2007-1749
				AUS20	AUS20	FKCI028-11	F	MNHN 2007-1806
				AUS23	AUS23	FKCI030-11	F	MNHN 2007-1813
				AUS45	AUS45	FKCI038-11	F	MNHN 2007-1881
				-	943LeMi	FKCI067-11	?	no
	L. nudifrons	ICEFISH 2004	Shag Rocks	LN2	1245LeNu	FK051-11	F	no
				LN3	-		F	no
				LN5	-		M	no
				-	1244LeNu	FKCI064-11	?	no
				-	1152LeNu	FKCI041-11	?	no
	L. squamifrons	POKER 2006	Kerguelen Islands	AUS3	AUS3	FKCI-033-11	M	MNHN 2007-17
				AUS24	AUS24	FKCI031-11	F	MNHN 2007-1814
				AUS25	AUS25	FKCI032-11	F	MNHN 2007-1815
				AUS46	AUS46	FKCI039-11	F	MNHN 2007-1885
				-	ICTI99	FKCI068-11	?	no
		ICEFISH 2004	Bouvet Island	LS4	1249LeSq	FKCI055-11	F	SAIAB 74977
				-	1250LeSq	FKCI056-11	?	no
				LS5	-		F	no
				LS6	1251LeSq	FKCI057-11	F	no
			South Sandwich Isl.	LS1	1252LeSq	FKCI058-11	M	no
				-	1253LeSq	FKCI059-11	?	no

Table I. - Continued.

Genus	Species	Survey	Locality	Chromosome sample ref	DNA sample ref	BOLD reference	Sex	Voucher
Patagonotothen								
	P. guntheri	ICEFISH 2004	Bouvet Island	PG2	1256PaGu	FKCI061-11	F	SAIAB 75162
				PG5	-		F	no
	P. ramsayi			PR1	-		F	no
			Falkland Islands	PR3	1262PaRa	FKCI062-11	F	no
	P. tessellata			PT5	-		M	no
				PT9	-		F	no
			Falkland Islands	PT2	1268PaTe	FKCI063-11	F	SAIAB 75143
	P. wiltoni		Falkland Islands	-	1255PaWi	FKCI060-11	?	no

fied by agarose gel electrophoresis, and the DNA fragments were purified with a QIAGEN MinElute column and kit. The probes were cloned into the M13 plasmid vector using the QIAGEN PCR Cloning kit. Positive clones were isolated, their inserts were amplified by PCR using M13L and M13R primers, and the amplicons were sequenced (Genoscreen). After checking the sequences, one clone for each rDNA probe was selected for reference, and the corresponding plasmids were purified using QIAGEN miniprep columns. The sequences of the 28S and 5S rDNA inserts were deposited in GenBank (5S: EF192604, 28S: EF192605). Probes were made by PCR from these plasmids using the M13L and M13R primers (final lengths: 28S, 1178 bp; 5S, 320 bp). Biotin-labeled 28S rDNA and digoxigenin-labeled 5S rDNA were synthesized by nick-translation (kit and protocol from Roche Diagnostics).

Hybridization of the probes to chromosomes and signal detection were performed according to Fischer *et al*. (2000). Chromosomes were denatured for intervals up to 1.15 min.

Karyotypes were obtained and FISH results analysed using a Zeiss Imager M1 fluorescence microscope equipped with Genus software (Genetix). Standard karyotypes were directly obtained from DAPI-stained chromosomes after FISH experiments.

Chromosomes were classified following the nomenclature of Levan (1964) as metacentric-submetacentrics (m-sm) or acrocentrics (a). Chromosome arm numbers (FN) are also provided.

RESULTS

Molecular phylogenetics

The majority rule consensus tree from the BI analysis of the COI dataset is presented in figure 1. All the nodes are strongly supported except for clade I. Clade A separates *E. maclovinus* from all other notothenioids except the Bovichtidae. Clade C groups *Gobionotothen* with the High Antarctic Clade and *Notothenia* spp. Clade E includes *Notothenia* spp. and the Channichthyidae (High Antarctic Clade), but the branch has a relatively low Bayesian posterior probability (0.85). Clade D consists of *Trematomus* spp., *Lepidonotothen* spp., and *Patagonotothen* spp. Clade G is composed of *Lepidonotothen* and *Patagonotothen* spp., but clade I separates *L. squamifrons* from the three other *Lepidonotothen* spp., *L. squamifrons* groups with the *Patagonotothen* spp. but with low Bayesian posterior probability support (0.52). Within clade J, *P. tessellata* is the sister group of all other *Patagonotothen* spp.

Cytogenetics

Scoring positive results from slide examination

Chromosomes were prepared on board ships during the Antarctic winter, the season when the highest mitotic activity in cells of notothenioid fishes is generally observed. However, we obtained only a few metaphase plates per slide, which precluded statistical analyses of our results. This might be due to the preparation of the chromosomes on-board, in sometimes very difficult field conditions, as opposed to inland-based laboratories. Once we estimated that we consistently observed the same chromosome counts, formulae, and ribosomal gene locations in several preparations from a specimen, we based our karyotypic and FISH analyses on the best-spread metaphase plate for each species. For most species, chromosomes from both males and females were examined, but differentiated sex chromosomes were not detected. We describe (or re-describe) karyotypes by species within genera. The karyotypes of species newly examined in this work are described in detail and compared to those previously established. We provide for each species the diploid number (2n), chromosome formula, arm number (FN), and 28S and 5S ribosomal gene positions. In the karyotypes (Figs 2, 3, 4), chromosomes are separated into rows of (m-sm) and (a) pairs in order of decreasing size. The chromosome pair bearing the 28S-5S ribosomal genes is shown in a square, with and without the FISH signals.

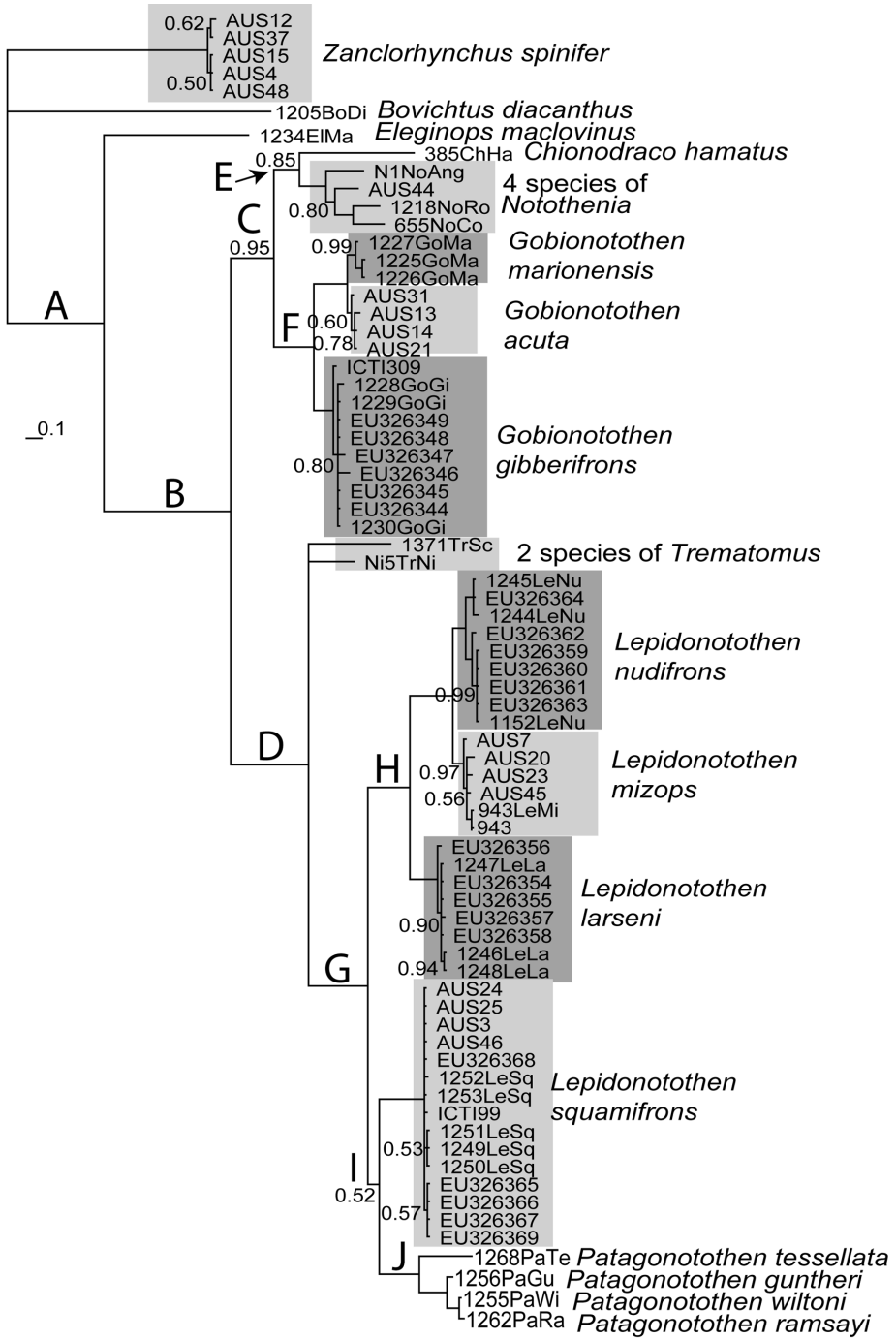

Figure 1. - Majority rule consensus tree from the BI analysis of the COI dataset (number of taxa = 73; number of characters = 684; posterior probability values are indicated next to the nodes; nodes without values indicated have a posterior probability of 1.00). Accession numbers starting with EU: sequences from GenBank.

GOBIONOTOTHEN SPP. (Fig. 2)

G. acuta (Fig. 2A)

Four females, Kerguelen: 2n = 48 (12 m-sm + 36 a); FN = 60; 28S and 5S rRNA gene clusters overlap in the heteromorphic, DAPI-negative arms of a small m-sm pair. The chromosomes of the first metacentric pair are twice as large as those of the next metacentric pair. The last two acrocentric pairs are extremely small (less than 1 μm).

These newly characterized karyotypes from Kerguelen specimens differ from those published for a single female specimen from Heard Island; 2n = 50 (6m + 8sm + 32a + 4B) by Ozouf-Costaz and Doussau de Bazignan (1987). The two

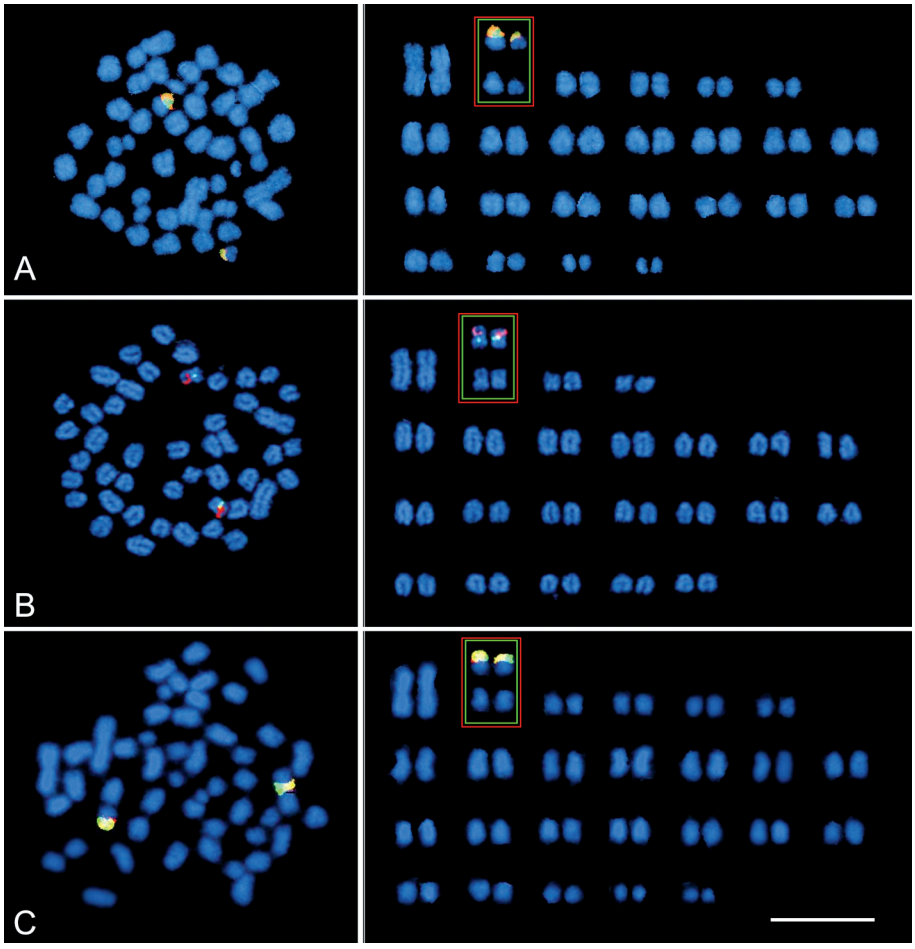

Figure 2. - DAPI-stained karyotypes of **A**: *Gobionotothen acuta*; **B**: *G. gibberifrons*; **C**: *G. marionensis*. Scale bar = 10 μm. Chromosomes are sorted by meta-submetacentric pairs (the first row), and acrocentric pairs (three next rows). In the frames, the 28s (green) and 5S (red) rDNA bearing pair are represented with and without FISH signals.

pairs of "micro-chromosomes" of the Heard specimen, designated "B", correspond to the very small acrocentrics of the Kerguelen specimens.

No male karyotype is available.

G. gibberifrons (Fig. 2B)

Two males, one female, South Georgia: 2n = 46 (8 m-sm + 38 a); FN = 54; 28S and 5S rRNA gene clusters overlap in the slightly heteromorphic, DAPI-negative arms of a small m-sm pair. As in *G. acuta*, chromosomes of the first metacentric pair are twice as large as those of the next one. The standard karyotype of *G. gibberifrons* was described by Phan *et al*. (1987) for South Shetland specimens of undetermined sex. They reported a slightly different formula (4m + 2 sm + 40 a). After re-examining their figure, we suggest that a small metacentric pair might have been counted as an acrocentric one, which explains the difference in the formula.

G. marionensis (Fig. 2C)

Two females, one male, South Sandwich and Candlemas Islands: 2n = 50 (12 m-sm + 38 a); FN = 54; 28S and 5S rRNA gene clusters overlap in the slightly heteromorphic, DAPI-negative arms of a small m-sm pair. As for other *Gobionotothen* spp., the first metacentric pair is twice the size of the next largest pair. The final two acrocentric pairs are extremely small (less than 1 μm), as in *G. acuta*.

***LEPIDONOTOTHEN* SPP.** (Fig. 3)
L. larseni (Fig. 3A)

One male, seven females, South Georgia: 2n = 48 (6 m-sm + 42 a); FN = 54; 28S and 5S rRNA gene clusters overlap in the slightly heteromorphic, DAPI-negative arms of a small m-sm pair. The three m-sm chromosome pairs are all small.

L. mizops (Fig. 3B)

Four females, Kerguelen Islands: 2n = 48 (6m-sm + 42 a); NF = 54. Karyotype and rRNA gene cluster positions are very similar to those of *L. larseni*. In the metaphase shown in Fig. 3, the rRNA signals were detectable on only one chromosome of the pair, presumably due to a low copy number on the other. Strong heteromorphism in ribosomal

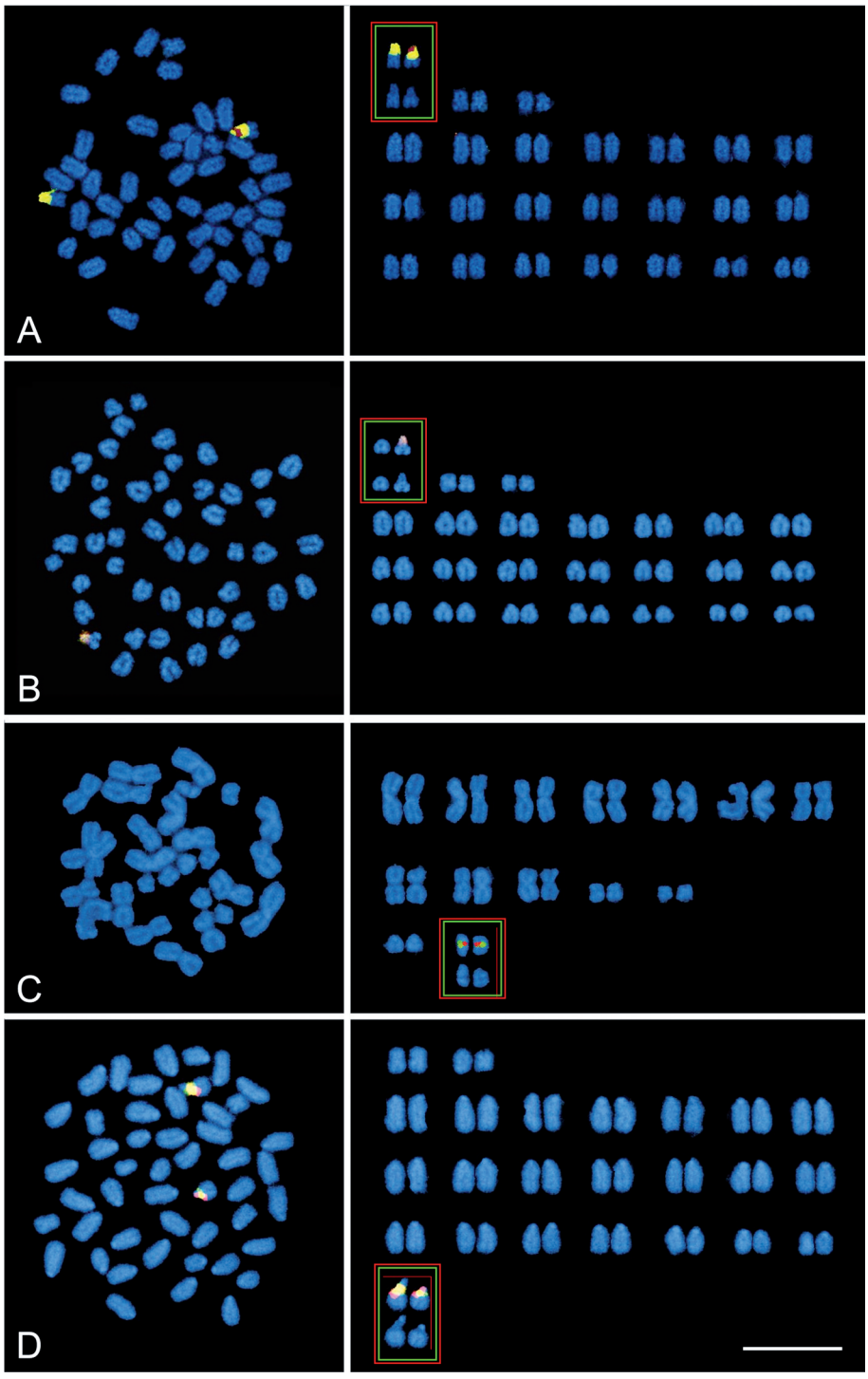

Figure 3. - DAPI-stained karyotypes of **A**: *Lepidonotothen larseni*; **B**: *L. mizops*; **C**: *L. nudifrons*; **D**: *L. squamifrons*. Scale bar = 10 μm. Chromosomes are sorted by meta-submetacentric pairs (the first row), and acrocentric pairs (three next rows). In the frames, the 28s (green) and 5S (red) rDNA bearing pair are represented with and without FISH signals.

gene patterns is a common feature in karyotypes of notothenioid species (Pisano and Ghigliotti, 2009).

The *L. mizops* karyotype had previously been established for four females and four males from Chiuchia Bank and Heard Island (Ozouf-Costaz and Doussau de Bazignan, 1987). They found the same chromosome number reported here, but the formula (4 m-sm + 44 a) differed slightly. Furthermore, but at that time, the 28S and 5S rRNA gene-bearing pair could not be identified.

L. nudifrons (Fig. 3C)

One male, two females, Shag Rocks: 2n = 28 (24 m-sm + 4 a); FN = 52; 28S and 5S rRNA gene clusters overlap in the peri-centromeric region of a small acrocentric pair. Among the 24 m-sm chromosomes, ten pairs are very large, and two are small.

L. squamifrons (Fig. 3D)

Three females and one male, Kerguelen Islands; 3 females, one male, Bouvet Island. No karyotype difference was observed among these specimens: 2n = 48 (4 m-sm + 44 a); FN = 52. 28S and 5S rRNA gene clusters overlap in the peri-centromeric region of a small acrocentric pair.

Ozouf-Costaz and Doussau de Bazignan (1987) had previously reported comparable results from three females and two males from Heard Island and Chiuchia Bank. The karyotype of *L. kempi* (one male and one female; Tangaroa 2004 Voyage; Ghigliotti, pers. comm.), a species now recognized as synonymous with *L. squamifrons* (Schneppenheim *et al*., 1994), is also congruent with that described here. Similar karyotypes and ribosomal gene patterns were also obtained from specimens of *L. squamifrons* from Heard Island (Pisano, pers. comm.; Aurora Australis, THIRST Voyage 1993).

***PATAGONOTOTHEN* SPP.** (Fig. 4)

P. guntheri (Fig. 4A)

Two females, Falkland/Malvinas Islands: 2n = 48 (4 m-sm + 44 a); FN = 52. 28S and 5S rRNA gene clusters overlap in the slightly heteromorphic, DAPI-negative arms of a small m-sm pair.

P. tessellata (Fig. 4B)

One male, one female, Falkland/Malvinas Islands: 2n = 48 (8 m-sm + 40 a); FN = 56. 28S and 5S rRNA gene clusters overlap in the slightly heteromorphic, DAPI-negative arms of a small m-sm pair.

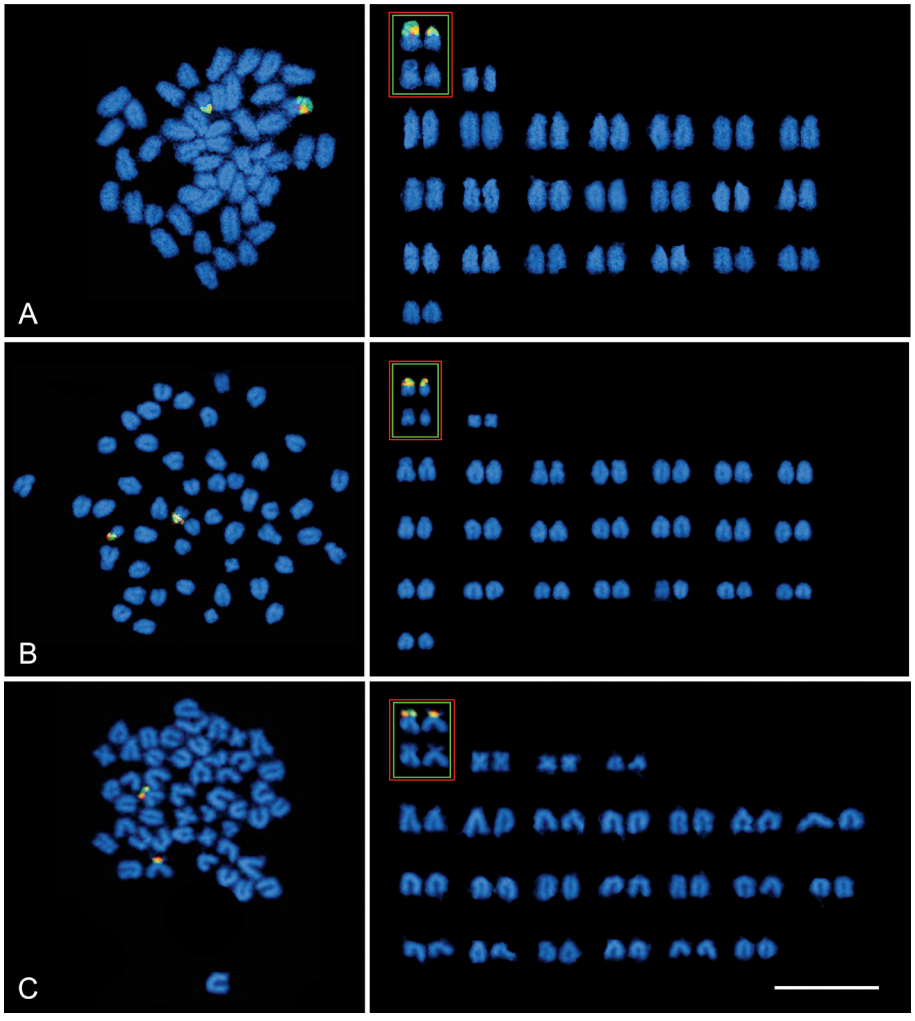

Figure 4. - DAPI-stained karyotypes of **A**: *Patagonotothen guntheri*; **B**: *P. ramsayi*; **C**: *P. tessellata*. Scale bar = 10 μm. Chromosomes are sorted by meta-submetacentric pairs (the first row), and acrocentric pairs (three next rows). In the frames, the 28s (green) and 5S (red) rDNA bearing pair are represented with and without FISH signals.

P. ramsayi (Fig. 4C)

Two females, Falkland/Malvinas Islands. 2n = 48 (4 m-sm + 44 a); FN = 52. 28S and 5S rRNA gene clusters are observed on a small m-sm pair in a position similar to that found for the other two *Patagonotothen* species.

The karyotype of this species (identical diploid number and formula) had already been established for specimens from Magellan Strait (Prirodina, 1984).

DISCUSSION

COI tree

As described in previous studies (Dettai *et al*., 2011), the COI tree (Fig. 1 and Fig. 5) clearly separates each species. The best sampled notothenioid genera are also recovered as monophyletic (*Gobionotothen, Patagonotothen*). *Lepidonotothen* is not supported as monophyletic, however, problems with this species had already been noted by Near and Cheng (2008), where it was, depending on the method and dataset, either the sister group of the *Lepidonotothen-Patagonotothen* clade or in a polytomy with members of both genera and of the genus *Trematomus*. The Nototheniidae are not monophyletic on this tree. The topology is identical to the one obtained by Near and Cheng (2008) for their nuclear gene S7, and compatible with the polytomy obtained by Sanchez *et al*., (2007) on several markers. While the phylogeny of notothenids needs additional investigation, the topology obtained is coherent with previous knowledge from independent molecular analyses.

Chromosomal characters

Teleost chromosomes are difficult to study because they are small and their DNA is homogeneous in base composi-

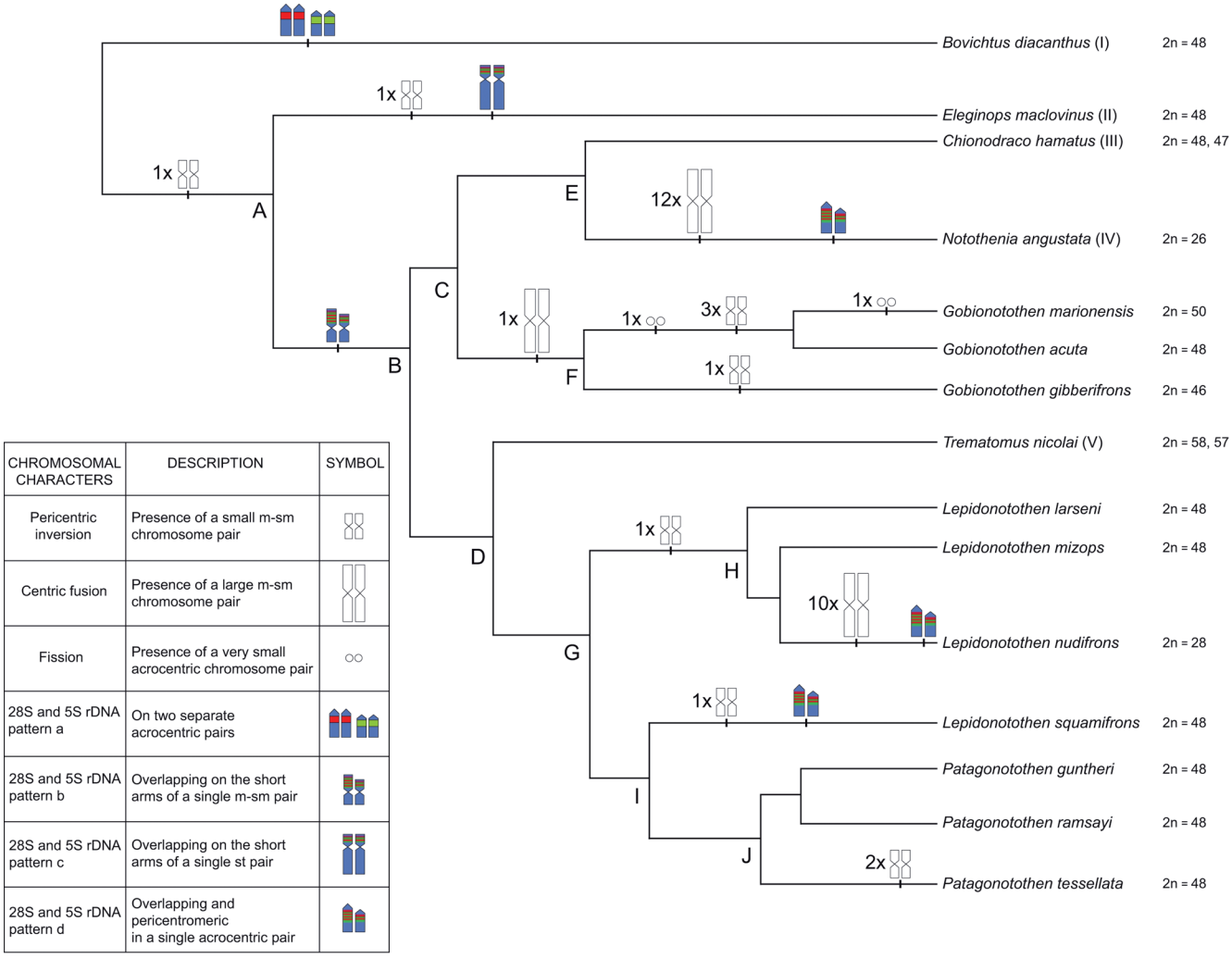

Figure 5. - Simplified phylogenetic tree with the chromosome characters plotted onto the branches. The cytogenetic features of species previously investigated were taken from literature: I: Mazzei *et al*., 2006, II: Mazzei *et al*., 2008, III: Mazzei *et al*., 2004, IV: Pisano and Ozouf-Costaz, 2000, V: Morescalchi *et al*., 1992.

tion, which restricts band resolution and contrast (Medrano *et al*., 1988; Hudson *et al*., 2005; Constantini *et al*., 2007). Furthermore, useful sets of chromosome-specific painting probes have yet to be generated, which enables analysis of interspecific chromosomal homology by *in situ* hybridization. In this study of species from three genera of the family Nototheniidae, we use changes in chromosomal characters (number and formulae) and variation in the chromosomal location and FISH-staining pattern of two molecular markers (28S and 5S rRNA genes) to compare karyotypes. When combined with our phylogenetic analysis, the results enable us to infer patterns of chromosomal change that may have played a role in the rapid speciation observed in the notothenioid suborder.

According to Jaillon *et al*., (2004), the ancestral chromosome number of teleosts is 2n = 48 acrocentric chromosomes. From changes in the chromosome numbers and formulae of the species examined in this and other studies, we have shown that three types of Robertsonian chromosomal rearrangements (Robertson, 1916) occurred as notothenioids diversified: 1) generation of large bi-armed chromosome(s) with a reduction of the diploid number (2n) and no FN change through fusion; 2) production of small bi-armed chromosome(s) without a change in diploid number but with an increase of FN by pericentric inversion; and 3) creation of small acrocentric chromosome(s) with increases in 2n and FN by fission (Gauthier, 2010). These three chromosome characters in various combinations, explain the changes in chromosome number and size observed among species of the nototheniid bush (Fig. 5).

The distribution of 28S and 5S rRNA genes shows four different patterns (Fig. 5): ***a*,** pericentromeric on two different acrocentric pairs in *Bovichtus*, as described by Mazzei *et al*., (2006); ***b***, overlapping in the long arms of a small meta-submetacentric pair (the most commonly observed pattern); ***c***, overlapping in the short arms of a subtelocentric pair in *Eleginops,* as described by Mazzei *et al*. (2008); and ***d***, overlapping and pericentromeric in a small acrocentric pair (*N. angustata*, *L. nudifrons*, and *L. squamifrons*). From these gene mapping patterns, we can postulate some primary chromosomal homologies.

Mapping chromosomal characters on the COI tree: evolutionary inferences

Although our data set is robust, we found the available ribosomal gene patterns and chromosome changes to be insufficiently informative relative to the number of species analysed to establish a parsimony-based chromosomal phylogeny. Thus, in accord with the strategy of Grandcolas *et al*., (2001), we mapped chromosomal characters (i.e., fusions, fissions, and inversions) and ribosomal gene patterns onto the COI tree (congruent with the topology obtained from nuclear gene sequences by Near and Cheng 2008) (Fig. 5) in order to: 1) establish whether some chromosomal synapomorphies support clades in the COI tree; and 2) deduce from the topology of the tree the order in which chromosomal rearrangements occurred. Because the karyotype of *Zanclorhynchus spinifer* is not yet available, we excluded this species from the reconstruction.

Overall, the chromosomal karyotypes of the notothenioids studied here are strikingly consistent with the COI molecular phylogenetic tree. With respect to the outgroup *Bovichtus*, the karyotype of the monospecific *Eleginops* (clade A) displays one pericentric inversion and the rRNA gene pattern ***c***. The clade *Notothenia*, represented here by *N. angustata*, is characterized by a high occurrence of centric fusions (hence, a correspondingly low chromosome number) (Pisano *et al*., 1998) and exhibits the ribosomal gene pattern ***d*** (Pisano *et al*., 2003). Clade F (*Gobionotothen*) is characterized by the presence of one shared, large metacentric pair that arose from a centric fusion and by the occurrence of several fissions and pericentric inversions that differentiate the three species; rRNA genes arrangement follows pattern ***b***. The non-ribosomal chromosomal karyotypes of species in Clade D (*Trematomus*, *Lepidonotothen*, and *Patagonotothen* spp.) are minor variants of the bovichtid karyotype (with the exception of *L. nudifrons*), and two rRNA gene patterns are observed, ***b*** and ***d*.**

Mapping of the rRNA gene patterns alone onto the COI tree enables us to infer the probable order of the rearrangements that gave rise to this pair in most nototheniids. The outgroup bovichtid clade has its rRNA genes encoded on separate chromosomes (pattern ***a***). Based on the topology of the tree, one might hypothesize that *E. maclovinus* evolved rRNA pattern ***c*** from the bovichtid pattern ***a*** by translocation of the 28S rRNA genes to the longer chromosome bearing the 5S genes followed by an intrachromosomal pericentric inversion. By contrast, most species of the large clade B possess overlapping 28S and 5S rRNA gene clusters located on the long arm of a single, slightly heteromorphic pair (pattern ***b***), which likely arose by translocation of the 5S genes to the smaller chromosome bearing the 28S genes, again followed by pericentric inversion. However, three species in clade B exhibit pattern ***d***: *Notothenia angustata* (clade E); *Lepidonotothen nudifrons* (clade H); and *L. squamifrons* (clade I). We propose that this pattern arose on three separate occasions by a second pericentric inversion in the chromosome pair showing the ***b*** pattern.

The occurrence ribosomal gene pattern ***d*** in three distinct clades of the COI tree is consistent with chromosomal and morphological characters of *N. angustata* and *L. squamifrons*. *Notothenia angustata* belongs to a clade in which the karyotypes of all species are characterized by low diploid chromosome numbers (2n = 22-26) and dominated by large metacentrics; the ***d*** type ribosomal gene-bearing pair is either acrocentric or fused to another autosome (Pisano *et*

al., 2003; Ozouf-Costaz & Pisano, pers. com.). In the COI tree, *L. squamifrons* is a singleton on its branch. Furthermore, *L. squamifrons* is the sister group of the *Patagonotothen* spp., with whom it shares a diploid number of 48 chromosomes that show minimal rearrangement.

Lepidonotothen nudifrons (clade H), by contrast, has a karyotype almost exclusively composed of large metacentrics, with 2n = 28, and ribosomal gene pattern ***d***. These features are very divergent from other species of clade H. *Lepidonotothen nudifrons* is a coastal species (3 to 400 m depth) whose distribution is restricted to the Scotia Arc and adjacent islands (South Georgia, South Shetlands, South Orkneys, South Sandwiches). Its sister species, *L. mizops*, is distributed around the Kerguelen Islands, Heard Island, Prince Edward Island and Crozet Island. *L. mizops* retains a diploid number of 48 chromosomes**.** We propose that the speciation event leading to *L. nudifrons* occurred in a small, isolated population that experienced a mutational cascade of centric fusions (Searle, 1998). Future development of reciprocal chromosome painting (i.e., cross-species chromosomal FISH) would facilitate an understanding of the divergence of *L. nudifrons* and *L. mizops* and other closely related notothenioid species.

This study shows that comparative cytogenetics can be combined with molecular phylogenetics to advance synergistically our understanding of the evolutionary relationships among the notothenioids. Our data show that most notothenioid clades, which in the past have been deduced by molecular and morphological phylogenetics, are supported by common derived chromosomal features. This observation suggests, in turn, that the notothenioid radiation may be driven in part by specific patterns of chromosomal rearrangements. Further investigation of this hypothesis is likely to contribute to our mechanistic understanding of adaptive radiations.

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