7 Molecular phylogeny and evolution of Notothenioid fish based on partial sequences of 12S and 16S ribosomal RNA mitochondrial genes

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ABSTRACT

The perciform suborder Notothenioidei is a highly diversified group of fish inhabiting the Southern Ocean. In terms of numbers of species and biomass, it represents an important element in the Antarctic marine environment. The present study investigates the phylogenetic relationships of 18 species of notothenioids from five families (Bovichtidae, Nototheniidae, Artedidraconidae, Bathydraconidae and Channichthyidae). Two Antarctic species from the family Zoarcidae (Perciformes) were included in the analysis as an outgroup. Phylogenetic analyses were based on partial sequences of the 12S and 16S mitochondrial ribosomal RNA genes. A total of 928 base pairs (bp) were sequenced for each of the 20 taxa.

Both distance and maximum parsimony-based methods were used to infer the phylogenetic relationships among and within families of notothenioids. The topology of the trees obtained from the molecular data did not significantly differ from that proposed on the basis of morphological data. The Bovichtidae appear to be the sister group to all other notothenioid families. The DNA analysis suggests that the families Nototheniidae and Bathydraconidae are paraphyletic. The short branch lengths displayed by the neighbour-joining tree might account for a radiation-like mode of evolution. The time of divergence among notothenioids was estimated on the basis of their nucleotide divergence.

Key words: Notothenioid evolution, molecular phylogeny, 12S and 16S rRNA, mitochondrial DNA.

INTRODUCTION

The perciform suborder Notothenioidei is a highly diversified group of fish inhabiting the waters around the Antarctic continent. In terms of numbers of species and biomass it represents the dominant element of the Antarctic fish fauna (Gon & Heemstra 1990, Eastman 1991, Miller 1993). Notothenioids show a remarkable degree of endemism (97% of the species), and are distributed nearly exclusively in the Antarctic marine waters (Eastman 1993, p. 55). This is a unique environment, characterized, especially in the high-Antarctic Zone, by temperatures as low as −2 °C, presence of sea ice, and large seasonal fluctuations of the primary production. The presence of a circum-Antarctic current, the Antarctic Convergence, reduces the exchange of surface water, thus partially isolating the Antarctic Ocean. These features, together with a decreased

availability of shelf habitats, seem to have reduced the opportunity for the fish fauna to diversify and colonize different habitats. Notothenioids have evolved several, remarkable, adaptations to the Antarctic conditions, enabling them to occupy successfully a large variety of ecological niches (Kock 1993, Eastman 1993, p.67). Among the physiological and molecular adaptations displayed by notothenioid fish, the most striking is certainly the presence of antifreeze glycopeptides (AFGPs), which are responsible for depressing the freezing point of the blood, thus ensuring that the fish do not freeze under Antarctic conditions (DeVries 1988, Cheng & DeVries 1991).

Also other relevant physiological features, such as blood viscosity, oxygen transport and the cardiovascular system, differ significantly in these fish from those in temperate species (Wells 1987, Macdonald & Wells 1991, Tota *et al.* 1991). In some Antarctic species haemoglobins have reduced affinity for oxygen; the extreme case is represented by the icefish (family Channichthyidae), which lack haemoglobin (Wells *et al.* 1990, di Prisco *et al.* 1990, 1991) and which have therefore been called 'white-blooded fish'. This major change in the blood biochemistry of Channichthyidae is allowed by the oxygen saturation of the Antarctic sea water, but whether it represents an adaptation or it is simply due to relaxed selection is difficult to say. On the contrary, neutral buoyancy, which is typical of some pelagic nototheniods (i.e. *Dissostichus* spp.), evolved from an ancestral benthic condition characterized by the absence of a swim bladder; this can probably be considered an adaptation to the pelagic niche (Eastman 1985).

The suborder Notothenioidei consists of six families (Bovichtidae, Nototheniidae, Artedidraconidae, Harpagiferidae, Bathydraconidae and Channichthyidae) with over 120 species (Eastman 1993, p. 59). The phylogenetic relationships among and within these families have been established, so far, solely on morphological characters (Iwami 1985). However, the complete lack of fossil records for these fish has made definition of their time and mode of evolution very difficult.

In the present study we report the molecular phylogeny of notothenioids based on the partial sequence of 12S and 16S rRNA mitochondrial genes (Bargelloni *et al.* 1994). This molecular approach provides an alternative hypothesis for the evolution of these Antarctic fish as well allowing comparison with phylogenies based on other characters. In addition, the DNA data permit (if the homogeneity of the nucleotide substitution rate is proven to hold by specific tests) timing of the evolutionary events of notothenioids, therefore providing insights into the mode of evolution, possibly related to paleoenvironmental events in Antarctica.

MATERIALS AND METHODS

Total genomic DNA was extracted from ethanol-preserved samples, as described elsewhere (Kocher *et al.* 1989). The species examined were the following: suborder Notothenioidei (family Bovichtidae) *Bovichtus variegatus* (family Nototheniidae) *Trematomus eulepidotus, T. hansoni, T. nicolai, T. pennellii, Pagothenia borchgrevinki, Notothenia coriiceps neglecta, Gobionotothen gibberifrons, Dissostichus mawsoni*; (family Artedidraconidae) *Histiodraco velifer, Pogonophryne scotti*; (family Bathydraconidae) *Gymnodraco acuticeps, Cygnodraco mawsoni, Parachaenichtys charcoti*; (family Channichthyidae) *Chaenocephalus aceratus, Chionodraco hamatus, Cryodraco antarcticus, Pagetopsis macropterus*. Two Antarctic species of Zoarcidae (Perciformes), *Lycodichthys dearborni* and *Pachycara brachycephalum*, were included in the study as outgroup.

The polymerase chain reaction (PCR) (Saiki *et al.*, 1988) was employed to amplify two segments of the mitochondrial DNA (mtDNA), from the 16S and 12S ribosomal RNA genes respectively. Double- and single-strand amplifications and direct sequencing were performed according to Patarnello *et al.* (1994). The PCR-primers used were 12Sa and 12Sb (Kocher *et al.* 1989) and 16Sa and 16Sb (Palumbi *et al.* 1991). Both strands of the amplified segments were sequenced; 375 base pairs (bp) of the 12S gene and 553 bp of the 16S gene were examined for each of the 18 taxa plus the two outgroups (GenBank Accession numbers Z32702-Z32739 and Z32747-Z32748). DNA sequences were aligned using a multiple-sequence alignment software (CLUSTAL: Higgins & Sharp 1988), with default settings. The alignment was unambiguous with the exception of a highly variable region of 45 bp in the 16S sequence (positions 223–267 in the dataset). The divergence times estimate among taxa was carried out both including and excluding this highly variable region of the 16S rRNA.

Phylogenetic analyses were performed by maximum parsimony (MP) implemented in PAUP (Swofford 1993). Due to the large number of taxa, heuristic search procedures were necessary to search for the most parsimonious tree(s). The reliability of the heuristic searches was improved by using the option 'random addition of taxa' with 100 replications in PAUP. Several different character-weighting schemes were used (Bargelloni *et al.* 1994).

Neighbour-joining analyses (NJ) (Saitou & Nei 1987) were carried out using the software package MEGA (Kumar *et al.* 1993). Different methods were used to estimate evolutionary distances accounting for multiple substitutions: Jukes–Cantor (Jukes & Cantor 1969), Kimura's two-parameter model (Kimura 1980), and Tamura–Nei's method (Tamura 1992).

Statistical confidence of MP and NJ evolutionary trees was assessed using bootstrapping (Felsenstein 1985), with 400 replications each.

The homogeneity of the rate was estimated using the relative rate test (Wilson *et al.* 1977); this approach tests whether the nucleotide substitution rates differ in two lineages using a third, 'reference' taxon; the advantage of such a test is that the knowledge of the divergence times between lineages is not required. The number of transversional substitutions per site was estimated using Kimura's formulas (Kimura 1980); the values calculated for each pairwise comparisons are reported in Table 7.1. Variances and covariances were determined according

Table 7.1. *Distance matrix. Genetic distances for pairwise comparisons of notothenioid mtDNA sequences. Percentage divergences based on all mutations are reported above the diagonal and those based on trasversions alone are presented below the diagonal. All distances were calculated using Kimura's two-parameter model (1980)*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 *Bovicthus variegatus*		17.64	18.25	17.80	18.52	17.48	16.85	17.44	18.07	16.57	16.57	16.52	18.05	17.91	16.70	16.39	16.55	16.69
2 *Trematomus eulepidotus*	4.96		0.93	0.46	1.16	0.93	3.54	4.04	4.77	4.28	4.04	4.28	5.90	5.52	4.39	4.52	4.27	4.89
3 *Trematomus hansoni*	5.09	0.11		1.16	1.39	1.39	3.90	4.16	4.65	4.78	4.53	4.52	6.02	5.77	4.63	4.76	4.51	5.13
4 *Trematomus nicolai*	4.96	0.00	0.11		1.16	0.93	3.54	3.79	4.52	4.28	4.04	4.03	5.90	5.52	4.51	4.64	4.39	4.76
5 *Trematomus pennellii*	5.47	0.46	0.35	0.46		1.39	3.90	4.52	5.01	4.89	4.65	4.64	5.76	5.88	5.00	5.13	4.88	5.50
6 *Pagothenia borchgrevinki*	4.96	0.23	0.35	0.23	0.69		3.53	3.78	4.27	4.28	4.03	4.02	5.63	5.39	4.63	4.26	4.51	4.88
7 *Notothenia coriiceps neglecta*	5.09	1.04	1.16	1.04	1.52	1.28		1.98	2.82	2.34	2.10	2.10	3.90	3.78	2.93	2.81	2.81	3.05
8 *Gobionotothen gibberifrons*	5.35	0.81	0.93	0.81	1.28	1.04	0.69		2.22	2.58	2.34	2.22	4.52	4.40	3.05	2.93	2.93	3.30
9 *Dissostichus mawsoni*	5.35	1.04	1.16	1.04	1.52	1.28	0.69	0.46		3.18	2.94	2.82	4.40	4.78	3.54	3.30	3.54	3.91
10 *Histiodraco velifer*	4.83	0.81	0.93	0.81	1.28	1.04	0.69	0.69	0.69		0.46	1.63	3.30	3.42	1.98	2.10	2.10	2.45
11 *Pogonophryne scotti*	4.96	0.69	0.81	0.69	1.16	0.93	0.58	0.58	0.58	0.11		1.39	2.93	3.30	1.98	1.86	1.86	2.22
12 *Gymnodraco acuticeps*	5.35	1.04	1.16	1.04	1.52	1.28	0.69	0.69	0.69	0.69	0.58		2.94	3.18	1.51	1.39	1.39	1.74
13 *Cygnodraco mawsoni*	5.47	1.04	1.52	1.40	1.63	1.63	1.28	1.04	1.04	1.04	0.93	0.81		3.30	3.53	2.93	3.42	3.78
14 *Parachaenichthys charcoti*	5.35	1.28	1.40	1.28	1.75	1.52	1.16	0.93	0.93	0.93	0.81	0.69	0.81		3.66	3.06	3.42	3.79
15 *Chaenocephalus aceratus*	5.09	1.52	1.63	1.52	1.99	1.75	1.40	1.16	1.16	0.93	1.04	0.93	1.28	0.93		0.81	0.35	1.16
16 *Chionodraco hamatus*	5.09	1.28	1.40	1.28	1.75	1.52	1.16	0.93	0.93	0.93	0.81	0.69	1.04	0.69	0.23		0.46	0.81
17 *Cryodraco antarticus*	5.09	1.28	1.40	1.28	1.75	1.52	1.16	0.93	0.93	0.93	0.81	0.69	1.04	0.69	0.23	0.00		0.81
18 *Pagetopsis macropterus*	5.22	1.40	1.52	1.40	1.87	1.63	1.28	1.04	1.04	1.04	0.93	0.81	1.16	0.81	0.35	0.11	0.11	

to Wu & Li (1985). Using *Bovichtus variegatus* as reference taxon, the homogeneity of the rate was statistically tested for all possible pairs of the remaining notothenioids; levels of significance were calculated using a standardized normal test (t test), with infinite degrees of freedom. Divergence times between taxa were calculated as the ratio of genetic distance (or mean genetic distance, when comparing clades with more than one taxon) and divergence rate.

RESULTS AND DISCUSSION

Phylogenetic evidence

Both parsimony and distance-based phylogenetic analyses yield an overall topology in good agreement with the phylogenetic pattern derived from cladistic analysis of morphological characters (Eastman 1993); the relationships among families inferred from molecular data are consistent with previous evidence. The family Bovichtidae appears to be the sister group to the other families of notothenioids, being highly divergent from them.

The taxonomical status of two families is questionable since both methods (PAUP and neighbour joining) indicate the families Nototheniidae and Bathydraconidae to be paraphyletic (Table 7.1, Fig. 7.1). Similarly, the subfamily Nototheniinae is an unnatural group because *G. gibberifrons* is more closely related to *D. mawsoni* than to *N. coriiceps* (Table 7.1, Fig. 7.1). This is not surprising, since nototheniid systematic relationships based on morphology were considered unsatisfactory, with revisions proposed recently (Balushkin 1990, 1991). Our molecular data support the splitting of the genus *Gymnodraco* from the rest of Bathydraconidae, previously proposed on the basis of morphological evidence by Hastings (1993, in Miller 1993). The neighbour-joining tree suggests that *Gymnodraco* is the sister group of the Channichthyidae (Fig. 7.1B). If this true, *Gymnodraco*, the only teleost with a single haemoglobin lacking the Bohr effect, should be regarded as an evolutionarily intermediate step toward the loss of haemoglobins in the Channichthyidae (Eastman 1993).

The short branch lengths at the inter-familial level displayed by the NJ tree (Fig. 7.1B), compared with the great distance separating *Bovichtus* from all the other notothenioids, suggest that rapid cladogenesis occurred long after the split of Bovichtidae, thus offering little time for mutations to accumulate.

Estimating time of divergence from DNA sequence data

Genetic distances between species can be used as a measure of time since their divergence; this is possible when the rate of genetic divergence is linear with time and homogeneous between taxa (molecular clock hypothesis). If the clock is valid, molecular data provide a valuable tool in timing evolutionary events.

However, even accepting the clock hypothesis, a problem still remains: calibrating the clock with absolute timing. This calibration can be accomplished when geological records for cladogenetic events are available; dates are usually provided by good fossil records of the taxa under investigation and/or by specific, well-established, geological events associated with separation of taxa (Caccone *et al.* 1994).

With regard to our data, evolutionary distances were calculated using transversions only, since transversions seem to evolve linearly with time (Miyamoto & Boyle 1989, Mindell &

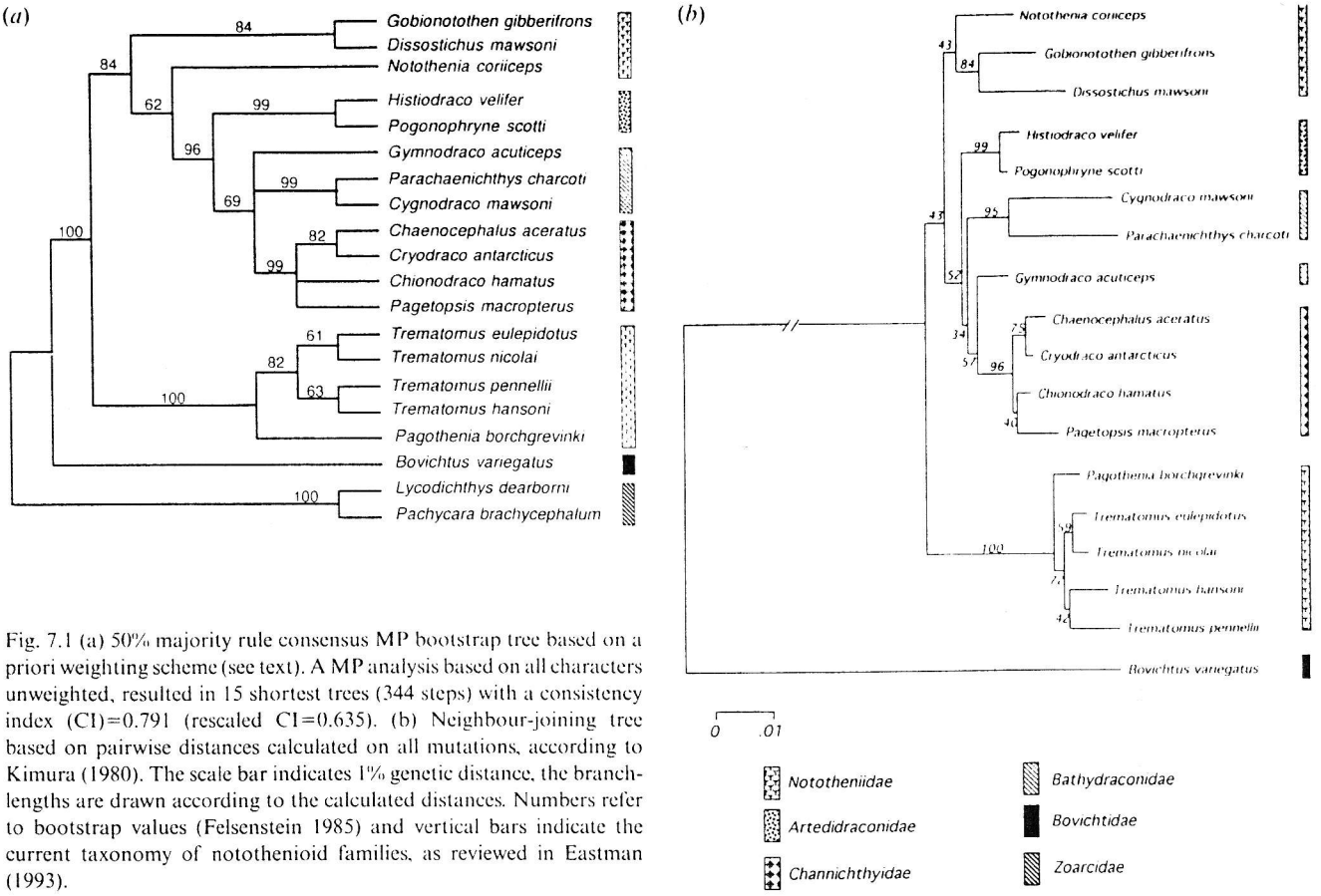

Fig. 7.1 (a) 50% majority rule consensus MP bootstrap tree based on a priori weighting scheme (see text). A MP analysis based on all characters unweighted, resulted in 15 shortest trees (344 steps) with a consistency index (CI)=0.791 (rescaled CI=0.635). (b) Neighbour-joining tree based on pairwise distances calculated on all mutations, according to Kimura (1980). The scale bar indicates 1% genetic distance, the branch-lengths are drawn according to the calculated distances. Numbers refer to bootstrap values (Felsenstein 1985) and vertical bars indicate the current taxonomy of notothenioid families, as reviewed in Eastman (1993).

Honeycutt 1990). Few minor differences in the rate were detected by the relative rate test, which involved the two Artedidraconidae spp. and *Cygnodraco mawsoni*. Therefore, rates were considered sufficiently homogeneous to be used in estimating time of divergence.

Two different estimates of divergence rates were applied to our data in order to calculate time of divergence between notothenioid species. A rate of 0.14% transversion (TV) per million years (My) was proposed for mountain newts (Caccone *et al.* 1994) and bovids (Allard *et al.* 1992); the substitution rate in newts was estimated using the same dataset (about 910 bp from 12S and 16S rRNA mitochondrial genes) as ours, whereas in bovids the complete sequence (2700 bp) of the 12S and 16S rRNA genes was used. Despite this difference in the length of the DNA segment considered, it is interesting that the rate proposed for newts and bovids are identical.

Using our data, we performed a transversion rate calibration with the complete separation of New Zealand from Antarctica (57 million years ago (Ma)) as the estimated time of divergence between *Bovichtus variegatus* and the rest of notothenioid taxa investigated. When our complete dataset was considered (928 bp), the mean nucleotide divergence between *Bovichtus* and the remaining species was 6.2% TV, yielding a rate of 0.11% TV/My which is very similar to that proposed for newts and bovids. When the reduced dataset (883 bp) was taken into account (excluding the highly variable sequence), the mean divergence of *Bovichtus variegatus* was 4.85% and the substitution rate was 0.085% TV/My, which represents a more conservative estimate. Due to the presence of a 45 bp unalignable and possibly hypervariable region, the complete dataset was regarded as less reliable and therefore the reduced dataset was used to calculate divergence times. Using the estimates proposed for newts and bovids (0.14% TV/My) as well as our more conservative estimates of 0.085% TV/My, times of divergence were calculated in relation to major events in notothenioid evolution (Table 7.2).

Tempo and mode of evolution of notothenioids

According to the distance-based phylogeny (NJ tree) *Bovichtus variegatus* diverged very early in the notothenioid evolution, probably after the separation of New Zealand from Antarctica (45–57 Ma).

Our estimate of the divergence time between *Dissostichus* and *Notothenia* ranges between 6–8 Ma (Table 7.2). It suggests that the northward expansion and successive regression of the Polar Front (6.5–5 Ma) possibly played a role as a vicariant factor in the distribution of species belonging to these two genera both outside and inside the Southern Ocean. Diversification of the Trematominae and Channichthyidae falls in a period (Mid Pliocene, 3.5–2.5 Ma) when partial deglaciation might have favoured speciation by providing new coastal and shelf- water habitats.

The short branching pattern displayed by the NJ tree for all the notothenioid taxa (except for *Bovichtus variegatus*) suggests

Table 7.2. *Major events in the evolution of notothenioids and their association with paleoclimatic and/or geological events. The estimate of the timing in notothenioid evolution was performed using a calibration of (a) 0.14% TV/My or (b) 0.085% TV/My (see text)*

Evolutionary event(s)	Mean divergence (a) (Ma)	Mean divergence (b) (Ma)	Associated paleoclimatic And/or geological events
Separation of *Bovichtus* from the rest of notothenioids	45	57	Complete separation of New Zealand from Antarctica (57 Ma)
Major divergence of notothenioid families (Trematominae versus remaining taxa, excluding Bovichtidae)	11	15	Development of East Antarctic sheet, continued drop in water temperatures, significant sea-ice formation (14–12 Ma)
Separation between *Notothenia* and *Dissostichus*	6	8	Northward expansion of Antarctic Polar Front (6.5–5 Ma)
Trematominae diversification	2.5–1.5	4.5–3	Partial deglaciation of Antarctica with relatively warm (2–6 °C) marine embayments; increase in
Channichthyidae diversification	2	2.5	shallow-water shelf habitat

rapid diversification of this group of fish, long after the separation of the Bovichtidae. On the basis of our estimates, this radiation-like mode of evolution possibly occurred 15–10 Ma, when a consistent sea-ice formation, drop in water temperatures and expansion of ice sheet played an important role in modifying the Antarctic climate (Eastman, 1993). These major changes in the Antarctic environment might have created empty niches to which very few non-notothenioid fish were able to adapt. If antifreeze glycopeptides (AFGPs) evolved only once in notothenioid evolution, long after the separation of Bovichtidae and before diversification of all the other notothenioids, as proposed by Bargelloni *et al.* (1994), AFGPs might have played a key role, among other features, in the adaptive radiation of notothenioid fishes.

ACKNOWLEDGEMENTS

We thank A. L. DeVries, E. Pisano, L. Camardella, G. diPrisco, and B. Dickson for providing us with samples. The present research was supported by the Italian PNRA (Programma Nazionale di Ricerche in Antartide) with grants to BB, TP and LB, by the Auckland University Research Committee (AURC) and the New Zealand Lottery Board with grants to PAR, and by US National Science Foundation with grant No. BSR-09107838 and BSR-9119867 to AM.

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