New Zealand Journal of Marine and Freshwater Research, 2006, Vol. 40: 299–304
0028–8330/06/4002–0299

Short communication

Lack of divergence in the mitochondrial cytochrome *b* gene between *Macruronus* species (Pisces: Merlucciidae) in the Southern Hemisphere

CARLOS OLAVARRÍA[1,2]
FERNANDO BALBONTÍN[3]
ROLANDO BERNAL[3]
C. SCOTT BAKER[1]

[1]School of Biological Sciences
The University of Auckland
Private Bag 92019
Auckland, New Zealand
email: c.olavarria@auckland.ac.nz

[2]Centro de Estudios del Cuaternario (CEQUA)
Punta Arenas, Chile

[3]Facultad de Ciencias del Mar
Universidad de Valparaíso
Casilla 5080, Reñaca
Viña del Mar, Chile

Abstract Two species of grenadier, the blue grenadier (or hoki) *Macruronus novaezelandiae* and the Patagonian grenadier *M. magellanicus*, have been recorded in the Southern Hemisphere with disjunct distributions along southern Australia–New Zealand and southern Chile–Argentina, respectively. The extent of genetic difference between these two putative species was examined using partial sequences (n = 44; 405 bp) of the mitochondrial DNA cytochrome *b* gene in a phylogenetic analysis and in an analysis of molecular variance (AMOVA). Our analysis showed a lack of genetic differentiation between species (F_{ST} = –0.02275, Φ_{ST} = –0.00250) and among stocks. This absence of genetic differences is consistent with recent larval and adult morphology data suggesting that the two species should be synonymised. A comparable lack of genetic isolation has been observed in other closely related taxa (genera *Merluccius* and *Micromesistius*) with similar disjunct distributions across the Southern Hemisphere.

M05055; Online publication date 3 May 2006
Received 22 August 2005; accepted 17 January 2006

Keywords *Macruronus novaezelandiae*; *Macruronus magellanicus*; Chile; New Zealand; mitochondrial DNA

INTRODUCTION

New Zealand and Chilean coasts share a significant number of genera and species of fish (Nakamura 1986). This provides a framework for addressing stock identities and systematic relationships of widely distributed species. For example, two genera with closely related species and discontinuous distributions across the South Pacific are *Merluccius* (hake) and *Micromesistius* (blue whiting). In a worldwide review of the genus *Merluccius*, Inada (1981) accepted one species of hake *Merluccius australis* (Hutton, 1872) for New Zealand–Australia and southern Chile–Argentina, based on morphological data. Subsequently, allozyme analysis considering initially two species, *Merluccius australis* from New Zealand–Australia and *M. polylepis* Ginsburg, 1954 from Chile–Argentina, found low levels of genetic divergence, leading to the suggestion of a single species and further subspecific recognition (Grant & Leslie 2001). In the example of the blue whiting *Micromesistius australis* (Norman, 1937), morphological analyses have separated it into two subspecies, *M. australis australis* off Chile, southern Argentina, and the Falkland Islands and *M. australis pallidus* from subantarctic waters off New Zealand (Inada & Nakamura 1975). This systematic relationship was later supported by mini- and microsatellite analysis (Ryan et al. 2002).

The genus *Macruronus* represents another example of taxonomic uncertainty for species distributed on the two sides of the South Pacific. Two or three species are currently proposed for this Southern Hemisphere genus (Inada 1990; Lloris et al. 2003). The hoki or blue grenadier, *M. novaezelandiae* (Hector, 1871) is distributed along southern Australia, including Tasmania and the south island of New Zealand (Lloris et al. 2003). This species was originally described for New Zealand

Fig. 1 General geographic distribution of *Macruronus novaezelandiae* and *M. magellanicus* (shaded). Location of sampling sites for *M. novaezelandiae* (AU, Australia; NZ, New Zealand, from Baker et al. (1995)) and *M. magellanicus* (CH, southern Chile). Map not to scale.

and Tasmania and later cited for Chile and Argentina (Günther 1880). A new species, the Patagonian grenadier *M. magellanicus* Lönnberg, 1907 was later described for the South American coast, based on differences of body proportions of a single damaged juvenile. This species is distributed along the coast of southern Chile, and in the Atlantic along the coast of Argentina extending north to southern Brazil (Lloris et al. 2003). Both species, *M. novaezelandiae* and *M. magellanicus*, are restricted to cold and temperate waters at depths of between 200 and 800 m (Lloris et al. 2003). They are among the most important fisheries for countries such as Chile, where the Patagonian grenadier fishery has had catches from 375 000 t in 1996 to 133 000 t in 2002 (SERNAPESCA 2003). In New Zealand, the hoki fishery has been one of the most important fisheries in recent years, with catches of over 200 000 t per year (NZ Ministry of Fisheries 2003). The third species of this genus, the cape grenadier *M. capensis* Davies, 1950 is restricted to the southern coast of Africa and not considered to be of commercial importance (Lloris et al. 2003).

The taxonomic relationship of the *Macruronus* species has been based entirely on morphometric analysis and the observed disjunct geographic distributions (Inada 1990). Nevertheless, a recent review of this work proposed that the variability in the meristic characters separate *M. novaezelandiae* and *M. magellanicus* only at a sub-specific level; retaining only *M. capensis* as a valid species (Lloris et al. 2003). However, because the work of Inada (1990) and Lloris et al. (2003) are both in the form of catalogues (the former of the order Gadiformes and the later of the family Merlucciidae) it is not possible to evaluate the analyses that led to their conclusions. Recently, a comparison of larval stages of *M. novaezelandiae* and *M. magellanicus* found identical pigmentation patterns, morphometric and meristic characters, discounting the current division into two species (Balbontín et al. 2004).

Here we investigate the systematic relationship of two species of the *Macruronus* genus. We compare sequences of mitochondrial DNA cytochrome *b* gene from *M. magellanicus* from the Chilean coast and *M. novaezelandiae* from New Zealand–Australia using new and previously published sequences (Baker et al. 1995). This molecular marker has been widely used in establishing systematic relationships of fish (Stepien & Kocher 1997), but has not been used to establish systematic relationship of the *Macruronus* genus.

MATERIALS AND METHODS

Dorsal muscles samples from 22 specimens of *Macruronus magellanicus* were obtained from fishing vessels in southern Chile (43°20´ to 51°S) between August and September 2001 (Fig. 1). Samples were stored in 80% ethanol until analysis. Genomic DNA was extracted using a standard phenol/chloroform extraction protocol (Davis et al. 1986). Symmetrical amplification of the mitochondrial DNA cytochrome *b* gene was performed via the polymerase chain reaction (PCR, Saiki et al. 1988) following standard protocols (Palumbi 1996). A 500 base pair (bp) portion of the mtDNA cytochrome *b* was amplified using the primers, tGludg (TGACTTGAARAACCAYCGTTG, Palumbi et al. 1991) and Cyb2 (CCCTCAGAATGA TATTTGTCCTCA, Kocher et al. 1989) following Baker et al. (1995). The PCR profile consisted of one initial cycle of strand denaturation at 94°C for 1 min, primer annealing at 48°C for 1 min,

and extension at 72°C for 1 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min (Baker et al. 1995). PCR amplification products were purified from primers and nucleotides using exonuclease I (Exo I) and shrimp alkaline phosphatase (SAP, Werle et al. 1994). The amplified fragments were sequenced in both directions with BigDye™ terminator chemistry on an ABI3100 DNA sequencer (Applied Biosystem). Sequencer (version 4.1.2, Genes Codes Co.) was used to align and edit the consensus sequences for each sample.

Sequences were aligned and compared to previously reported cytochrome *b* sequences of *M. novaezelandiae* obtained at three fishing grounds around New Zealand (west coast of South Island (n = 9), Cook Strait (n = 9), and Chatham Rise (n = 1)) and one in Australia, off the west coast of Tasmania (n = 8) (Baker et al. 1995). Comparisons of the two sets of sequences were performed using MacClade (version 4.0, Maddison & Maddison 2000) to identify variable sites and define haplotypes.

The phylogeny of the *Macruronus* haplotypes was reconstructed using the Neighbor-Joining method (Saitou & Nei 1987) and parsimony, as implemented in PAUP* (version 4.0b10, Swofford 2002). Phylogenies were rooted with a *Merluccius merluccius* sequence as outgroup (GenBank accession code AF120096, Verardi & Bullini unpubl. data). The best fit model of molecular evolution for maximum likelihood was evaluated using Modeltest (version 3.06, Posada & Crandall 1998). A F81 model of sequence evolution was selected with estimated nucleotide frequencies: A = 0.2554, C = 0.2677, G = 0.1651, and T = 0.3117. For the Neighbor-Joining method, minimum evolution was used as the default optimality criterion. For parsimony, heuristic search conditions were starting trees obtained by stepwise addition with 10 random sequence addition replicates and tree bisection reconnection (TBR) branch swapping, with searches limited to 100 rearrangements for each replicate. The robustness of phylogenies was examined by bootstrap resampling, with 1000 replicates for Neighbor-Joining and 1000 full heuristics for parsimony.

Genetic diversity for each species was estimated at haplotype and nucleotide level using Arlequin (version 2.0, Schneider et al. 2000). Geographic differentiation among haplotypes frequencies and nucleotide variation was quantified using analysis of molecular variance (AMOVA, Excoffier et al. 1992) as implemented in Arlequin, using nucleotide differentiation (Φ_{ST}) and haplotype frequency differences (F_{ST}). We assigned portions of total variation to divergence either between species (*M. magellanicus* and *M. novaezelandiae*) or within populations (Australia and New Zealand for *M. novaezelandiae* and Chile for *M. magellanicus*). The significance of the observed Φ_{ST} and F_{ST} values were tested using 20 000 random permutations of the data matrix.

Macruronus magellanicus haplotypes were submitted to GenBank with the accession codes DQ364239–DQ364241.

RESULTS

A 405 bp segment of the mtDNA cytochrome *b* gene was successfully sequenced for 17 of the 22 *Macruronus magellanicus* specimens collected off Chile. Two variable sites defined three haplotypes, two of which were represented by only one individual. The analysis carried out by Baker et al. (1995), of a comparable 425 bp segment of the cytochrome *b* gene, from 27 *M. novaezelandiae* specimens collected on three fishing grounds around New Zealand and one off the west coast of Tasmania, identified five haplotypes defined by four polymorphic sites.

Combining the previously published sequences from Australia and New Zealand with those from Chile revealed seven haplotypes among the total sample of 44, each differing by only a single base pair. The most common haplotype from *M. magellanicus* (88.2%) matched the most common haplotype from *M. novaezelandiae* (Mno-A, 85.2%; Fig. 2). When *M. novaezelandiae* population samples from Australia and New Zealand were pooled and compared to those from Chile, both species showed similar genetic diversity at the haplotype (*M. magellanicus,* h = 0.228 ± 0.130; *M. novaezelandiae,* h = 0.279 ± 0.112) and nucleotide level (*M. magellanicus,* π = 0.06%; *M. novaezelandiae*, π = 0.07%). Both Neighbor-Joining and parsimony reconstructions showed no genetic divergence between *M. magellanicus* and *M. novaezelandiae*, with no clustering of haplotypes by geographic origin or species (Fig. 2).

An AMOVA confirmed the absence of differentiation between the two species at both haplotype and nucleotide level (F_{ST} = –0.023, P = 0.662 ± 0.003; Φ_{ST} = –0.003, P = 0.427 ± 0.003). Pair-wise tests of differentiation also failed to find significant differences among the two stocks of *M. novaezelandiae* and one of *M. magellanicus* (Table 1). However, the power of our test for stock differences was limited by a small sample size.

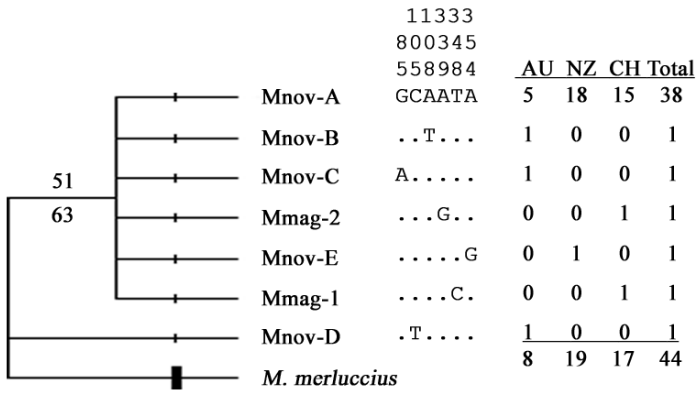

Fig. 2 Parsimony reconstruction of *Macruronus* mtDNA cytochrome *b* haplotypes. Bootstrap support is shown when higher than 50% (for Neighbor-Joining above branches and for parsimony below branches). Crossbars represent one change. Thick crossbar represent 69 changes of *Macruronus* compared with *Merluccius merluccius*. Polymorphic sites and their location are indicated. Haplotype frequencies are also shown.

Table 1 Pair-wise test of differentiation of mtDNA cytochrome *b* sequences among two stocks of *Macruronus novaezelandiae* and one of *M. magellanicus*, based on Φ_{ST} (above the diagonal) and Φ_{ST} (below the diagonal) values. Probability (*P*) of obtaining greater values by chance alone of 20 000 random permutations of the data matrix ($P < 0.05$) is shown in bold.

Species	Stock	Australia ($n = 8$)	New Zealand ($n = 19$)	Chile ($n = 17$)
M. novaezelandiae	Australia	–	0.175 **0.068 ± 0.002**	0.072 **0.165 ± 0.003**
M. novaezelandiae	New Zealand	0.078 **0.065 ± 0.002**	–	–0.013 **0.590 ± 0.004**
M. magellanicus	Chile	0.043 **0.167 ± 0.003**	0.003 **0.448 ± 0.003**	–

DISCUSSION

No evidence of divergence was found in our phylogenetic or AMOVA analyses of the mtDNA cytochrome *b* gene of the two currently recognised species, *M. novaezelandiae* and *M. magellanicus*. Further, there was only a low level of population differentiation evidenced by rare haplotypes. Based on this absence of genetic divergence, consideration should be given to synonymising the two currently recognised species, as suggested previously by Lloris et al. (2003), based on the absence of morphological divergence.

Larger sample sizes and other more variable genetic markers regions, such as the mitochondrial DNA control region or nuclear microsatellite DNA should be used in future analyses to establish relationships at a population or stock level. Around New Zealand, at least two breeding stocks have been identified based on morphometric comparisons (Livingston & Schofield 1996), although mtDNA RFLPs and cytochrome *b* sequences failed to reveal any population subdivision (Baker et al. 1995; Smith et al. 1996). Off Chile, the Patagonian grenadier is thought to form a single stock, based on the absence of differences in allozymes, mtDNA RFLP, morphologic and parasitological analyses (Galleguillos et al. 1998). In the Argentinean southwestern Atlantic, it has been suggested that stock differentiation could be present among coastal and offshore samples, based on ecological and morphological data, although mtDNA RFLP analysis did not detect differentiation (D'Amato et al. 1999).

Finally, we note that molecular markers, such as the cytochrome *b* used here, are needed to resolve the systematic status of the cape grenadier (*M. capensis*), which is still considered a valid species despite the limited number of studied specimens (Lloris et al. 2003). Further studies could contribute to the understanding of the interesting pattern of geographic

isolation but absence of genetic divergence among these Southern Hemisphere fish species.

ACKNOWLEDGMENTS

We thank L. Adasme for collection of the Patagonian grenadier samples during a survey supported by project FIP 2001–19 of the Chilean Fisheries Research Fund. Funding for genetic analysis was provided by a New Zealand Marsden Fund grant to C. S. Baker. C. Olavarría is supported by scholarships from the University of Auckland and the Centro de Estudios del Cuaternario. We thank A. Hickey for laboratory assistance and E. Newcombe, D. Steel, A. Hickey, S. Caballero, and S. Lavery for reviewing early versions of this manuscript. Sadly, in the winter of 2002 one of the co-authors (Rolando Bernal) passed away after a long illness. We acknowledge Rolando's efforts to undertake this work during its earlier stages. *To the memory of Rolando Iván Bernal Deramond (1970–2002).*

REFERENCES

Baker CS, Perry A, Chambers GK, Smith PJ 1995. Population variation in the mitochondrial cytochrome *b* gene of the orange roughy *Hoplostethus atlanticus* and hoki *Macruronus novaezelandiae*. Marine Biology 122: 503–509.

Balbontín F, Uribe F, Bernal R, Braun M 2004. Descriptions of larvae of *Merluccius australis*, *Macruronus magellanicus*, and observations on a larva of *Micromesistius australis* from southern Chile (Pisces: Gadiformes). New Zealand Journal of Marine and Freshwater Research 38: 609–619.

D'Amato ME, Lunt DH, Carvalho GR 1999. Microsatellite markers for the hake *Macruronus magellanicus* amplify other gadoid fish. Molecular Ecology 8: 1086–1088.

Davis LG, Dibner MD, Battey JF 1986. Basic methods in molecular biology. New York, Elsevier Science Publishing. 388 p.

Excoffier L, Smouse PE, Quattro JM 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479–491.

Galleguillos R, Montoya R, Troncoso L, Oliva M, Oyarzún C 1998. Identificación de unidades de stocks en el recurso merluza de cola en el área de distribución de la pesquería. Fondo de Investigación Pesquera, Valparaíso, Chile, Proyecto FIP 96–30. 81 p.

Grant WS, Leslie RW 2001. Inter-ocean dispersal is an important mechanism in the zoogeography of hakes (Pisces: *Merluccius* spp.). Journal of Biogeography 28: 699–721.

Günther A 1880. Report on the shore fishes procured during the voyage of the H.M.S *Challenger* in the years 1873–1880. Reports on the scientific results of the voyage of the H.M.S. *Challenger* 1: 1–82.

Inada T 1981. Studies on the Merlucciid fishes. Far Seas Fisheries Research Laboratory Bulletin 18: 1–172.

Inada T 1990. Family Merluccidae. In: Cohen DM, Inada T, Iwamoto T, Scialabba N ed. Gadiform fishes of the world (Order Gadiformes). An annotated and illustrated catalogue of cods, hakes, grenadiers and other gadiform fishes known to date. Rome, FAO Fisheries Synopsis 125. Pp. 319–346.

Inada T, Nakamura I 1975. A comparative study of two populations of the gadoid fish *Micromesistius australis* from the New Zealand and Patagonian-Falkland regions. Far Seas Fisheries Research Laboratory Bulletin 13: 1–26.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences of the United States of America 86: 6196–6200.

Livingston ME, Schofield KA 1996. Stock discrimination of hoki (*Macruronus novaezelandiae*, Merluccidae) in New Zealand waters using morphometrics. New Zealand Journal of Marine and Freshwater Research 30: 197–208.

Lloris D, Matallanas J, Oliver P 2003. Merluzas del mundo (Familia Merlucciidae). Catálogo comentado e ilustrado de las merluzas conocidas. FAO Catálogo de Especies para los Fines de la Pesca No. 2. Roma, FAO. 57 p.

Maddison DR, Maddison WP 2000. MacClade: analysis of phylogeny and character evolution. Sunderland, Massachusetts, Sinauer Associates Inc.

Nakamura I 1986. Important fishes trawled off Patagonia. Japan Marine Fishery Resource Research Center (JAMARC). 369 p.

NZ Ministry of Fisheries 2003. Export case study: hoki. Ministry of Fisheries of New Zealand. http://www.starfish.govt.nz/economic/facts/fact-hoki.htm [accessed 16 June 2003].

Palumbi SR 1996. Nucleic acids II: the Polymerase Chain Reaction. In: Hillis D, Moritz C, Mable BK ed. Molecular systematics. Sunderland, MA, Sinauer Associates Inc. Pp. 205–248.

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G 1991. The simple fool's guide to PCR. Honolulu, Department of Zoology, University of Hawaii.

Posada D, Crandall K 1998. MODELTEST: testing the model of DNA susbstitution. Bioinformatics 14: 817–818.

Ryan AW, Smith PJ, Mork J 2002. Genetic differentiation between the New Zealand and Falkland Islands populations of southern blue whiting *Micromesistius australis*. New Zealand Journal of Marine and Freshwater Research 36: 637–643.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491.

Saitou N, Nei M 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425.

Schneider S, Roessli D, Excoffier L 2000. Arlequin. A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

SERNAPESCA 2003. Anuario estadístico de pesca 2002. Valparaíso, Chile, Servicio Nacional de Pesca, Ministerio de Economía, Fomento y Reconstrucción. 156 p.

Smith PJ, McVeagh SM, Ede A 1996. Genetically isolated stocks of orange roughy (*Hoplostethus atlanticus*), but not of hoki (*Macruronus novaezelandiae*), in the Tasman Sea and southwest Pacific ocean around New Zealand. Marine Biology 125: 783–793.

Stepien CA, Kocher TD 1997. Molecules and morphology in studies of fish evolution. In: Kocher TD, Stepien CA ed. Molecular Systematics of Fishes. Sydney, Academic Press. Pp. 1–11.

Swofford DL 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods). Sunderland, MA, United States, Sinauer Associates.

Werle E, Schneider C, Renner M, Volker M, Fiehn W 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Research 22: 4354–4355.