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BRIEF COMMUNICATION

DNA barcoding highlights a cryptic species of grenadier *Macrourus* in the Southern Ocean

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Although three species of the genus *Macrourus* are recognized in the Southern Ocean, DNA sequencing of the mitochondrial COI gene revealed four well-supported clades. These barcode data suggest the presence of an undescribed species, a conclusion supported by meristic and morphometric examination of specimens.

Key words: Antarctic fisheries; COI; mitochondrial DNA; species identification.

The grenadiers or rattails (family Macrouridae) are a large family of >300 species of mostly bentho-pelagic predators and scavengers, found in all oceans from the upper continental slope down to the abyssal depths (Cohen *et al*., 1990; Froese & Pauly, 2006; Iwamoto & McMillan, 2008). The genus *Macrourus* is a small group of four bentho-pelagic species found on the upper and middle continental slope in cold temperate and polar waters (Cohen *et al*., 1990), where they feed on a wide range of fishes and invertebrates (Marriott *et al*., 2003; Morley *et al*., 2004). Three species have been recognized in the Southern Ocean (Iwamoto, 1990; Eschmeyer & Fricke, 2009): the circumpolar Whitson's grenadier *Macrourus whitsoni* (Regan), the ridge-scaled grenadier *Macrourus carinatus* (Günther) and the bigeye grenadier *Macrourus holotrachys* Günther from the south-west Atlantic Ocean. A fourth species, the roughhead or onion-eye grenadier *Macrourus berglax* Lacépède, is found in the

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North Atlantic Ocean in cold temperate to Arctic waters. The three Southern Ocean species are morphologically similar and the taxonomic status of the Southern Ocean species has been confused, until recently, in part because of a paucity of comparative material (Cohen *et al*., 1990). Some characters show overlap, and identification of species had been based on relatively few specimens, using a combination of characters including geographic and depth distributions (Cohen *et al*., 1990). One key character, the presence or absence of squamation on the underside of the head, requires careful examination, but clearly distinguishes the naked *M. holotrachys* from the scaled *M. whitsoni* and *M. carinatus* (Cohen *et al*., 1990). Specimens of the variable *M. whitsoni* are easily misidentified by non-specialists, especially when identifications are made at sea, but can be distinguished from *M. carinatus* by their smaller more delicate scales, deeper habitat and apparent distributions (Cohen *et al*., 1990; Marriott *et al*., 2003; Laptikhovsky, 2005).

Targeted fisheries have developed for *M. carinatus* and *M. holotrachys* on the Patagonian slope of the South Atlantic Ocean (Cohen *et al*., 1990; Laptikhovsky *et al*., 2008). Grenadiers are important by-catch species in the toothfish *Dissostichus* spp. longline and trawl fisheries in the Southern Ocean with reported catches of all grenadiers across the area managed by the Convention from the Conservation of Antarctic Marine Living Resources (CCAMLR) exceeding 1500 t in the 2008–2009 season, the majority being captured on the Kerguelen Plateau, around South Georgia, and in the Ross Sea (Duhamel *et al*., 1997; Morley *et al*., 2004; Hanchet *et al*., 2008; CCAMLR, 2010). *Macrourus whitsoni* is the dominant by-catch species of the *Dissostichus* spp. longline fishery in and near the Ross Sea accounting for 8% of the total landed mass, and has been recognized as a single species (Hanchet *et al*., 2001, 2008, 2009). *Macrourus berglax* is targeted in the North Atlantic Ocean (Lorance *et al*., 2008), where it is also taken as a by-catch in longline fisheries in the Barents Sea (Dolgov *et al*., 2008) and around east Greenland (Fossen *et al*., 2003).

As part of the International Polar Year (IPY), a large number of Southern Ocean fish specimens were collected and tissue samples were taken for DNA barcoding. The barcode identification system is based on diversity in a single region of the mitochondrial DNA, the cytochrome *c* oxidase I gene (COI; Hebert *et al*., 2003; Ratnasingham & Hebert, 2007), and has proved to be a controversial initiative (Moritz & Cicero, 2004; Rubinoff *et al*., 2006). Species recognition using barcodes relies on different species having different unique sequences or different assemblages of closely related sequences. Intraspecific variation or genetic distance is thus generally much less than interspecific variation, enabling species identification and highlighting possible cryptic species (Waugh, 2007). While substantial overlap in intra and interspecific variation has been reported in some marine gastropods (Meyer & Paulay, 2005) and in corals (Shearer & Coffroth, 2008), in marine fishes *c*. 98% of species tested to date can be distinguished by COI barcodes (Ward *et al*., 2009). The *Macrourus* COI sequence data indicated four well-supported DNA clades among the three recognized Southern Ocean *Macrourus*. Here, these data are presented and evidence provided for a new undescribed species, a conclusion subsequently supported by meristic and morphological differentiation of the new species.

Specimens of *Macrourus* caught on commercial and research vessels in the Southern Ocean (Fig. 1) were frozen whole at sea for onshore processing, except those on the IPY-CAML R.V. *Tangaroa* survey which were tissue sampled at sea. Muscle

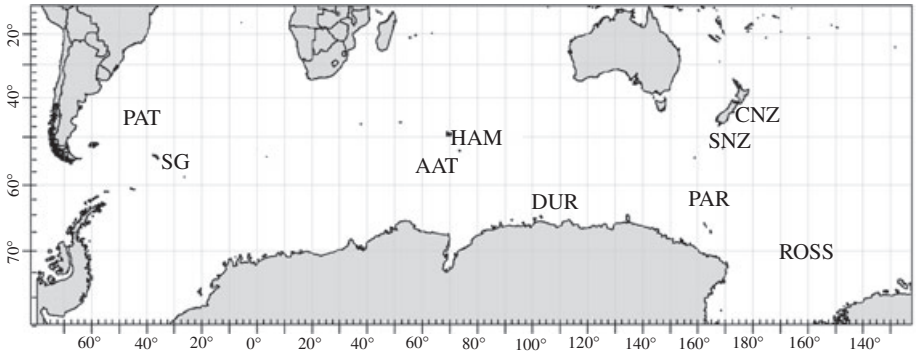

FIG. 1. Locations of *Macrourus* specimens sampled for DNA barcoding in the Southern Ocean: AAT, Australian Antarctic Territory; CNZ, Chatham Rise, New Zealand; DUR, Durmont D'Urville Sea; HAM, Heard and McDonald Islands; PAR, Pacific Antarctic Ridge; PAT, Patagonian Shelf; ROSS, Ross Sea region; SG, South Georgia; SNZ, Southern Plateau, New Zealand.

samples from fresh or thawed specimens were stored in 90% ethanol and the specimens then fixed in 10% formalin, prior to storage in 70% isopropanol or 70% ethanol, and registration in one of three collections: the Museum of New Zealand Te Papa Tongarewa (NMNZ), the Australian Antarctic Division, Australia and the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP) fish collection, Argentina. A set of 61 tissue samples from registered specimens was used to establish reference COI sequences for Southern Ocean *Macrourus* in the Barcode Of Life Database (BOLD; www.barcodinglife.org). Following the initial COI barcode results, the reference specimens were carefully re-examined and some identifications were revised. An additional 80 specimens, identified as *M. whitsoni*, were collected through the New Zealand Ministry of Fisheries Observer programme in the Ross Sea region over the summer 2008–2009. Muscle samples were taken from frozen specimens, and whole specimens retained for meristic counts and morphological analyses. Following the DNA results, which indicated two taxa in the set of *M. whitsoni* specimens, the frozen specimens were thawed and examined for characters in a blind test without initial reference to the individual DNA results. Three characters that were applicable for at-sea identifications were selected: number of pelvic fin rays, body colour, and relative size and number of teeth rows on the lower jaw.

The DNA was extracted from a sub-sample of muscle tissue from each of the reference specimens using an automated glass-fibre protocol (Ivanova *et al*., 2006). The 650 base pairs (bp) barcode region of COI was amplified under standard conditions using the primer cocktail FishF1t1 and FishR1t1 (Ivanova *et al*., 2007). Polymerase chain reaction (PCR) products were visualized on a 1·2% agarose gel E-GelH (Invitrogen; www.invitrogen.com) and sequenced in both directions, using the sequencing primers M13F and M13R (Ivanova *et al*., 2007), and the BigDyeH Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.; www.appliedbiosystems.com) on an ABI 3730 capillary sequencer following the manufacturer's instructions. Sequences were deposited in the BOLD Data system (Ratnasingham & Hebert, 2007) in the public project 'Rattails in the Southern Ocean'; BOLD accession numbers are given in Fig. 2.

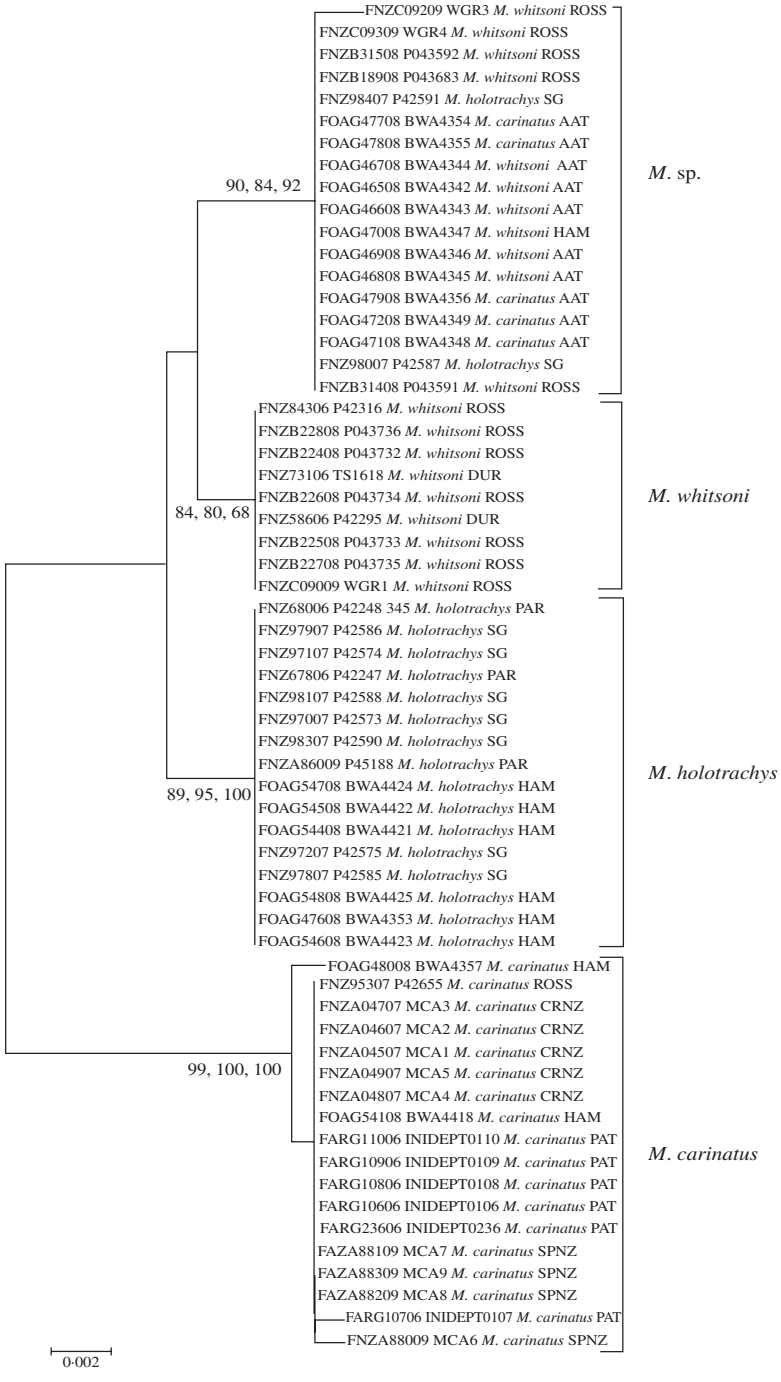

FIG. 2. Relationships of COI sequences from Southern Ocean *Macrourus* specimens. Barcode of Life Data (BOLD) accession numbers are given for each specimen. Numbers at nodes are bootstrap percentages (>60%) after 1000 replicates for maximum parsimony (MP), maximum likelihood (ML) and Bayesian posterior probabilities; scale bar represents an interval of the transition model TIM2. Location codes as in Fig. 1.

Sequences were aligned in CLUSTAL in MEGA v4 (Kumar *et al*., 2004) and sequence divergences within and among taxa were calculated using the TIM2 + G, equal base frequencies distance model (Posada, 2003), using MEGA v4. Initial neighbour-joining (NJ) clustering used the BOLD management & analysis system; subsequent maximum parsimony (MP) and maximum likelihood (ML) trees were built using phylogenetic analysis using parsimony (PAUP; Swofford, 2003) with heuristic searches, employing tree bisection-reconnection branch swapping; support for each internode was evaluated by 1000 bootstrap replications (Felsenstein, 1985). Bayesian phylogenetic analyses were estimated with MrBayes v3.0 (Huelsenbeck & Ronquist, 2001). Four simultaneous Monte-Carlo chains were run for 1×10^7 generations, saving the current tree for every 1000 generations. Consensus trees with posterior probabilities were created with a burn-in value equal to 1000 (the first 1000 trees were discarded). Nucleotide substitution models were selected in jModeltest v0.1.1 (Posada, 2008) using Akaike information criterion (AIC) and Bayesian information criterion (BIC); the transition model TIM2 + G was selected by both criteria.

The second set of *M. whitsoni* tissue samples collected in the Ross Sea region, 2008–2009, was tested with restriction fragment length polymorphism (RFLP) test that distinguished individuals in the two clades (the upper two clades of Fig. 2) containing specimens initially identified as *M. whitsoni* and the putative new species *M*. sp. DNA was extracted as above and amplified with COI primers (Folmer *et al*., 1994), following standard methods (Smith *et al*., 2008). The amplified products were digested with the restriction enzyme TAQ1 that recognizes diagnostic bases in the two clades; two TAQ1 restriction sites are present in the *M. whitsoni* clade and three sites in the *M*. sp. clade. Enzyme digestions were performed in 20 μl volumes for a minimum of 4 h, following manufacturer's recommendations (New England BioLabs; www.neb.com). The digested products were separated in 1·4% agarose gels and run at 60 V for 2 h; a DNA size ladder was included in each gel to estimate size of the amplified fragments. The amplified products were detected with ethidium bromide that was incorporated into the gel and viewed under UV light (312 nm). Molecular identification of six specimens was confirmed with COI sequencing.

Aligned sequences were obtained for 651 bp of COI from the reference set of 61 tissue samples of *Macrourus* from the Southern Ocean. Thirty-nine bases were variable and 16 were parsimony informative in both the Southern Ocean and the total data sets; all substitutions occurred in the third nucleotide position within codons, and there were no amino acid substitutions. Phylogenetic analyses (ML, MP and Bayesian) produced trees with similar topologies with four well-supported clades (Fig. 2) with high bootstrap values (ML and MP) and posterior probabilities (Bayesian), indicating four rather than three species in the Southern Ocean. The four clades contained specimens initially identified as (1) *M. holotrachys*, *M. whitsoni* and *M. carinatus*; (2) *M. whitsoni* and *M. carinatus*; (3) *M. holotrachys* and (4) *M. carinatus* and *M. holotrachys*. On the basis of the COI sequence results, the Southern Ocean specimens were re-examined and some were found to have been misidentified; the four clades (Fig. 2) were reduced to specimens of (1) *M. holotrachys*, *M. whitsoni* and *M. carinatus*; (2) *M. whitsoni*; (3) *M. holotrachys* and (4) *M. carinatus*. Thus, three of the four clades were reduced to single species clades: *M. whitsoni*, *M. carinatus* and *M. holotrachys*. The first clade contained specimens originally identified from all three Southern Ocean species (Fig. 2;

M. sp.) and allocated to species in part on capture locations (*M. whitsoni* from AAT, HAM and ROSS; *M. carinatus* from AAT and *M. holotrachys* from SG) following Cohen *et al*. (1990); all of these specimens were subsequently re-identified as belonging to a new and hitherto undescribed species.

Sequence divergence was low within each of the four clades ranging from 0·00 to 0·02% (Table I). The mean overall diversity, 1·1%, was also shallow but was at least ×5–10 the within-species diversities. Pair-wise species comparisons ranged from 0·6 to 2·0%; comparisons including *M. carinatus* showed the greatest divergences (1·8–2·0%; Table I). It has been suggested that the barcode gap between species should be based on the smallest rather than mean interspecific distances (Meier *et al*., 2008); these values are shown in parentheses in Table I. For the *Macrourus* COI data set there is little difference between the mean and smallest values due to the low intraspecific divergences in two clades (*M. carinatus* and *M*. sp.) and 0 values in the other two (*M. whitsoni* and *M. holotrachys*). The low intraspecific COI variation (0·0–0·2%) is similar to the lowest levels of conspecific COI variation in other marine fishes (Ward *et al*., 2005; Steinke *et al*., 2009*a*; Zemlak *et al*., 2009). The interclade divergence between the *M. whitsoni* clade and the *M*. sp. clade was ×6 and ×5 the smallest, intraclade divergences and was equivalent to the divergence between *M. holotrachys* and *M. whitsoni* (Table I). Although there is no absolute divergence value that can be employed as a species criterion, relatively deep divergences among COI haplotypes within nominal species typically highlight cryptic fish species, rather than individual or population differentiation within species (Steinke *et al*., 2009*b*; Ward *et al*., 2009; Zemlak *et al*., 2009). Around 98% of marine fish species tested to date have been distinguished by COI barcodes, the few exceptions reflecting recent radiations, introgressive hybridization or misidentifications (Ward *et al*., 2009). Low COI sequence divergences (0·5–4·9%) were reported among 12 species of *Bathyraja* in the North Pacific Ocean (Spies *et al*., 2006) and a low COI sequence divergence (0·5%) was indicative of cryptic species of *Bathyraja* isolated on plateaux and shelf regions in the Southern Ocean (Smith *et al*., 2008). For some new provisional fish species, COI divergences have been supported with additional mitochondrial DNA (mtDNA) markers (Smith *et al*., 2008; Ward *et al*., 2008). In general, the evolutionary signal from mtDNA loci is robust and reflects patterns of population history and species limits that are rarely contradicted by analyses with nuclear markers (Zink & Barrowclough, 2008).

Specimens provisionally identified as *M. whitsoni* in the *Dissostictus* spp. longline fishery in the Ross Sea region were divided into two haplotype groups, based on

TABLE I. Nucleotide distances (Kimura 2-parameter *d*) within and between species of *Macrourus* (values in parentheses indicate lowest interspecific nucleotide distances)

		Between species		
	Within species	*M.* sp.	*M. whitsoni*	*M. holotrachys*
M. sp.	0·0002	—		
M. whitsoni	0·0000	0·006 (0·005)	—	
M. holotrachys	0·0000	0·008 (0·008)	0·006 (0·006)	—
M. carinatus	0·0006	0·020 (0·018)	0·018 (0·015)	0·020 (0·018)

the RFLP test that recognized a diagnostic base position 327 (TGG–TGA; Fig. 2). The blind test of three phenotypic characters of the same Ross Sea region specimens showed that individuals fell into one of two groups that corresponded with the DNA haplotypes: one with 9 (rarely 10) pelvic fin rays, a pale body colour and a single row of relatively long teeth, and a second group with 8 (rarely 7) pelvic fin rays, a dark body colour and ≥ 2 rows of finer teeth (Table II). There were no differences between sexes for the three selected characters. Scale row counts and number of pyloric caeca differed in ranges, but showed overlap and were not diagnostic. The pale morph corresponded with the one of the syntypes of *M. whitsoni* (British Museum of Natural History – BMNH 1912.7.1.87) examined (P. J. McMillan, unpubl. data), consequently specimens in this clade were recognized as *M. whitsoni*; specimens of the dark morph corresponded with the initial mixed species clade, which was recognized as *M*. sp. (see Fig. 2). Interestingly, Cohen *et al*. (1990) had commented on two colour morphs among their specimens of *M. whitsoni*, which were dominated by the dark morph. Iwamoto (1990) reported only the dark morph in alcohol preserved specimens, but which had the relatively wide range of pelvic fin ray counts (7–9) covering both *M. whitsoni* and *M*. sp. (Table II). Neither Cohen *et al*. (1990) nor Iwamoto (1990) had examined the teeth rows.

The COI, meristic and morphometric differences among the Ross Sea specimens initially identified as *M. whitsoni* provide strong evidence for two sympatric phylogenetic species, one of which is *M. whitsoni* and the other currently unrecognized. Specimens of both species have been captured over the same depth range, *M. whitsoni* (920–1655 m) and *M*. sp. (695–1671 m), and even on the same longline set in the *Dissostictus* spp. fishery. The shallow sequence divergences probably indicate either recent evolutionary divergence or slow nucleotide substitution at COI in this genus. The presence or absence of squamation on the underside of the head, in conjunction with characters from this study, counts of pelvic fins rays, tooth rows and colour (Table II), provide effective field characters for distinguishing *Macrourus* spp., where multiple species are likely to co-occur in by-catch in Southern Ocean fisheries.

Eight specimens of the North Atlantic Ocean *M. berglax* had also been barcoded (BOLD records SCAFB987-07, SCAFB480-07, CMNAF022-06, CMNAF014-06, SCFAC598-06, SCFAC273-06, SCAFB256-07 and GenBank EU148231.1); these barcodes were identical to the South Atlantic *M. holotrachys*. Both species are characterized by mostly lacking scales on the underside of the head (in *M. carinatus*, *M. whitsoni* and *M*. sp., swaths of scales are present on the underside of the head, except for an area in front of the mouth, Table II), but appear to differ in the number of pyloric caeca (*M. berglax* 19–20, *M. holotrachys* 8–16) and number of pelvic fin rays (*M. berglax* usually 8, *M. holotrachys* usually 9). The key provided by Cohen *et al*. (1990), however, was based on few specimens of *M. berglax*, and the authors commented that characters must be checked with an adequate specimen series (Cohen *et al*., 1990). Given that COI distinguishes other taxa in the genus *Macrourus*, it is possible that *M. berglax* and *M. holotrachys* represent populations of an anti-tropical species, but this needs to be tested with additional genetic markers and more detailed morphological analyses. Certainly there are many examples of fishes with anti-tropical distributions in temperate regions, *e.g. Sardinops* and *Engraulis* (Okazaki *et al*., 1996; Grant *et al*., 2005).

TABLE II. Key diagnostic characters for species of *Macrourus* from Cohen *et al.* (1990) and Iwamoto (1990), and from barcode identities: pelvic fin rays – common number (and rare); teeth rows in lower jaw (S, single row with well-formed teeth; M, 3–5 rows with fine teeth)

Species	Distribution	Depth range (m)	Pelvic fin ray	Colour	Teeth rows	Pyloric caeca	Underside head	References
M. berglax	Temperate to Arctic North Atlantic Ocean	100–1000	8 (7–9)	Grey, darker on ventral trunk; anal fin dark edged	n.d.	19 or 20	Almost or entirely naked	Cohen *et al.* (1990)
M. holotrachys	Patagonian slope, South Georgia	300–1200	9 (8)	Light to medium brown, greyish brown; fins darker	n.d.	8–16	Entirely naked or 1–3 scales	Cohen *et al.* (1990)
M. carinatus	Sub-Antarctic, temperate waters South America, South Africa, New Zealand	300–1100	8 (9)	Medium brown to straw; fins darker	n.d.	13–21	Scaled	Cohen *et al.* (1990)
M. whitsoni	Circumpolar Antarctic waters, Falkland shelf	400–3185	n.d.	Dark brown to swarthy; some much paler	n.d.	18–28	Scaled	Cohen *et al.* (1990)
M. whitsoni	Circumpolar Antarctic waters, Falkland shelf	400–3185	8 (7–9)	Dark brown to swarthy in alcohol	n.d.	n.d.	Scaled	Iwamoto (1990)
M. whitsoni ($n = 23$)	Ross Sea region	920–1655	9 (10)	Silvery-grey to mid-brown = pale morph	S	15–26	Scaled behind snout	This study
M. sp. ($n = 54$)	Ross Sea region	695–1671	8 (7)	Mid-brown to blackish-brown = dark morph	M	20–37	Scaled behind snout	This study

n, numbers examined for this study from Ross Sea region; n.d., no data.

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