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New tools (DNA barcoding), old hypothesis: the case of the taxonomic identity of the Argentine hakes (Actinopterygii: *Merluccius*)

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The present study evaluated the possible occurrence of cryptic species among Merluccidae from Argentina by examining sequences of cytochrome c oxidase subunit I (*coI*) mtDNA. This approach can discriminate *Merluccius hubbsi* and *Merluccius australis*; specimens with morphological diagnostic characters of *Merluccius patagonicus* formed a cohesive cluster with *M. hubbsi* specimens. BIN analysis confirmed the effectiveness of barcoding within a global context.

Key words: *coI*; fish taxonomy; Merlucciidae; Merluccius patagonicus; mitochondrial DNA.

The genus *Merluccius* has long been recognized as comprising two nominal species in Argentine waters, Argentine hake *Merluccius hubbsi* Marini 1933 and southern hake *Merluccius australis* (Hutton 1872) (Cousseau, 2010). Recently, Patagonian hake *Merluccius patagonicus* Lloris & Matallanas 2003 was described as a new species occurring in Argentina. According to Lloris & Matallanas (2003) *M. patagonicus* differs from *M. hubbsi* by a combination of morphometric, meristic and morphological characters. A comprehensive morphological study, however, found no evidence of the presence of *M. patagonicus* in Argentina, concluding that *M. patagonicus* is a junior synonym of *M. hubbsi* (Díaz de Astarloa *et al.*, 2011).

Merluccius spp. is one of the most heavily exploited groups of demersal fishes (Inada, 1981; Moyle & Cech, 1996; Lloris *et al.*, 2003). Despite their great commercial importance, taxonomic ambiguities still exists for the genus *Merluccius*, mainly because of the high intraspecific variation and low interspecific differences which make species discrimination challenging (Inada, 1981; Lloris *et al.*, 2003; Matallanas & Lloris, 2006). Accurate identification is required for sustainable exploitation of

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fishing resources (Lleonart *et al.*, 2006), as mislabelling may hide exploitation of endangered species (Marko *et al.*, 2004).

When morphology-based approaches alone cannot provide suitable identifications the practice of an integrative taxonomy (Dayrat, 2005; Will *et al.*, 2005; Padial *et al.*, 2010) that draws data from different sources is promising (Pires & Marinoni, 2010). One important point of this proposal is the integration of molecular and morphological data (Pires & Marinoni, 2010). DNA barcoding has been considered as an efficient aid to traditional taxonomy (Hebert & Gregory, 2005; Hajibabaei *et al.*, 2007; Ferri *et al.*, 2009), designed to facilitate fast and accurate species identification, from a short, standardized DNA sequence (Hebert *et al.*, 2003*a*, *b*; Miller, 2007). It is based on the premise that every species will most likely have a unique DNA barcode and that genetic variation between species exceeds that within species (Hebert *et al.*, 2003*a*, *b*; Luo *et al.*, 2011). As a consequence, DNA barcoding has the potential to aid taxonomic investigations by enhancing the discovery of new species and facilitating the identification of unknown specimens with complex morphology (Hebert *et al.*, 2003*a*), allowing the clarification of problems of synonymy (Pires & Marinoni, 2010) and recognition of cryptic species (De Broyer & Danis, 2011; Grant *et al.*, 2011).

The genetic relationship among species of *Merluccius* have been examined based on mitochondrial genes (Quinteiro *et al.*, 2000, 2001; Campo *et al.*, 2007; Roa-Varón & Ortí, 2009; Silva-Segundo *et al.*, 2011) and nuclear markers (Roldán *et al.*, 1999; Grant & Leslie, 2001; Campo *et al.*, 2009; Roa-Varón & Ortí, 2009). In most of the above mentioned studies, the existence of two major lineages of hake, American and Euro-African, previously suggested by Inada (1981) was confirmed. The phylogenetic relationships between Euro-African species are clearly defined, but those of the American group are much more complex and have not yet been convincingly resolved (Roldán *et al.*, 1999; Quinteiro *et al.*, 2000; Grant & Leslie, 2001; Campo *et al.*, 2007, 2009; Silva-Segundo *et al.*, 2011).

The aim of this study was to evaluate the possible occurrence of cryptic species among species of Merlucciidae that inhabit Argentina. To reach this objective, sequences of cytochrome c oxidase subunit I (*coI*) of individuals morphologically identified as *M. australis* or *M. hubbsi* were examined; from the latter, specimens having some diagnostic characters cited for *M. patagonicus* were selected. Sequences were also compared with available barcode data from sequences of *Merluccius* species from other regions to explore the effectiveness of DNA barcoding to discriminate these species.

Specimens of *Merluccius* were collected from the Argentine Sea from 36°45′ to 55°04′ S, on board the R.V. *Dr. Eduardo L. Holmberg* of the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP) and by commercial fishing fleets of Ushuaia port. Identifications were conducted based on Inada (1981) and Lloris & Matallanas (2003). A total of 14 individuals were selected from 229 individuals for genetic analysis, six *M. australis* and eight *M. hubbsi* [Barcode of Life Data (BOLD) and GenBank accession numbers are given in Table I]. Specimens of *M. hubbsi* with almost one of the morphological diagnostic characters mentioned by Lloris & Matallanas (2003) for describing *M. patagonicus* as a new species for Argentinean waters (lower jaw longer than that of *M. hubbsi*; opercular membrane oblique; upper profile of the head with a depression; 123–126 lateral line scales; sagitta otolith with excisura; hyomandibula intermuscular and pterygoid processes unequally longer; urohyal anterodorsal process inclination angle acute) were selected. Of the latter, six specimens presented two to

TABLE I. List of Argentinean specimens of *Merluccius* barcoded. Barcode of Life Data Systems (BOLD) specimen numbers given, along with GenBank accession numbers and geographic locality

Species	BOLD			GenBank Acc. Number	Collection sites
	Museum ID	Sample ID	Process ID		
M. australis	UNMDP 0108	UNMDP-T 0108	FARGB 868–12	KM255101	off southern Patagonia
	UNMDP 0112	UNMDP-T 0112	FARGB 872–12	KM255105	off southern Patagonia
	UNMDP 0120	UNMDP-T 0120	FARGB 880–12	KM255102	off southern Patagonia
	UNMDP 0122	UNMDP-T 0122	FARGB 882–12	KM255106	off southern Patagonia
	UNMDP 0124	UNMDP-T 0124	FARGB 884–12	KM255103	off southern Patagonia
	UNMDP 0125	UNMDP-T 0125	FARGB 885–12	KM255097	off southern Patagonia
M. hubbsi	UNMDP 0776	INIDEP-T 0776	FARG 718–09	HM421964	off southern Patagonia
	UNMDP 0777	INIDEP-T 0777	FARG 719–09	HM421965	off southern Patagonia
	UNMDP 0778	INIDEP-T 0778	FARG 720–09	HM421966	off southern Patagonia
	UNMDP 0779	INIDEP-T 0779	FARG 721–09	HM421967	off southern Patagonia
	UNMDP 0780	INIDEP-T 0780	FARG 722–09	HM421968	off southern Patagonia
	UNMDP 0781	INIDEP-T 0781	FARG 723–09	HM421969	off Buenos Aires Province
	UNMDP 0782	INIDEP-T 0782	FARG 724–09	HM421970	off Buenos Aires Province
	UNMDP 0783	INIDEP-T 0783	FARG 725–9	HM421971	off Buenos Aires Province

six of those characteristics specified in Table II. The specimens were labelled and their photographs were retained as e-vouchers (Monk & Baker, 2001).

A sample of white muscle tissue was excised from each specimen and preserved in 100% ethanol at −20 °C. DNA extraction, polymerase chain reaction (PCR) and sequencing of the *coI* gene were performed according to standard DNA barcoding protocols (Ivanova *et al.*, 2006). Amplification of the 5′ barcode region of *coI* was attempted using C_FishF1t1/C_FishR1t1 primer cocktails developed for fishes (Ward *et al.*, 2005; Ivanova *et al.*, 2007). Extraction and amplification were achieved at the International Barcode of Life Argentinean reference Barcode Laboratory of CONICET at the Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina. Sequencing was accomplished in the Canadian Centre for DNA Barcoding (CCDB) in Ontario, Canada. All sequence assemblies, as well as electropherogram (trace) files, primer sequences and specimen data were deposited in Southwestern Atlantic Hakes (code SAH) project at www.boldsystems.org (BOLD; Ratnasingham & Hebert, 2007). This includes digital images of the morphological voucher specimens, sex, total and standard lengths as well as GPS co-ordinates for all collection localities.

The sequences obtained in this work were compared with publicly available (BOLD, www.boldsystems.org) *coI* sequences (>500 bp) of *Merluccius* conspecific specimens (14 specimens of *M. hubbsi* from Argentina and Brazil waters and seven individuals of *M. australis* from Argentina and New Zealand waters) (Table S1, Supporting Information). The *coI* sequence of one specimen of Atlantic cod *Gadus morhua* L. 1758 (GenBank Acc. No. KC015378) was chosen from McCusker *et al.* (2012), extracted from BOLD and added to the analysis as an outgroup (Table S1).

DNA sequences were aligned with SeqScape 2.1.1 software (Applied Biosystems, Inc.; www.lifetechnologies.com) and posterior analyses were performed with MEGA 5.05 software (Tamura *et al.*, 2011). Sequence divergences were calculated using the Kimura-2-parameter (K2P) distance (gamma parameter set at 1) model (Kimura, 1980), as it is commonly used for describing differences among species in DNA barcoding studies. Additionally, the sequences divergences were analysed with a Tamura-Nei distance model (Tamura & Nei, 1993), for comparison purposes, since it is the model most widely used in *Merluccius* DNA studies. A neighbour-joining tree of K2P distances (K2P–NJ) (Saitou & Nei, 1987) was created, to provide a graphic representation of divergences between species. Robustness of the tree was tested using bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

The barcode index number (BIN) was employed in order to explore the DNA barcoding effectiveness not only to discriminate worldwide *Merluccius* spp., but also to verify species identifications (Ratnasingham & Hebert, 2013). Sequences were automatically assigned to a BIN on the BOLD Workbench 3.6 (www.boldsystems.org; analyses performed on 21 Jul 2014). *coI* sequences were obtained from the 14 targeted specimens. Mean sequence length was 624 bp (range: 501–652 bp). No insertions, deletions or stop codons were found (supporting that all of them constitute functional mitochondrial *coI* sequences). Moreover, all the amplified sequences were > 500 bp (86% were >600 bp).

The specimens of *M. hubbsi* examined in this study formed a cohesive cluster which substantially diverged by a pronounced congeneric divergence (K2P: 5·1%; Tamura-Nei: 5·2%) from that of *M. australis* (Fig. 1). Previous DNA mitochondrial studies [cytochrome b (*cytb*), control region and 16S rDNA] also pointed out high genetic distances between *M. hubbsi* and *M. australis* (Tamura-Nei: 7·8% in

TABLE II. Diagnostic characters according to Lloris & Matallanas (2003) of *Merluccius hubbsi* (MH) and *Merluccius patagonicus* (MP) in the studied specimens

Specimen (Process ID)	LSL (% HL)	Opercular membrane		Upper profile of the head		Lateral line scales		Sagitta otolith excisura		Hyomandibula intermuscular and pterygoid processes lengths		Urohyal anterodorsal process inclination angle	
		O	Pa	D	S	123–126	133–144	P	A	UL	EL	A	O/R
FARG 718-09	MP		MH		MH		MH	MP			MH		MH
FARG 719-09	MP		MH		MH		MH	MP		MP			MH
FARG 720-09	MP		MH		MH	MP		MP		MP			MH
FARG 721-09	MH		MH		MH	MP		MP		MP			MH
FARG 722-09	MP		MH	MP		MP		MP		MP		MP	
FARG 725-09	MP	MP		MP		MP			MH	MP		MP	

LSL, lower snout length; HL, head length; O, oblique; Pa, parallel; D, with depression; S, straight; P, present; A, absent; UL, unequally longer; EL, equally longer; A, acute; O/R, obtuse/right.

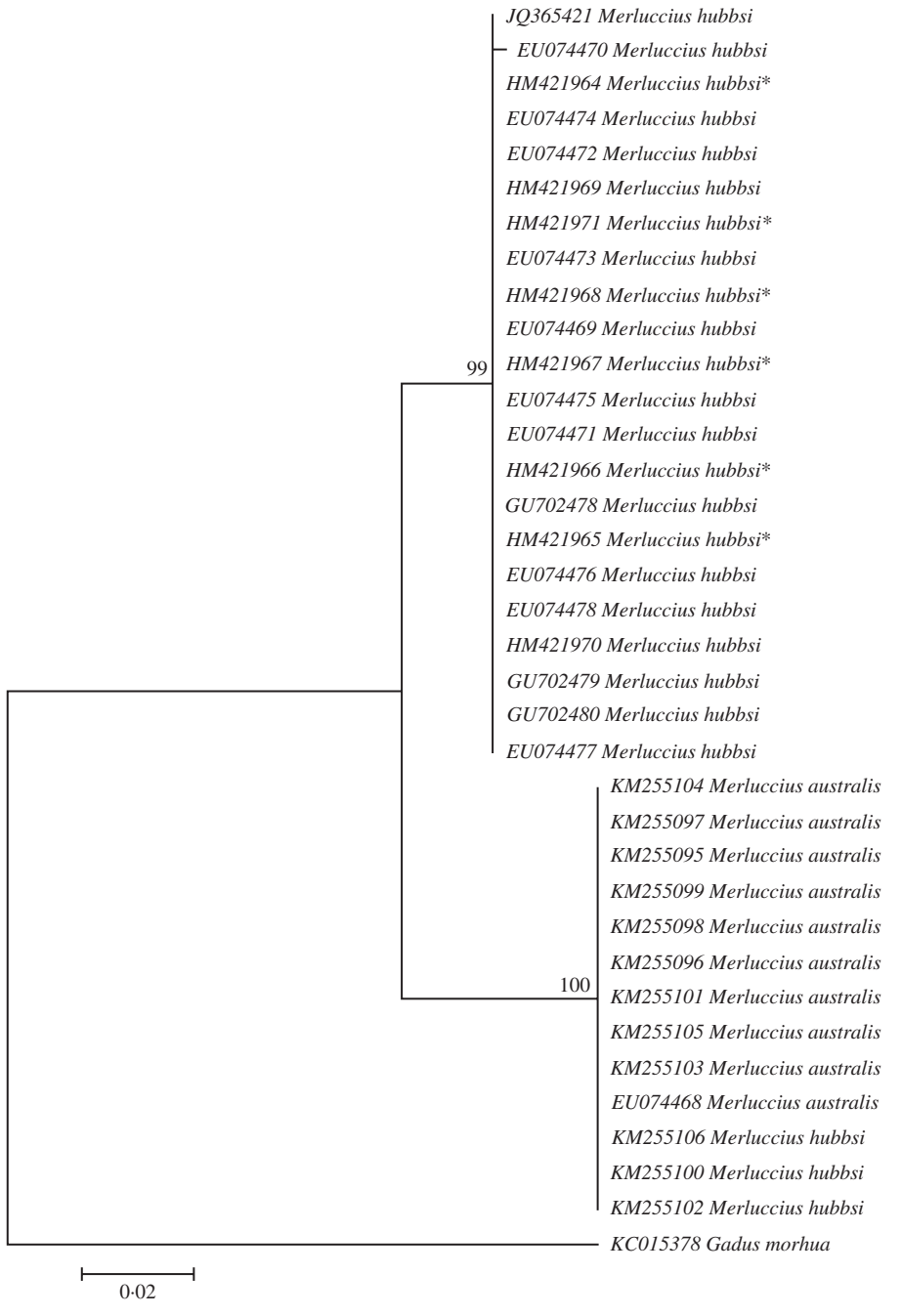

FIG. 1. Consensus neighbour-joining tree of Kimura-2-parameter distances of cytochrome c oxidase subunit I (*coI*) sequences of *Merluccius hubbsi* and *Merluccius australis* from Argentina and those barcode compliant records publicly available on Barcode of Life Data Systems (BOLD). *Gadus morhua* was used as an outgroup. Numbers at branches represent bootstrap values (only values >90 are given). Code numbers represent GenBank Accession Numbers. *, specimens of *M. hubbsi* with diagnostic characters described for *M. patagonicus*.

Quinteiro *et al.* (2000); K2P: 4·2% in Campo *et al.* (2007); Tamura-Nei: 1·7 to 4·8% in Silva-Segundo *et al.* (2011)).

All the specimens of *M. australis* exhibited a unique haplotype with null (0%) K2P and Tamura-Nei distances. The K2P–NJ distance tree showed that these specimens grouped together and substantially diverged from those of *M. hubbsi* (Fig. 1). Also the eight sequences of *M. hubbsi* were identical. The NJ tree of *coI* sequences divergences (K2P) (Fig. 1) indicated that these specimens formed a cohesive cluster with the *coI* sequences of the same species obtained from the publically accessible section of BOLD. The divergence between all the studied sequences was very low, averaging 0·02% with both distance models (K2P and Tamura-Nei). Comprehensive molecular analysis of *Merluccius* found one (Quinteiro *et al.*, 2000) or two haplotypes (Silva-Segundo *et al.*, 2011) in *M. hubbsi*, separated by a relatively low intraspecific distance (Tamura-Nei: 0·2%). In the present study, three closely related haplotypes were found, two of them from specimens from BOLD (GenBank Acc. No. EU074470 and EU074473), differing by one nucleotide each and other haplotype shared by the remaining specimens. Accordingly, low divergences have been found between specimens in both species by means of other DNA mitochondrial sequences analyses [Quinteiro *et al.*, 2000 (Tamura-Nei: 0·5%); Silva-Segundo *et al.*, 2011 (Tamura-Nei: 0·2%)] and trough nuclear markers (Roldán *et al.*, 1999; Grant & Leslie, 2001; Roldán & Pla, 2001).

No intraspecific sequence variation between the specimens that showed morphological features of *M. patagonicus* and the majority of the specimens of *M. hubbsi* examined here and extracted from BOLD were obtained (Fig. 1). According to Hebert *et al.* (2004) genetically divergent specimens could be flagged as putative species if they displayed ten-fold intraspecific differentiation from the mean the group under study. Therefore the specimens of *M. hubbsi* we examined would not qualify for distinct species status.

Any assessment on the reliability of DNA barcoding for assigning individuals to species should include comparisons with sister species. Such an assessment would require that all members of a genus be examined, and that taxa be included from more than one geographic region (Moritz & Cicero, 2004). The analysis of DNA barcode sequences with varying techniques for cluster recognition provides an efficient approach for recognizing putative species (operational taxonomic units, OTU) (Kekkonen & Hebert, 2014). The BIN system is a persistent registry for animal OTUs recognized through sequence variation in the *coI* DNA barcode region (Ratnasingham & Hebert, 2013). Since OTUs show high concordance with species, this system can be used to verify species identifications (Ratnasingham & Hebert, 2013). The BOLD published records of the 12 currently recognized species of *Merluccius* (Inada, 1981; Lloris *et al.*, 2003) represent nine barcode clusters or BINs, indicating a high degree of accuracy in species identification belonging to this genus. Seven of the OTUs were taxonomically concordant representing distinct species, while two of them formed multispecies complexes; Panama hake *M. angustimanus* Garman, 1899 ($n = 1$), South Pacific hake *M. gayi* (Guichenot 1848) ($n = 7$) and *M. productus* (Ayres 185) ($n = 39$) grouped together in one BIN (BOLD:AAB5094) and also one specimen of the Senegalese hake *M. senegalensis* Cadenat 1950 and 76 individuals of the European hake *M. merluccius* (L. 1758) clustered together (BOLD:AAA8613). Adequate sample sizes are critical for any effort to delineate species (Kekkonen & Hebert, 2014); the discordant BINs (BOLD:AAB5094 and BOLD:AAA8613) clustered species that were represented by

only one individual with species with >30 or 70 specimens. Furthermore, the BIN system is built on prior studies that have established that most animal species show <2% intraspecific variation in *coI*, but >4% divergence from their nearest neighbour (Ratnasingham & Hebert, 2013). The within-BIN maximum K2P distances were <2% for the present study (1·57 for BOLD:AAB5094 and 1·16 for BOLD:AAA8613), agreeing with previous studies that revealed low divergences between *M. angustimanus*, *M. gayi* and *M. productus* (Silva-Segundo *et al.*, 2011) and between *M. merluccius* and *M. senegalensis* (Quinteiro *et al.*, 2001; Campo *et al.*, 2007). On the other hand, the BIN analysis recognized two taxonomic units in the sequences studied here, which agree with the current taxonomic classification. *Merluccius australis* specimens were assigned to the same BIN (BOLD:AAB2174) as specimens of *M. hubbsi*, which also included those with *M. patagonicus* characters in a unique BIN (BOLD:AAM2029).

Present analyses strongly suggest that only two species of *Merluccius* inhabit Argentine waters: *M. hubbsi* and *M. australis*, since no evidence of cryptic diversity was found. Accordingly, it is recommended that *Merluccius patagonicus* remain as junior synonym of *M. hubbsi*. *coI* barcoding can be taken up as a complementary approach for resolving unambiguous identification of species of *Merluccius* not only from Argentinean waters, but also from around the world, with applications for the management and conservation of these important fisheries resources.

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Supporting Information

Supporting Information may be found in the online version of this paper:

Table S1. Specimens information from Barcode of Life Data Systems (BOLD) of publicly available compliant sequences of *Merluccius* spp., indicating museum, process and sample IDs, GenBank accession numbers, and location of capture. Additional details are available on the BOLD website.

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