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[Note]

Mislabeling of Two Commercial North American Hake Species Suggests Underreported Exploitation of Offshore Hake

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Abstract.—Mislabeling of North American merlucciid hakes in stock surveys and commercial market samples was detected by employing nuclear 5S ribosomal DNA (rDNA) and mitochondrial cytochrome b variation as molecular markers. Results showed that offshore hake *Merluccius albidus* is sold in European markets but is labeled as the morphologically similar silver hake *M. bilinearis*, which is the target species of the fishery. This suggests that offshore hake may be inadvertently included within silver hake landings, as the two species overlap in the southern area of silver hake distribution (approximately 41°–35°N latitude near North American coasts). An inexpensive and technically easy technique based on polymerase chain reaction (PCR) amplification of a fragment of 5S rDNA and visualization of PCR products in agarose gels is recommended for routine species assignation in landings for purposes of exploitation estimates and for authentication of commercial hake species.

The silver hake *Merluccius bilinearis* and offshore hake *M. albidus* are members of the large, globally distributed genus *Merluccius* or true hakes (Alheit and Pitcher 1995). Silver hake, or whiting, are distributed on the continental shelf of the northwest Atlantic Ocean ranging from Newfoundland to South Carolina (Helser et al. 1995; Morse et al. 1999). Offshore hake are also distributed over the continental shelf and slope of the northwest Atlantic Ocean, but extend farther south to the Caribbean Sea, the Gulf of Mexico, and coastal countries of Surinam and French Guyana (Chang et al. 1999). The two species are similar physically and can only be distinguished by differences in the number of gill rakers and lateral line scales (Ginsburg 1954; Markle 1982; Cohen et al. 1990). Because of their physical similarity and range overlap, only recent landings (since 1994) differentiate the two species; therefore, historical collections may have included both species.

More than 1,416,000 metric tons of silver hake have been landed since 1980 (data source: www.fao.org/fishery/statistics/en). Offshore hake are generally caught as bycatch in silver hake fisheries, and reported landings since 1994 represent only 0.07% of the total catch of both species. Export to Europe is the main commercial outcome for these two species. Spain is Europe's top hake market (Alheit and Pitcher 1995) and the main importer of North American hake. Hake is the most popular and expensive fresh fish in Spain; increasing U.S. fish exports (Escudero 2002) include silver hake, which is generally sold fresh in whole pieces.

European regulations require labeling of commercial fish samples with product identification to species level and inclusion of both commercial and scientific names on the label (European Directives CE 104/2000 and CE 2065/2001; Spanish National Directive RD 121/2004). A tool for species validation and species certification is needed for hakes with overlapping distributions that can be caught together in commercial fisheries, as is also the case for other members of this genus, like the cape hake *M. capensis* and deepwater hake *M. paradoxus* from the African coast (Alheit and Pitcher 1995). Because of their morphological similarity, silver hake and offshore hake can be easily misidentified in

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landings, and thus the labeling of commercial products is not accurate at present. Moreover, exploitation assessments are not possible unless all catches are correctly identified.

Several methods have been described for species-specific identification of hakes. One method is based on protein analysis employing MALDI-TOF methodology (Carrera et al. 2006). Other methods employ DNA as the target molecule, following generally at least three steps: (1) DNA extraction, (2) polymerase chain reaction (PCR) amplification of target sequences, and (3) sequence analysis for determining the species, which can be carried out by direct sequencing (Pepe et al. 2005, 2007), restriction digestion (Quinteiro et al. 2001; Pérez et al. 2005), visualization of the PCR product in acrylamide (Castillo et al. 2003; Perez and Garcia-Vazquez 2004) or in nondenaturing polyacrylamide gels (Chapela et al. 2007), or by capillary electrophoresis (Machado-Schiaffino et al. 2008). The weaknesses and relative advantages of these methods, all of them 100% accurate, are mostly related to costs and requirements of technical expertise and equipment. To date, the simplest, quickest, and easiest method for species identification in molecular biology with minimal requirements in technical expertise and price is simple PCR amplification followed by visualization of the amplified product on agarose gels. This method is so easy that it is employed in undergraduate teaching laboratories as an example of applied molecular biology (e.g., Moran and Garcia-Vazquez 2006). The utility of PCR for species-specific identification has never been tested for North American hake species. The objective of the present study was to apply PCR plus agarose gel methodology to develop a species-specific molecular marker for inexpensive, easy, fast, and 100% reliable identification of the two North American Atlantic hake species, silver hake and offshore hake. Species-specificity was confirmed by employing a different marker (mitochondrial DNA sequences). The need for its application in fish markets was tested by analyzing (1) catches obtained in extensive hake surveys in U.S. waters and (2) commercial products sold in various localities of Spain, the main European importer of hake.

Methods

Study Site and Sampling

Reference samples.—Thirty adult individuals each of silver hake and offshore hake were caught at random from different distribution areas in the context of the National Marine Fisheries Service (NMFS) Northeast Fisheries Science Center (NEFSC) bottom trawl survey (Azarovitz 1981) of 2004. The specimens were morphologically examined in detail in situ. The species was determined based on morphological characteristics, mainly by the number of gill rakers (Chang et al. 1999). A sample of tissue (approximately 3 g of gill tissue) was taken and preserved in 95% ethanol for genetic analyses.

Survey samples.—A total of 291 samples were collected from the NMFS-NEFSC bottom trawl survey. Fish were morphologically identified by visual examination as 224 offshore hake and 67 silver hake. They were weighed and measured, and a small sample of tissue (1 g of gill or muscle) was preserved in 95% ethanol. In U.S. waters, silver hake are managed as two separate stocks (northern and southern stocks); the boundary dividing the stocks is located between Nantucket Shoals and the northern edge of Georges Bank (Lock and Packer 2004). A total of 51 sampling stations equally distributed across the northern (27 stations) and southern (24 stations) distribution areas were considered to provide extensive spatial coverage of each species within North American waters. The samples examined in this study were collected in spring, fall, and winter surveys in 2004 and 2005.

Market samples.—Commercial samples ($n = 164$ commercial lots, each lot being a package or box containing a number of specimens) were purchased in different Spanish markets from five regions in the Spanish Peninsula (Asturias, Basque Country, Alicante, Galicia, and Madrid). All were sold as fresh hake. The species name marked on the label was noted regardless of whether it contained the scientific name or not. The geographic origin of the product (country of origin or catch area) was also recorded. Three to twelve individuals per commercial lot were sampled (approximately 3-mm^3 muscle tissue biopsy stored in pure ethanol) and genetically analyzed.

Genetic Analysis

DNA extraction and analysis.—The DNA was extracted from tissue samples (approximately 1 mm^3 per individual) following a Chelex-based protocol (Estoup et al. 1996). A species-specific marker for easy and inexpensive species identification, a region of 5S ribosomal DNA (rDNA) comprising partial coding sequence and complete nontranscribed spacer (NTS) was amplified using the primers 5S-A (5′-TACGCCC-GATCTCGTCCGATC-3′) and 5S-B (5′-CAGGCT-GGTATGGCCGTAAGC-3′) as described by Pendas et al. (1995). Polymerase chain reactions were performed in a GeneAmp PCR System 2400 (Perkin Elmer Cetus). Each PCR (20-μL total volume) contained 0.5 units (U) of Taq Polymerase (Promega, Madison, Wisconsin), Promega Buffer 1×, 2.5-mM $MgCl_2$, 250 μM of each deoxynucleotide triphosphate (dNTP), 1 μM of each primer, and 2 μL of sample

DNA (approximately 2 ng of DNA). Reaction conditions were as follows: first an initial step of denaturation at 95°C for 5 min; 35 cycles consisting of denaturation at 95°C for 20 s; annealing at 65°C for 20 s and extension at 72°C for 30 s; and a final extension at 72°C for 20 min. After the amplification, the PCR products were run through agarose gels of 2.5% density (80 V) for 20 min and visualized by staining with 3 μL of ethidium bromide (10 mg/mL). The size of the amplified fragments was estimated by comparison with a standard 100-base-pair (bp) DNA marker (Promega).

To obtain the complete sequence of the 5S rDNA region, the primers 5S-C (5′-AAGCTTACAGCACCTGGTATT-3′) and 5S-MD (5′-TTCAACATGGGCTCCGACGGA-3′; Perez and Garcia-Vazquez 2004) were employed. The whole region includes the complete coding sequence and the NTS. The PCRs were carried out on reaction mixtures containing approximately 5 ng of extracted hake DNA template, 10-mM tris-HCl (pH 8.8), 2.5-mM $MgCl_2$, 50-mM KCl, 0.1% Triton X-100, 1 μM of each primer, 1 U of DNA Taq Polymerase (Promega), and 250 μM of each dNTP in a final volume of 40 μL. The PCR was performed using the GeneAmp PCR System 2400 (Perkin Elmer Cetus) with the following conditions: an initial denaturing step at 95°C for 5 min; 35 cycles of denaturing at 95°C for 20 s; annealing for 20 s at 65°C and an extension at 72°C for 30 s; and a final extension at 72°C for 20 min. The PCR products were visualized in 50-mL 2.5% agarose gels with 3 μL of 10-mg/mL ethidium bromide solution. Stained bands were excised from the gel, and DNA was purified with a Wizard SV Gel and PCR Clean-Up System (Promega) before sequencing. Automated fluorescence sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with the BigDye 3.1 Terminator system at the DNA Sequencing Unit, University of Oviedo, Spain. Both strands (forward and reverse) of each DNA fragment were sequenced.

A second, mitochondrial genetic marker was employed to validate the nuclear marker in the 30 reference samples from each species, as well as for double-checking the species in mislabeled commercial or survey samples. A 230-bp sequence of mitochondrial cytochrome b was PCR amplified with the primers H_{15149} and L_{14841} described by Kocher et al. (1989) under the PCR conditions described in Campo et al. (2007).

Sequence editing.—The sequences obtained were edited using BioEdit Sequence Alignment Editor software (Hall 1999). Sequences were aligned with the Clustal W application (Thompson et al. 1994) included in BioEdit.

Results

Species-Specific Markers in Reference Samples

In the 60 individuals from reference samples (examined in detail for morphological characteristics), two main fragments (plus a few faint secondary bands) were obtained from PCR amplification of the 5S ribosomal RNA genes employing the primers 5S-A and 5S-B designed by Pendas et al. (1995). The main bands were approximately 593 and 383 bp long for offshore hake and 703 and 586 bp long for silver hake (Figure 1). Exact band sizes were calculated based on the combination of both DNA strand sequences (direct and reverse) and the position of the primers.

Complete sequences of the 5S rDNA region are available at the GenBank database (www.ncbi.nlm.nih.gov/; accession numbers EU919180 and EU919181 for offshore hake; EU919182 and EU919183 for silver hake).

The sequences obtained for each species contained complete 120-bp coding regions plus the NTS. Differences in sequence length were due to insertions–deletions in the NTS. The coding regions were identical, indicating that they correspond to functional genes and not pseudogenes. The existence of more than one functional locus for this gene is common in fish species (Martins and Galetti 2001).

The reference individuals exhibiting the 5S RNA amplification patterns described above for each species were analyzed for a second marker, the mitochondrial cytochrome b. Cytochrome b polymorphisms have been previously published for these species (Campo et al. 2007) and can be found at GenBank (EF362891-3 and EF362884-6 for offshore hake and silver hake, respectively). The 30 individual offshore hake exhibited a 5S rDNA pattern with 593- and 383-bp-long bands and also contained cytochrome b sequences identical to those previously described for this species (EF362891-3). All 30 silver hake samples had the typical pattern of 703- and 586-bp-long 5S rDNA amplification fragments and exhibited cytochrome b sequences identical to one of the cytochrome b haplotypes previously described for the species (EF362884-6). Therefore, the species identification was confirmed with one nuclear marker and one mitochondrial marker, and the 5S rDNA marker can be considered validated as a tool for differentiating offshore hake and silver hake.

Genetic Identification of Trawl Survey Samples

In the trawl survey (Table 1), a total of 145 and 146 individuals were analyzed for the northern and the southern areas, respectively. In the northern area, all the individuals sampled were morphologically identi-

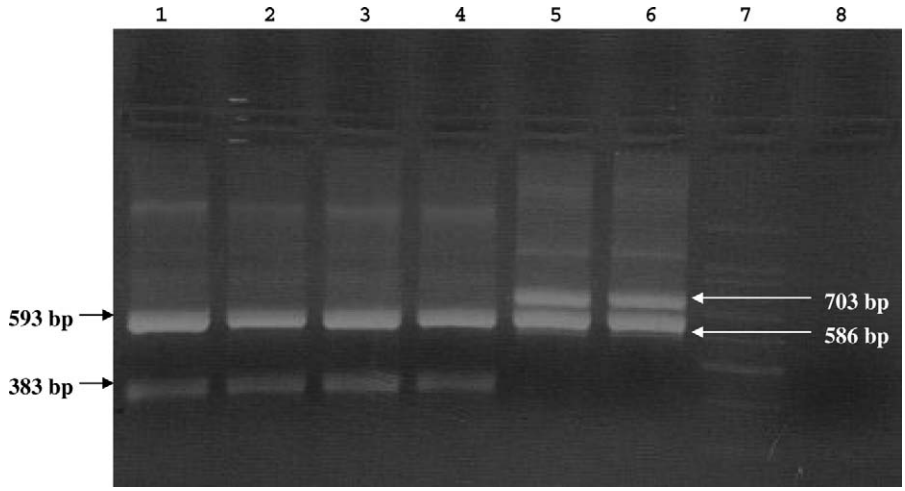

FIGURE 1.—Agarose gel showing polymerase chain reaction amplification products of offshore hake (lanes 1–4) and silver hake (lanes 5–6). Lane 7 shows a 100-base-pair (bp) marker (Promega Bench Top DNA Ladder), and lane 8 is the negative control. Main amplification fragment sizes are marked with arrows.

fied as silver hake. The genetic markers confirmed the visual species determination, as all of the fish exhibited genetic features corresponding to that species. In the southern area, 79 individuals were morphologically classified as silver hake and 67 as offshore hake. Genetic analyses showed that five individuals were mislabeled. One individual (B71-8) morphologically identified as silver hake exhibited a 5S rDNA pattern typical of offshore hake, and four individuals (A90-1, A90-14, A105-2, and A206-1) morphologically classified as offshore hake were identified as silver hake based on the 5S rDNA amplification fragment sizes. Genetic species misidentification was confirmed by the cytochrome b gene sequence, which in the five cases corresponded to the species identified with the 5S rDNA. In total, southern stock samples yielded 3.4% misidentification (Table 1), with 1.27% and 5.97% mislabeling for silver hake and offshore hake, respectively.

Genetic Identification of Market Samples

Many labels of the 164 commercial lots purchased in Spain were not accurate (Table 2). Seven lots did not follow the European and national legislation about labeling, being generically marked as "American hakes" with "Northwest Atlantic" as geographical origin. The genetic marker evidenced that all of them contained silver hake. The remaining 157 lots, which were labeled with the scientific name, had all been imported as silver hake, as stated on the labels. The 75 lots with a geographical origin given as Canada were genetically silver hake, as confirmed from the 5S rDNA PCR pattern. However, a proportion as high as 12.2% of hakes caught in Massachusetts waters (from the commercial label) exhibited a PCR pattern corresponding to offshore hake, indicating mislabeling (Table 2).

Discussion

Offshore hake has rarely been considered as a target for species identification employing molecular markers because its commercial value is considered marginal; the main hake species caught in North American waters is the silver hake. The results of this study, however, suggest that offshore hake is being exploited together with silver hake, and their catches are probably

TABLE 1.—Morphological and genetic identification of offshore hake and silver hake in northern and southern U.S. sampling areas. Misidentification is the number (and percent) of individuals erroneously assigned to a species using morphological traits.

	Morphological identification		Genetic identification		Misidentification (%)		
Area in U.S. waters	Silver hake	Offshore hake	Silver hake	Offshore hake	Silver hake	Offshore hake	Total
Northern	145	0	145	0	0	0	0
Southern	79	67	82	64	1 (1.27%)	4 (5.97%)	5 (3.4%)

TABLE 2.—Genetic analysis of commercial North American hake samples sold in Spanish markets. Geographic origin and species name are reported as marked on the label (N = number of hake lots analyzed; genetic identification was by 5S ribosomal DNA amplification; mislabelling = proportion of sample lots mislabeled or incompletely labeled).

			Genetic identification		
Origin on label	N	Species on label	Silver hake	Offshore hake	Mislabeling
Canada	75	Silver hake	75	0	0
Massachusetts	82	Silver hake	72	10	12.2%
Northwest Atlantic	7	American hake	7	0	Incomplete labeling

underreported because fish of both species are identified as silver hake. Official data report that offshore hake represent less than 1 in 1,000 of the hake caught in North American Atlantic waters; however, at least in the southern area where the two species overlap, misidentification seems to occur. In commercial products sold in Spain, the most important market for hakes, our study shows that a proportion of hake imported from North American countries as silver hake was actually offshore hake. Based on our survey of commercial products (Table 2), unreported offshore hake would be 12% of the total imports from Massachusetts. Misreports are probably inadvertent because the two species are morphologically similar and offshore hake are relatively rare; even in scientific surveys, a certain level of visual misidentification has been detected (e.g., 3% in our results; Table 1).

Mislabeling is an important problem in fisheries management. It not only defrauds consumers but could also adversely affect estimates of stock size if it influences the reporting of catch data used in fisheries management (Marko et al. 2004). If the level of unreported offshore hake detected in silver hake commercial lots in Spain (12.2%) was similar in all southern stock catches, more than 11,000 metric tons of offshore hake could have been unknowingly caught during the last 10 years. It is difficult to quantify the potential impact of such inadvertent harvest on the offshore hake stock because its status is not assessed by NMFS (Chang et al. 1999). However, our results highlight the need for the implementation of a method for species verification of hake catches as there is a serious risk of unknown and uncontrolled exploitation of offshore hake in North American waters.

With respect to the technique applied in this study, it is the simplest and cheapest in molecular biology because its requirements (in terms of equipment, cost, and technical expertise) are minimal. It is simpler than any other technique described in the few articles about molecular methodologies for hake identification that included offshore hake. Restriction enzyme-based techniques (Pérez et al. 2005) take longer and involve one more step because they require additional time for enzymatic digestion. Another method that can distinguish the two species includes direct electrophoresis of PCR amplification products on nondenaturing polyacrylamide gels (Chapela et al. 2007), but this method requires several hours more for separating single strands of DNA than that required for separating PCR products in agarose gels (<20 min). Methods based on real-time PCR are now available for identification of fish species (e.g., Lopez and Pardo 2005; Trotta et al. 2005) and could be easily developed for all hake species. Real-time PCR methods are short and easy, although their disadvantage is the cost of real-time platforms required for genotyping. Finally, mitochondrial single-nucleotide polymorphisms based methodology (Machado-Schiaffino et al. 2008) is fast and technically easy, but it also needs expensive equipment such as an ABI PRISM 3100 Genetic Analyzer and adequate software like GeneScan (Applied Biosystems) to determine amplicon sizes and their fluorescent-label identity by capillary electrophoresis. The method described in the present study, however, is reproducible even in the most modest laboratory because all the equipment needed is inexpensive (one thermocycler and power supply for electrophoresis plus an ultraviolet illuminator for reading the gels) and minimal expertise is required for the entire process. About 2 h are required for manual DNA extraction, an additional 2 h are needed for PCR amplification, and no more than 20 min are required for visualization in agarose gels. This time could be shortened further if the process is robotized for routine analysis of commercial samples. Even with the manual process we described, commercial products do not need to be immobilized for more than one working day to obtain 100% reliable species identification. This is about the same time necessary for sanitary inspection, chemical analyses of trace pollutants, and other necessary controls for exported seafood. The described protocol also enables the separation of other hakes (Perez and Garcia-Vazquez 2004) and unrelated gadid species (Moran and Garcia-Vazquez 2006) and could be employed for routine quality

controls when equipment for real-time PCR or proteomics is not available.

In conclusion, with a cheap, technically easy, and quick genetic marker, we have revealed misidentification of the two North American Atlantic species of *Merluccius*, offshore hake and silver hake, in both fisheries-independent surveys and commercial lots exported to Spain. Possible underreported exploitation of offshore hake could be deduced from these results. Implementation of molecular methodology for species identification is recommended for better management of merlucciid hake fisheries and trade in North America.

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