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Detection of Mislabeling in Hake Seafood Employing mtSNPs-Based Methodology with Identification of Eleven Hake Species of the Genus *Merluccius*

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Species-specific DNA-based tags are valuable tools for the management of both fisheries and commercial fish products. Eleven hake species of the genus *Merluccius* have been identified employing mtSNPs-based methodology. The method is highly reproducible, fast, and technically easy. It is a reliable tool, allowing for routine analysis of commercial seafood. It can be applied by nonexperts in genetics because both laboratory handling and interpretation of results are easy and direct. The convenience of routine surveys in fish markets has been clearly established with a survey of commercial hake batches imported in south Europe. A total of 40 commercial processed hake were analyzed in this study. More than 20% of mislabeling has been detected.

KEYWORDS: Hake; *Merluccius*; species identification; commercial samples; control region; mitochondrial single nucleotide polymorphism; SNaPshot

INTRODUCTION

Mechanisms for certification of the species present in commercial food constitute an added value in the food market. Clear and reliable species identification is crucial for food allergy prevention, for example. The same mechanisms can be employed for detection of food mislabeling that can reduce the commercial value of a product. They can be applied also in fisheries management (e.g., for certification of the species in landings). Among the various methods employed for species identification and certification, genetic markers are of highest relevance. Many species-specific DNA-based tags have been developed to species identification in seafood, from simple techniques of PCR-fragment size determination (*1, 2*) to PCR-RFLP (restriction fragment length polymorphism (*3*)) to direct sequencing of target DNA fragments (*4*).

Hakes are among the most heavily fished demersal finfish groups (*5*). There are 12 species morphologically described for the genus *Merluccius* (*6*), distributed in Atlantic and Mediterranean European waters (*Merluccius merluccius*), Atlantic African coasts (going southward they are *M. senegalensis*, *M. polli*, *M. capensis*, and *M. paradoxus*), along Atlantic American coasts except in equatorial waters (*M. bilinearis* and *M. albidus* in the north, *M. hubbsi* and *M. australis* in the south entering the southern Pacific around the Patagonia), along Pacific American coasts (from north to south *M. productus*, *M. angustimanus*, *M. gayi*, and *M. australis*), and as far as the east of New Zealand (*M. australis*). In some regions, two species morphologically similar are caught together in commercial fisheries (e.g., *M. capensis* and *M. paradoxus* in African waters (*5*)). Deliberate or inadvertent mislabeling is possible in hake commercialization, both fresh (for species caught in the same areas) and processed (headed and gutted, fillets, fishsticks, surimi, etc.). There are a suite of DNA-based markers for identification of most hake species, generally based on DNA extraction, PCR amplification of target DNA fragments, and further determination of the species based on sequence analysis employing various methods: direct sequencing (*4, 7*), restriction digestion (*3, 8*), and visualization of the PCR product in agarose (*9*), acrylamide (*2, 10*), or nondenaturing polyacrilamide gels (*11*). All these methods allow identification of hakes for fisheries control or detection of commercial fraud in seafood. However, their use has not been fully implemented in real life because there are no data about the actual extent of mislabeling of commercial hake products in different markets.

In this study, we have applied a novel DNA-based methodology of single nucleotide polymorphisms (SNP) analysis (SNaPshot) for a field study of mislabeling in hake products commercialized in international markets. SNP are becoming popular molecular markers because they are abundant in all genomes and rapid and efficient assays exist for genotyping (*12*). Actually, SNPs methodology has already been applied in other fishes such as salmonids (*13*) and eels (*14*). The principal aim of this study was to explore the extent of mislabeling existing in European hake markets, applying mitochondrial single nucleotide polymorphism (mtSNPs) methodology as a tool for rapid and accurate identification of hake species from the *Merluccius* genus. A mitochondrial control region has previously been used as a successful molecular marker to discriminate species belonging to the *Merluccius* genus (*8*). Species misla-

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Table 1. Species Analysed, Number of Individuals Sequenced, and Number of Haplotypes per Species Found in the Control Region with the Program Collapse 1.2 (*20*)

species	individuals sequenced	haplotypes
M. productus	20	3
M. angustimanus	2	1
M. gayi	16	5
M. australis	9	7
M. bilinearis	26	9
M. albidus	18	8
M. hubbsi	19	5
M. merluccius	10	4
M. senegalensis	12	2
M. polli	11	3
M. capensis	13	4
M. paradoxus	13	2

Table 2. Genetic Assignment of Commercial Samples ($n = 40$) Employing the mtSNP-Based Methodology

sample batch	species on the label	seafood type	origin on the label	genetic assignment
1−3	*M. capensis/paradoxus*	frozen fillets	SE Atlantic	*M. paradoxus*
4−5	"55% hake"	fishsticks		*M. hubbsi*
6	*M. hubbsi*	frozen fillets	SW Atlantic	*M. hubbsi*
7[a]	*M. capensis*	frozen fillets	SE Atlantic	*M. paradoxus*
8	*M. australis*	frozen fillets	SE Pacific	*M. australis*
9−11	*M. hubbsi*	frozen fillets	SW Atlantic	*M. hubbsi*
12−14	*M. capensis/paradoxus*	frozen fillets	SE Atlantic	*M. paradoxus*
15−17	*M. hubbsi*	frozen fillets	SW Atlantic	*M. hubbsi*
18−20	*M. australis*	frozen fillets	SE Pacific	*M. australis*
21−22	"55% hake"	fishsticks		*M. hubbsi*
23−24	*M. capensis/paradoxus*	frozen fillets	SE Atlantic	*M. paradoxus*
25[a]	*M. capensis/paradoxus*	frozen fillets	SE Atlantic	$^2/_3$ *M. paradoxus* and $^1/_3$ *M. hubbsi*
26−27[a]	*M. capensis*	frozen fillets	SE Atlantic	*M. paradoxus*
28−29[a]	*M. capensis/paradoxus*	fishsticks		*M. hubbsi*
30−32	*M. capensis/paradoxus*	frozen fillets	SE Atlantic	*M. paradoxus*
33	"European hake"	frozen whole fish		*M. merluccius*
34−35	"Cape hake"	frozen whole fish		*M. capensis*
36	*M. capensis/paradoxus*	frozen fillets	SE Atlantic	*M. paradoxus*
37	*M. capensis*	frozen fillets	SE Atlantic	*M. capensis*
38[a]	*M. capensis*	frozen fillets	SE Atlantic	*M. paradoxus*
39−40	*M. capensis/paradoxus*	frozen fillets	SE Atlantic	*M. paradoxus*

[a] Mislabeled samples.

beling is assumed to exist preferentially in imported hake because only one species, *Merluccius merluccius*, occurs in European waters; thus, no labeling mistakes are expected in products derived from European fisheries unless deliberate fraud is committed. On the basis of this rationale, the exploration of mislabeling was focused on imported seafood labeled as hake. Sampling was centered in Spain because this country supports the highest trade (particularly imports) of hake in Europe (*15*).

MATERIALS AND METHODS

Samples Analyzed. *Noncommercial Samples.* For all *Merluccius* species, a number of 9−26 adult individuals per species were collected except for *M. angustimanus*, for which only two samples were obtained (**Table 1**). The individuals were sampled from different regions (at least two or three individuals per region) to cover the geographical distribution range of each species. They were obtained in the context of various research cruises. They were taxonomically classified and identified based on morphological characteristics. Tissue samples (muscle or gill biopsies) were obtained from each individual, preserved in ethanol, and stored at 4 °C until analysis. A total number of 169 *Merluccius* individuals were collected for this study.

Commercial Fish Food. Three different samples (each sample being one fillet, fishstick, or individual) were analyzed per batch in a total of 40 commercial processed hake batches (**Table 2**). Samples were obtained from commercial markets for a preliminary survey of the level of mislabeling existing in imported seafood hake products in south

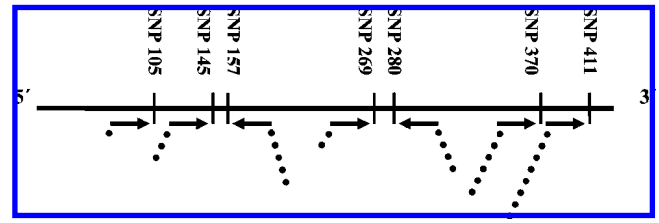

Figure 1. Schematic diagram of SNPs location in the control region sequence. The number of each primer name indicates the nucleotide position in the sequence (5′−3′). Each dot represents a 5′-GACT motif.

Europe. Three types of commercial hake products were considered: whole pieces commercialized as "frozen hake", frozen hake fillets, and precooked hake (fishsticks). All the commercial batches analyzed were clearly and unambiguously labeled as "Hake", and 33 of them exhibited the scientific name of the species on the label. The hake batches analyzed were obtained from 10 different commercial suppliers in five Spanish cities (each of them with >300 000 inhabitants) covering reasonably the main selling points of imported hake.

DNA Extraction and PCR Amplification. Total DNA was extracted from samples of the 12 hake species using a fast and relatively cheap Chelex Resin protocol (*16*). PCR amplification of the mitochondrial control region was performed employing the primers MmerHK01 and MmerHK02 (*17*). In addition, amplification of the mitochondrial control region of other species belonging to the Merluccidae (*Macrouronus magellanicus*) and Gadidae (*Gadus morhua*) family were carried out in order to check the occurrence of false positives. PCR amplifications were carried out on reaction mixtures containing approximately 50 ng of extracted hake DNA template, 10 mM Tris−HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 40 pmols of each primer, 1 unit of Taq DNA Polymerase (Promega, Madison, WI), and 250 μM of each dNTP in a final volume of 40 μL. PCR was performed using the GeneAmp PCR system 2400 by Perkin-Elmer Cetus with the following conditions: an initial denaturing step at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 53 °C for 30 s, and an extension at 72 °C for 30 s, ending with a final extension at 72 °C for 10 min.

DNA Purification and Sequencing. PCR products were visualized in 2% agarose gels with 3 μL of 10 mg/mL ethidium bromide (expected amplicon size around 480 bp). Stained bands were excised from the gel, and DNA was purified with an Eppendorf PerfectPrep Gel CleanUp kit prior to sequencing. Automated fluorescence sequencing was performed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems) with BigDye 3.1 terminator system, at the Sequencing Unit of the University of Oviedo (Spain).

Mitochondrial SNPs Design. A total of 169 sequences were obtained and edited employing the BioEdit Sequence Alignment Editor software (*18*) and aligned with the ClustalW application (*19*). The different haplotypes of each species were obtained with the program Collapse 1.2 (*20*) (**Table 1**). Species-specific sites (invariant within hake species and different for different hake species) were identified. A total of seven polymorphic sites between species without variation in the downstream and upstream adjacent 20 base pairs (bp) were chosen in order to design primers for PCR (**Figures 1** and **2**). Primers were designed on the cDNA strand (3′−5′) when the distance between two polymorphic sites was smaller than 20 bp. A "pigtail" (having a different number of GACT motif repeats) was added to the 5′ end of each primer in order to differentiate the amplicons by size (**Table 3**).

SNPs Multiplex Amplification. The control region for each sample was PCR amplified using the primers MmerHK01 and MmerHK02 (described above) and quantified in 2% agarose gels using a low mass ladder DNA (Invitrogen). SNaPshot multiplex system (ABI PRISM) was employed to amplify SNPs, based on determination of fragment sizes and fluorescent label identity by capillary electrophoresis at multiple SNP sites. PCR SnaPshot assays were carried out on reaction mixtures containing approximately 3 μL of the control region amplicons (0.2 pmol), 5 μL of SnaPshot reaction mix (Taq polymerase, buffer, and fluorescently labeled ddNTPs), and 1 μL of primers mix (final concentration 0.5 μM) in a final volume of 10 μL. PCRs were performed

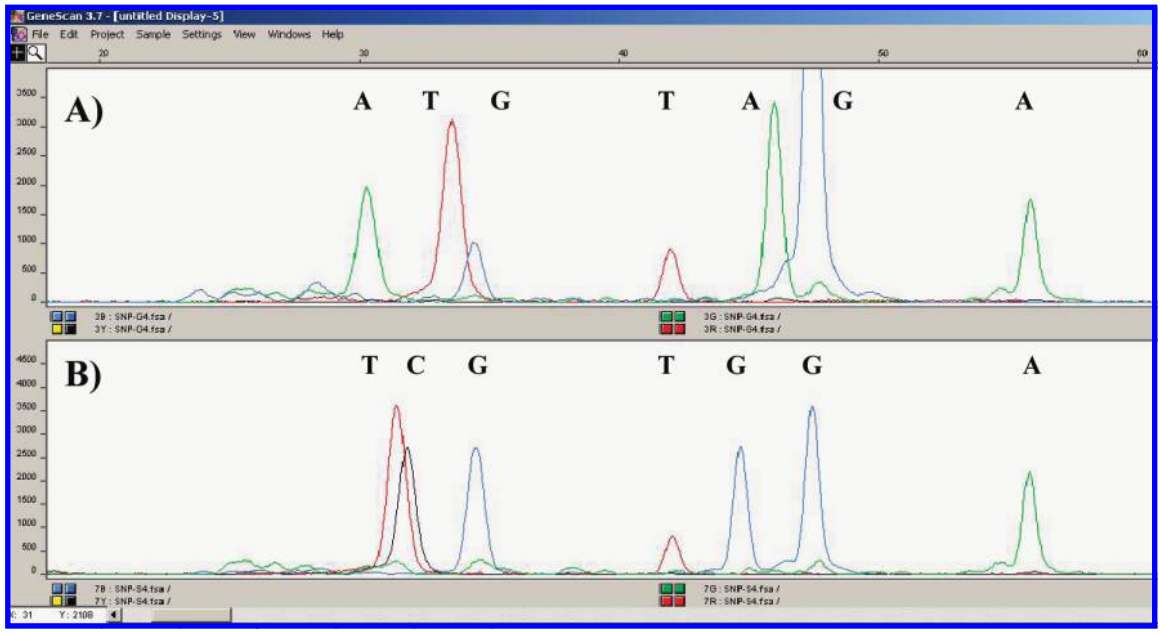

Figure 2. Electropherogram of SNaPshot products (seven polymorphic sites considered) for two *Merluccius* species: (**A**) *M. gayi*; (**B**) *M. senegalensis*. Fluorescence peaks in red, green, black, and blue correspond to thymine, adenine, cytosine, and guanine, respectively.

Table 3. Details of Each SNP[a]

	primer sequence (5′−3′)	5′-GACT repeats	expected amplicon size	observed amplicon size
SNP105	TTCTCATATTATGCTCATGA	1	24	30
SNP269	TTGCCCCTTAGAGAGAACGC	2	28	32
SNP145	ACTTTAAGCCCTTCTAACAT	3	32	36
SNP280	GCAGTCATATATTCAAATTTC	4	37	42
SNP157	AACTTGCTCTTGGTATAAAG	5	40	46
SNP370	TATGGACCTGAAGCTAGGCA	6	44	48
SNP411	AGTGTAAGGTCAAGGGTACT	8	52	55

[a] SNP name, primer sequence, number of 5′-GACT repeats, and expected and observed amplicon size.

Table 4. Nucleotide Bases Present at Each SNP Site for 12 Hake Species Considered

species	SNP105	SNP269	SNP145	SNP280	SNP157	SNP370	SNP411
M. bilineariz	A	C	G	T	A	C	A
M. productus	A	C	G	T	A	G	A
M. angustimanus	A	C	G	T	A	G	A
M. gayi	A	T	G	C or T	A	G	A
M. hubbsi	A	C	A	C or T	A	G	G
M. australis	A	C	A	C	A	G	A
M. albidus	A	C	T	T	A	G	A
M. senegalensis	T	C	G	T	G	G	A
M. merluccius	T	T	G	T	A	G	A
M. capensis	T	C	G	T	A	G	A
M. paradoxus	T	C	T	C	A	C	A
M. polli	T	C	G	C	A	C	A

using the GeneAmp PCR system 2400 by Perkin-Elmer Cetus with the following conditions: an initial denaturing step at 96 °C for 3 min, followed by 25 cycles of denaturing at 96 °C for 10 s, annealing at 50 °C for 5 s, and an extension at 60 °C for 30 s, ending with a final extension at 60 °C for 1 min. After SNPs amplification, a treatment with alkaline phosphatase (Calf intestinal, CIP) was applied in order to remove phosphoryl groups from nonincorporated labeled ddNTPs. PCR products were incubated with 1 unit of CIP (New England BioLabs) at 37 °C for 1 h followed by 15 min of inactivation at 75 °C.

The amplicon size and its fluorescent label were determined with an ABI PRISM 3100 genetic analyzer (Applied Biosystems). SNPs were genotyped employing the GeneScan software (Applied Biosystems). Each sample was genotyped twice in order to confirm the results.

Mixtures of control region amplicons of two species were carried out (equal amount of DNA of each species) in order to test if the mtSNPs-based methodology was sensitive enough to identify different species in the same fish product. Species from either geographically close (*M. albidus*−*M. bilinearis*) or overlapping areas (*M. capensis*−*M. paradoxus*; *M. australis*−*M. hubbsi*, and *M. australis*−*M. gayi*) were chosen to prepare the mixtures.

RESULTS

The control region sequences obtained for the 169 noncommercial hake adults exhibited variation within-species, as expected. A total number of 53 haplotypes were found. They are available at the GenBank database (http://www.ncbi.nlm.nih.gov/), corresponding to the accession numbers EF362839 to EF362881 and EU410446 to EU410455. The seven species-specific points chosen for SNP amplification by SNaPshot multiplexing allowed unambiguous identification for 10 *Merluccius* species, with exception in the differentiation of *M. productus* and *M. angustimanus*, for which they exhibited identical nucleotides at the seven sites (**Table 4**).

Both *Macruronus magellanicus* and *Gadus morhua* samples did not amplify using the primers MmerHK01 and MmerHK02. More than 10 samples were tested for each species. In each PCR, both negative and positive controls were employed to discard contamination and verify that amplification occurred properly. In the case of *Macruronus magellanicus*, mitochondrial control region sequences were not available in GenBank for aligning them with our *Merluccius* sequences. Control region sequences of *Gadus morhua* were available in GenBank (accession number NC002081). Several mismatches (more than eight in each case) were detected when primers MmerHK01 and MmerHK02 were aligned with the *Gadus morhua* control region sequence. Low homology of the primers here employed (homologous for *Merluccius* species) with the target DNA is likely the main cause of failure of PCR amplification in other species.

Clear species-specific patterns of fluorescence were revealed by SNP multiplexing. As expected for the SNaPshot methodology, the fluorescence peak obtained for the amplicon of a given size appeared of a slightly longer size. This is due to different

migration of labeled dideoxynucleotides in the system of capillary electrophoresis with respect to normal nucleotides. The difference between the actual size and the size estimated from the emitted fluorescence was always the same for all the assays and all the samples. Real sizes of 24 (SNP105), 28 (SNP269), 32 (SNP145), 37 (SNP280), 40 (SNP157), 44 (SNP370), and 56 (SNP411) bp were read as 30, 32, 36, 42, 46, 48, and 55 bp, respectively (**Table 3**). All *Merluccius* species (except *M. angustimanus*) were identified based on the SNP panel with the SNaPshot methodology assayed here.

Mislabeling was detected in various commercial samples analyzed (**Table 2**): five batches of frozen fillets and two batches of fishsticks. The global mislabeling of products labeled with the scientific name was 21.2% (7 out of 33 batches). The two batches of fishsticks labeled as *M. capensis*/*M. paradoxus* actually contained *M. hubbsi*. In one batch of hake fillets labeled as *M. capensis*/*M. paradoxus* (batch 25 in **Table 2**), $^1/_3$ of the fillets analyzed were *M. hubbsi*, and the rest were *M. paradoxus*. Finally, three batches of fillets labeled as *M. capensis* were *M. paradoxus*.

Some commercial batches exhibited inaccurate or unspecific labels, due to lack of information on species names or simply to incertitude of the taxonomic classification of the hake (i.e., *M. capensis*/*M. paradoxus*). In the seven batches where scientific names were absent, labeled generically as "Hake", "European hake", or "Cape hake" (batches 4−5 and 21−22, 33, and 34−35, respectively), the species was determined unequivocally. They were *M. hubbsi*, *M. merluccius* and *M. capensis*, respectively. Three batches ambiguously labeled as *M. capensis*/*M. paradoxus* contained only *M. paradoxus*.

In the mixtures of different species, the two species of each mix were correctly identified in all cases. This confirms that mtSNP-based methodology was sensitive enough to detect more than one species in the same seafood product. Most potential mixtures could be theoretically resolved with this methodology because there are single points species-specific. For example, guanine in position 411 (SNP411) detects *M. hubbsi*, and guanine in position 157 (SNP157) indicates presence of *M. senegalensis*.

DISCUSSION

The most striking result of this study was the existence of a high level of mislabeling in commercial products containing hake. This evidence confirms the urgency of implementing systems for controlling the species and, more generally, ensuring traceability in fish markets.

The origin of the mislabeling (inadvertent or deliberate) is not easy to unravel. In some cases, it is likely inadvertent, for example when *M. capensis* is confused with *M. paradoxus*. The two species are caught simultaneously in commercial fisheries and only minor morphological differences exist between them (*21*). It is easy to understand that mislabeling occurs when the classification is based on visual identification in this particular case. In other cases, however, the explanation of mislabeling is not so easy. When American species are labeled as African ones (e.g., *M. hubbsi* labeled as *M. capensis*/*M. paradoxus*), the mistake is of continental level. The mislabeling is likely originated in a step of the commercial chain (distribution centers, selling points) after catching, not during the fisheries or at landing.

The genetic marker employed in this study is robust enough for identification of all the hake species commercialized in Europe. Although the seven SNPs considered in the method developed here did not allow differentiation between *M. angustimanus* and *M. productus*, *M. angustimanus* is not important for European hake markets. On the other hand, these two species are phylogenetically very close (*22*); thus, high similarity between the sequences of the control region of both species is not surprising.

The SNaPshot method is highly accurate and robust, and genotyping has been easily carried out on precooked seafood in this study. Other more classic techniques are generally time-consuming (i.e., restriction fragment length polymorphism typing) and may require high quantities of DNA template (*23*). Because of its easy execution and interpretation of results, the method developed in the present study could be recommended for routine analysis of commercial hake samples aimed to the rapid identification of the species. It can be carried out with no need of advanced knowledge and/or high technical expertise in molecular biology.

In conclusion, employing SNaPshot methodology, we have detected more than 20% mislabeling in hake products commercialized in European markets. This discovery emphasizes the need of implementing traceability methods for quality control of seafood in Europe.

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