JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2007,** *55,* 6903−6909 **6903**

A Rapid Methodology for Screening Hake Species (*Merluccius* Spp.) by Single-Stranded Conformation Polymorphism Analysis

MARIA JOSE CHAPELA, ANA SÁNCHEZ, MARIA ISABEL SUÁREZ, RICARDO I. PÉREZ-MARTÍN, AND CARMEN G. SOTELO*

Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas, Eduardo Cabello 6, Vigo 36208 Pontevedra, Spain

An accurate screening method for hake species identification based in single-stranded conformation polymorphism analysis is presented. The differentiation of 11 species of the *Merluccius* genus and another five species of the Gadiformes order was studied. For this purpose, two fragments of the cytochrome b gene were sequenced; the first is the 5′-end, a fragment of 465 bp (Kocher fragment), and the second is the 3′-end of the cytochrome b, a 588 bp fragment (SB fragment). These two fragments were amplified, denatured, and submitted to native nondenaturing polyacrylamide gel electrophoresis. Results show that with this technique and both fragments, all of the species studied can be unequivocally identified. The validation of the methodology was carried out with 24 commercial hake products showing good performance of the technique for species identification in commercial products. Results show that all species were identified. This technique has advantages over other published methods, because only one polymerase chain reaction step is needed, saving time and money, and it decreases the time needed for hake species identification in food products, making it especially suitable as a screening methodology when a high number of samples should be analyzed in routine examinations.

KEYWORDS: Species identification; traceability; hake; *Merluccius*; cytochrome b; sequencing, SSCP

INTRODUCTION

The group of bony fish known as hakes includes 13 species belonging to the *Merluccius* genus (Subfamily Merlucciinae). They are widely distributed throughout both hemispheres; it is one of the groups of fish most intensively captured, with catches of around 1200000 tons per year. Their quality and commercial value can be very different depending on the species. In European markets, *Merluccius merluccius* are mostly sold as a fresh whole fish, while other species from other geographical locations are sold as frozen commercial products in different presentations. Overexploitation of fisheries and the commercialization of different types of hake products underline the need for methods that unequivocally identify the existing species of the group. Sometimes in this group of species, morphological characteristics are not evident in these cases, or when morphological characters are removed, genetic characters must be employed for species identification (*1*).

European legislation establishes that seafood products should be labeled indicating the commercial designation of species, the production method, and the catch area (Directive 2000/13/EC, EU Commission Regulation No. 2065/2001). So, in order to enforce these labeling rules, faster and cheaper analytical methods should be available to allow the verification of seafood species and also to speed up the process of inspection of imported seafood at customs and points of entry.

The development of hake species identification methods has been reported previously, including some of our own previous work in collaboration with other European laboratories. Polymerase chain reaction−restriction fragment length polymorphism (PCR-RFLP) is the technique most often chosen for developing such identification techniques. Hold et al. (*2*) describe the development of a single PCR-RFLP method for identifying most of the relevant fish species, including nine species of hake.

The Hold et al. method (*2*) requires the use of six restriction enzymes to complete the authentication protocol of 34 fish species; however, *M. merluccius* and *Merluccius senegalensis* cannot be identified employing it.

Other methods have been developed for hake species identification based on the PCR-RFLP technique. Some of them are based on mitochondrial DNA (*3*). Pérez et al. (*4*−*6*) based their methodology on an ITS1-rDNA fragment treated with four restriction enzymes. Also, Pérez and García Vázquez (*7*) identified eight species of hake by combining PCR-RFLP of the 5srRNA and the mitochondrial cytochrome b gene fragment; this last was one needed for *M. merluccius* and *Merluccius capensis* identification. All of these methodologies are based on the application of the PCR-RFLP technique, which involves at least a three-step protocol, including the PCR step for at least

* To whom correspondence should be addressed. Tel: +34-986-214471. Fax: +34-986-292-762. E-mail: carmen@iim.csic.es.

10.1021/jf070132c CCC: $37.00
Published on Web 07/24/2007

Table 1. Hake Species and Individuals Used for the Study[a]

species	common name	key	N KF	H KF	N SBF	H SBF	source
M. albidus	offshore hake	MALB	8	3	6	2	University of Kansas (United States)
M. australis	southern hake	MAUS	6	1	8	1	fishing companies
M. bilinearis	silver hake	MBIL	6	2	6	5	University of Kansas and Fisheries and Oceans Canada
M. capensis	cape hake	MCAP	5	4	7	5	Instituto das Pescas of Angola and a fishing company
M. gayi	South Pacific hake	MGAY	7	2	7	2	Fish market of Valparaíso (Chile) and Del Mar University (Chile)
M. hubbsi	Argentine hake	MHUB	9	2	3	1	Marine Research Institute CSIC (Spain)
M. merluccius	European hake	MMER	6	5	7	5	Fish market Vigo (Spain)
M. paradoxus	deep-water cape hake	MPAR	3	2	3	1	Marine Research Institute CSIC (Spain)
M. polli	Benguela hake	MPOL	8	6	2	2	Instituto das Pescas of Angola
M. productus	North Pacific hake	MPRO	8	3	11	2	Northwest Fisheries Science Center (United States)
M. senegalensis	Senegalese hake	MSEN	10	2	9	1	Oviedo University (Spain)
M. magellanicus	Patagonian grenadier	MMAG	14	1			Instituto Nacional de Pesca (Uruguay) and a fishing company
M. novaezelandiae	blue grenadier	MNEO	5	1			National Institute of Water and Atmospheric Research (New Zealand)
T. chalcogramma	Alaska pollock	TCHA	1	1	1	1	Marine Research Institute CSIC (Spain)
P. pollachius	pollack	PPOL			1	1	Marine Research Institute CSIC (Spain)
P. virens	pollock	PVIR	1	1	1	1	Marine Research Institute CSIC (Spain)
total			97		72		

[a] N KF, number of individuals sequenced for the Kocher fragment; H KF, Kocher haplotypes of sequences used for the SSCP study; N SBF, number of individuals sequenced for the SB fragment; and H SBF, SB fragment haplotypes of sequences used for the SSCP study.

2 h and the digestion with restriction enzymes for more than 5 h prior to electrophoresis.

Another methodology, aimed at revealing nucleotide differences, is the analysis of single strand conformation polymorphism (SSCP), which is a faster, cheaper, and more reliable alternative to PCR-RFLP species identification. SSCP analysis was first described by Orita et al. (*8*) in a study to detect mutations, but since its publication, it has also been employed to identify different types of species. Species differences in the sequence of a particular amplicon are revealed by differences in electrophoretic mobility of each of the strands of the amplicon previously denatured. The suitability of the analysis depends on the characteristics of the DNA fragment selected for the amplification, that is, the absence of intraspecific variability, the number of nucleotide substituions among species, etc. (*9*).

SSCPs have been employed previously to identify bacteria (*10*), parasites (*11*−*13*), and mollusc populations (*14*, *15*). Also, SSCP has been used as a tool for species identification in fish and meat (*16*−*18*) and in seafood products, such as those made from eels, salmon, caviar, tuna (*19*−*22*), and molluscs (*23*, *24*).

In this work, we present the development of an SSCP technique for hake species identification, with the aim of offering a cheaper and easier way to perform analyses than those previously described, especially for laboratories that must analyze a high number of samples in a short period of time and that perhaps lack experience in working with DNA.

MATERIALS AND METHODS

Collection of Authentic Hake Species and Commercial Samples. Eleven different hake species and another five species of the Gadiformes order (*Theragra chalcogramma*, *Pollachius pollachius*, *Pollachius virens*, *Macruronus magellanicus*, and *Macruronus novaezelandiae*) were studied. Most of them are commonly employed in the elaboration of fish products commonly labeled as hake. Scientific and common names of hakes and their sources are shown in **Table 1**. Frozen commercial samples were obtained in local markets. Products included frozen fish pieces and reconstituted precooked products (**Table 2**). Both types of samples were kept frozen until analyzed.

DNA Extraction. DNA was extracted from 0.2 g of thawed muscle that was digested overnight in a thermoshaker at 56 °C with 860 μL of lysis buffer (1% sodium dodecyl sulfate, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, and 10 mM Tris-HCl, pH 8), 100 μL of 5 M guanidium thiocyanate, and 40 μL of proteinase K (20 mg/mL). After 3 h, extra proteinase K (40 μL) was added to the solution and it was left overnight. After digestion, DNA was isolated employing the kit Wizard DNA Clean-Up System (Promega) following the manufacturer's instructions. The DNA solution was collected and stored at −20 °C.

Table 2. List of Commercial Hake Commercial Products Used in the Validation Test

type of hake products	key
extruded	CP1
hake with sauces	CP2, CP5
sticks	CP3, CP22
precooked	CP6, CP7, CP9, CP10, CP4, CP24
bellies	CP8
fillets	CP11, CP13, CP14, CP15, CP21
loins	CP12, CP18, CP23
center cuts	CP16, CP17
tails	CP19, CP20

DNA Amplification. Two different cytochrome b fragments were amplified (**Figure 1**). Kocher et al. (*25*) primers were used to amplify a 464 bp region of the cytochrome b. Another pair of primers, which we later employed (SBH-15486 and SBL-14899), were designed in our lab based on *Gadus morhua* complete mitochondrial DNA from Genbank (NC002081). SBH-15486 5′-GCAGTTTCATKCAAGGGC-CTTATT-3′ and SBL-14899 5′-CCCTTTGTTGTTGCTGCTTTTACA-3′ were designed to amplify a region of 588 bp of the right domain of the cytochrome b. PCR reactions were performed in a final volume of 25 μL using Ready-to-Go PCR beads adding primers and DNA. Amplification with Kocher et al. primers was carried out with a preheating step of 5 min at 94 °C, then 35 cycles of 40 s at 94 °C, 80 s at 53 °C, 80 s at 72 °C, and a final extension step of 7 min at 72 °C. Amplification with SB primers was carried out with a preheating step of 5 min at 94 °C, then 35 cycles of 35 s at 94 °C, 35 s at 50 °C, 40 s at 72 °C, and a final extension step of 7 min at 72 °C. PCR was checked in 2% agarose gels.

DNA Sequencing. Twenty microliters of PCR was treated with 3 μL of ExoSAP-IT (Amersham Biosciences). The mixture was incubated at 37 °C for 30 min and then at 80 °C for another 15 min. Sequencing reactions were prepared with Big Dye (Applied Biosystems) following the manufacturer's instructions. The conditions of the sequencing reactions were a preheating step of 3 min at 94 °C, then 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C. The extension products were purified using a precipitation procedure, and the pellet obtained was stored at −20 °C. Electrophoresis was carried out in an ABI PRISM 310 DNA Sequencer (Applied Biosystems). The collected data were processed using the software BIOEDIT with CLUSTAL used to align sequences (*26*) and MEGA to estimate genetic distances and construct phylogenetic trees (*27*).

Preparation of Single-Stranded DNA (ssDNA). Amplicons were denatured by mixing 5 μL of the PCR product with 15 μL of denaturing

Figure 1. Cytochrome b gene scheme showing the locations of the primers Kocher H and L amplifying Kocher fragment and SB H 15486 and SB L 14898 amplifying the SB fragment.

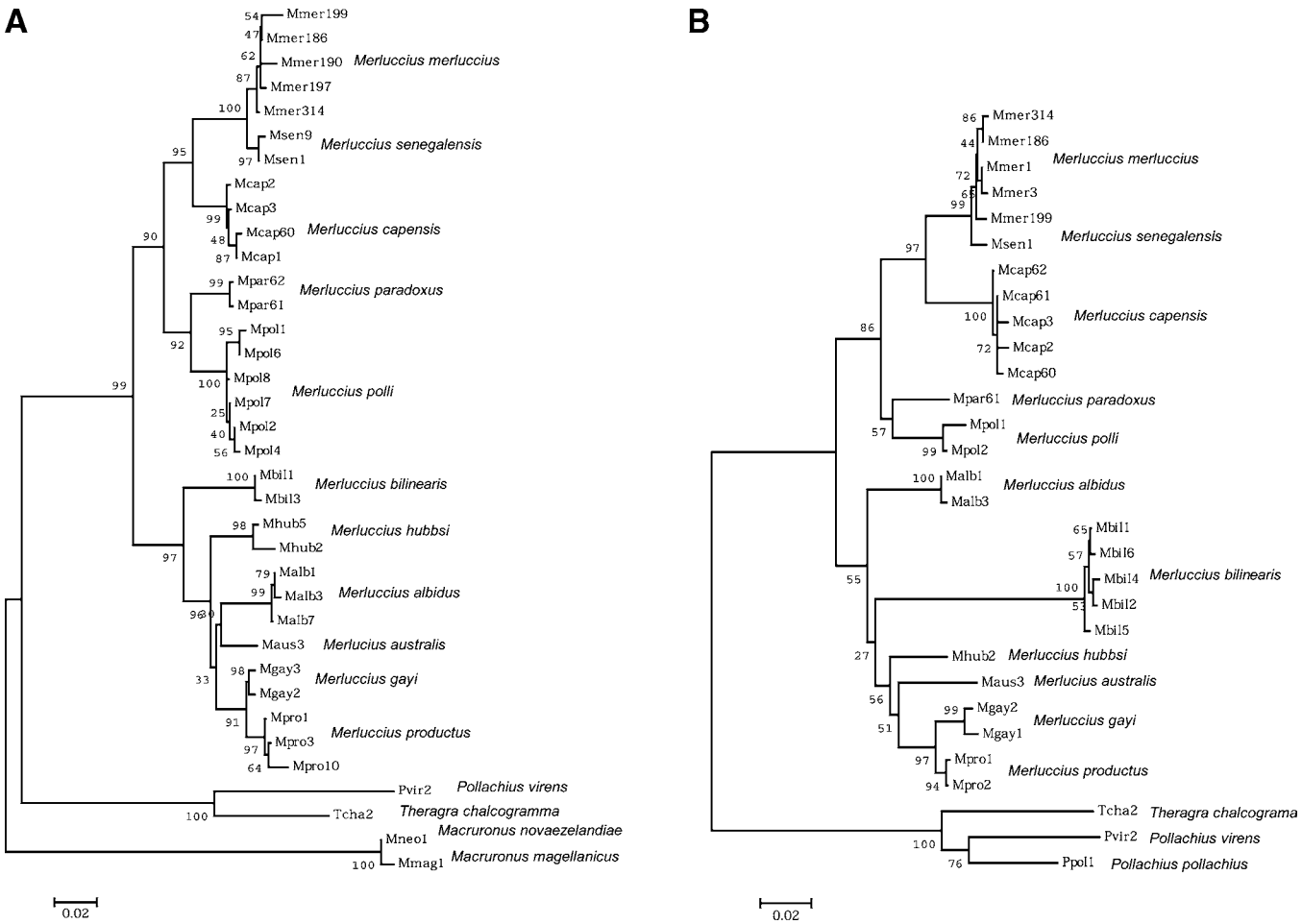

Figure 2. (**A**) Phylogenetic tree constructed from Kocher fragment using Tamura–Nei distances and the neighbor-joining method. Numbers indicate the bootstrap results. (**B**) Phylogenetic tree elaborated from SB fragment using Tamura–Nei distances and the neighbor-joining method. Numbers indicate the bootstrap results.

solution [19 volumes 95% (w/v) formamide and 1 volume of 0.2 M NaOH] and heating the mixture for 5 min at 95 °C. Afterward, samples were placed in iced water.

Polyacrylamide Gel Electrophoresis (PAGE). ssDNA was separated by native PAGE using the manufactured gels CleanGel HP 10% from ETC (ETC Elektrophorese-Technik, Germany); 6 μL of cooled DNA solution was applied without delay to the sample wells of a ClenGel HP 10%, which had been rehydrated with Delect Buffer. Rehydration of the gel (with 40 mL of buffer) and soaking of electrode strips with buffers were performed according to the instructions given by ETC (ETC Elektrophorese-Technik Germany). The conditions of electrophoresis were 200 V, 20 mA, and 10 W for 10 min; 375 V, 30 mA, and 20 W for 50 min; and 450 V, 30 mA, and 20 W for 90 min. The total electrophoresis time was 150 min, and the temperature of the thermostated plate employed was 10 °C.

After electrophoresis, gels were silver stained following the protocol outlined in the application manual AN 1018 02/02 of ETC (ETC Elektrophorese-Technik Germany).

RESULTS AND DISCUSSION

Mitochondrial DNA Sequences of Hake Species. Two cytochrome b fragments were studied for the development of this SSCP method for identification of 11 hake species. **Table 1** presents the species (11 hakes and five other Gadiformes) employed for the development of the technique.

One of the fragments studied was 465 bp corresponding to the 5′-end of the whole cytochrome b gene (from position 14305 to position 14771) (**Figure 1**); this fragment (Kocher fragment) was obtained after amplification using the primers described by Kocher (*25*). Sequences obtained with the amplified fragments were analyzed in terms of genetic distance measurement, and afterward, phylogenetic trees were constructed based upon these distances. The technique, forensically informative nucleotide sequencing (FINS), previously described by Barlett and Davidson (*28*), indicates the resolution level of species differentiation that could be achieved with this fragment. **Figure**

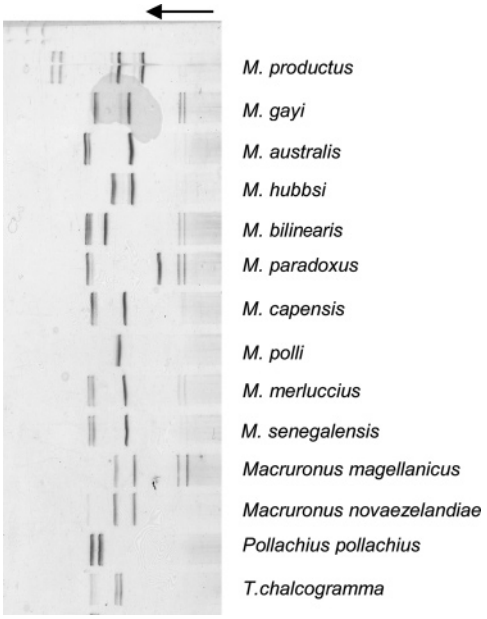

Figure 3. SSCP analysis of Kocher fragment of 14 species, hake, and other gadoids, showing the interspecific variability. The arrow indicates the migration direction.

2 A shows the level of resolution obtained with the use of the Kocher fragment for the identification of 11 hakes. In this case, the phylogenetic tree shows that each species, including the other Gadiformes, is well-differentiated, and two major clades of hake have been detected that correspond to the American group of hakes (*Merluccius productus*, *Merluccius gayi*, *Merluccius australis*, *Merluccius hubbsi*, *Merluccius albidus*, and *Merluccius bilinearis*) and the Euro-African group (*Merluccius paradoxus*, *M. capensis*, *M. merluccius*, *Merluccius polli*, and *Merlucccius senegalensis*). The other two clades correspond to the other Gadiformes (*Pollachius* sp. and *T. chalcogramma*) and to *Macruronus* sp.

The analysis of the fragment sequences also permitted the estimation of intraspecific variability, which, in some cases, could have interfered with the correct identificacion using a technique such as SSCP. Sequences of specimens of the same species were analyzed for finding haplotypes using the "number of differences" option from MEGA (*27*), and only these different haplotypes were used for SSCP analysis (**Table 1**).

A second fragment of 588 bp corresponding to the 3′-end of the whole cytochrome b gene was also sequenced (SB fragment) (**Figure 1**). Primers used for the amplification of this fragment were designed using a GeneBank sequence of *G. morhua* (NC002081). The sequences were also analyzed in terms of genetic distance measurement and phylogenetic tree construction. **Figure 2B** shows the level of resolution obtained with the use of the SB fragment for hake species identification. In this case, the phylogenetic tree shows the same results found with the Kocher fragment; again, two major clades were detected, one of which corresponds to the American group of hakes (*M. productus*, *M. gayi*, *M. australis*, *M. hubbsi*, *M. albidus*, and *M. biliniaris*) and the other to the Euro-African group (*M. paradoxus*, *M. capensis*, *M. merluccius*, *M. polli*, and *M. senegalensis*).

SSCP Analysis of Cytochrome b Sequences of Hake Species. Kocher (465 bp) and SB (588 bp) fragments obtained from the DNA amplification of authenticated hake and gadoid species were melted to ssDNA and separated by native nondenaturing PAGE. SSCP profiles are usually characteristic, and every sample presents two bands of ssDNA, although in some cases up to three bands can be detected. This would indicate the presence of several stable conformations of ssDNA in the gel (*21*). There are two possible explanations: (i) ssDNAs are

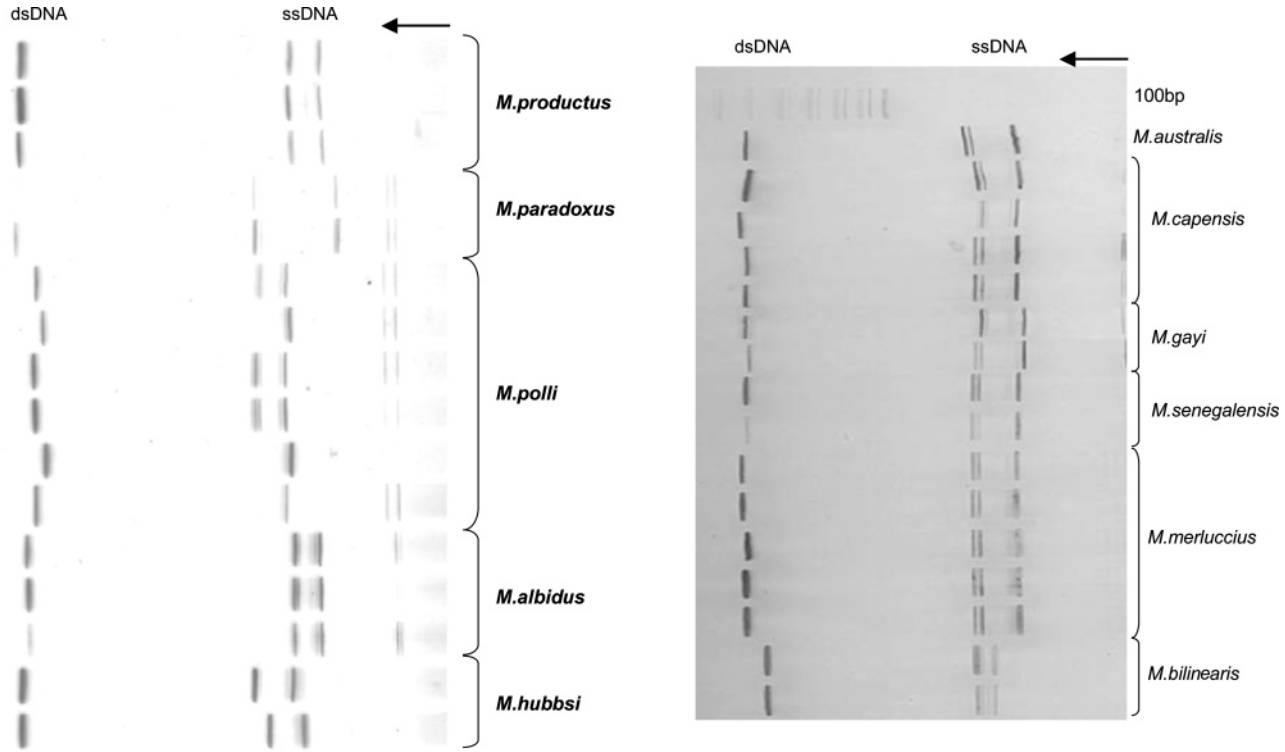

Figure 4. (**A**) SSCP analysis of Kocher fragment showing the intraspecific variability and differences due to individuals of the species *M. productus*, *M. paradoxus*, *M. polli*, *M. albidus*, and *M. hubbsi*. The arrow indicates the migration direction. (**B**) SSCP analysis of Kocher fragment showing the intraspecific variability differences due to individuals of the species *M. capensis*, *M. gayi*, *M. senegalensis*, *M. merluccius*, and *M. bilinearis*. The arrow indicates the migration direction.

in a state of transition between two or more conformations because of small differences in electrophoresis conditions along the gel, and (ii) ssDNAs are in equilibrium between two or more conformations (*29*, *30*). Also, it has been detected that the double-stranded DNAs (dsDNAs) belonging to different species present a slightly different mobility. This phenomenon possibly indicates that there were some differences among the dsDNA conformers (*31*).

In the case of the profiles obtained with the Kocher fragment, results showed that the patterns were species-specific for six of the 11 species of the *Merluccius* genus analyzed (**Figures 3** and **4A**) and for three of the other Gadiformes studied (*Macruronus* spp., *P. pollachius*, and *T. chalcogramma*). Most of the species of the American group of hakes can be identified. In the Euro-African group, three species, out of five, have the same pattern of bands and remained unidentified after the SSCP analysis: *M. merluccius*, *M. senegalensis*, and *M. capensis*. Besides, *M. gayi* presents a very similar SSCP profile as the *M. merluccius*, *M. senegalensis*, and *M. capensis* group.

In some cases of sympatric species, such as South African hakes (*M. capensis* and *M. paradoxus*) and South Atlantic hakes (*M. australis* and *M. hubbsi*), the SSCP patterns are different, and this allows their always difficult morphological identification. Different haplotypes of the same species show low intraspecies variability of the ssDNA patterns (**Figure 4A**,**B**). Only a small amount of intraspecies variability in *M. hubbsi* and in *M. polli* was detected. In each case, two different band patterns are obtained; however, differences between both patterns allow their identification from other hake species (see **Figure 4A**).

Because the *M. merluccius*, *M. capensis*, *M. senegalensis*, and *M. gayi* group was not fully differentiated using the Kocher fragment, the second cytochrome b fragment, SB, was studied. SSCP profiles obtained with the SB fragment show two main ssDNA bands. As can be seen in **Figure 5**, all of the species from the Euro-African group of hakes were identified, including the three species mentioned above, *M. merluccius*, *M. senegalensis*, and *M. capensis*. *M. merluccius*, which is considered the most highly valued species of the group (*1*), has a characteristic band of slow mobility, which allows its unambiguous differentiation from the rest of species studied. Also, *M. gayi*, whose Kocher SSCP pattern was similar to the merluccius-senegalensis-capensis, can also be differentiated using this fragment. However, in the case of other hake species, SSCP profiles are not fully specific, such as *M. productus* and *M. albidus*, *M. gayi* and *M. bilinearis*, *M. paradoxus* and *M. hubbsi*, and *M. australis* and one haplotype of *M. capensis*. Therefore, this fragment should only be used to clarify the identity of *M. capensis*, *M. senegalenis*, *M. merluccius*, and *M. gayi* only after the SSCP profiles from Kocher have been obtained.

Identification of Commercial Samples of Hakes Using SSCP Analysis. DNA extracted from 24 commercial food products containing hake (**Table 2**) was successfully amplified with both primers. In some cases, even DNA from thermally processed products was employed as a template.

Commercial products (**Table 2**) were analyzed using SSCP analysis of the Kocher fragment first. The pattern obtained allowed for the identification of the species employed in the manufacture of each product, while there were only a handful of commercial samples, CP3, CP16, and CP20, which needed to be checked also with the SB fragment. Identification made for these samples is shown in **Figure 6**.

Commercial samples CP1, CP5, CP7, CP8, CP10, CP11, CP12, CP14, and CP17 were identified as *M. paradoxus*; samples CP2, CP4, CP6, CP9, CP13, CP15, CP18, CP19, CP21, CP22, CP23 were identified as *M. hubbsi*; and the two haplotypes found for this species were detected in commercial products. CP24 was identified as *M. productus*. Only three out of 24 products (CP3, CP16, and CP20) needed to be analyzed with the SB fragment (**Figure 7**). CP16 (*M. australis*) was analyzed because, although the pattern of *M. australis* is specific, in some cases, it could be mistaken with the *M. merluccius*, *M. senegalensis*, *M. capensis*, and *M. gayi* group. CP3 (*M. gayi*) and CP20 (*M. capensis*) need the use of the SB SSCP analysis to choose between the four possibilities of the Kocher fragment (*M. meluccius*, *M. senegalensis*, *M. capensis*, and *M. gayi*).

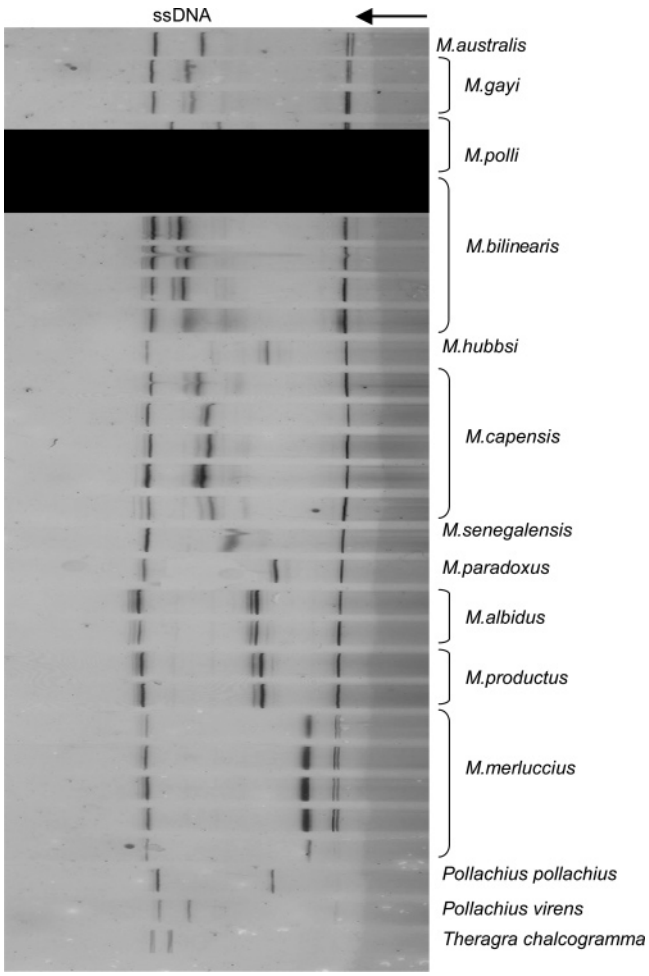

Figure 5. SSCP analysis of SB fragment showing the interspecific variability. The arrow indicates the migration direction.

To confirm the identification that had been done employing the SSCP technique, the commercial products were also identified employing FINS. Unknown sample sequences from commercially products can be identified by measuring their level of similarity against the pool of reference sequences, whereby identifying the closest match between the unknown sequence and a reference sequence allows you to identify the species. In this study, the first half cytochrome b gene fragment amplified in commercial products was sequenced and analyzed by phylogenetic tree construction. **Figure 8** shows, as an example, the phylogenetic tree obtained for the identification of commercial product CP1: The same analysis was done for all of the other commercial samples. Results obtained show that the 24 products analyzed were unequivocally identified employing the SSCP technique.

Sequencing is an accurate technique for species identification, but sample preparation and sequence analysis are time-consum-

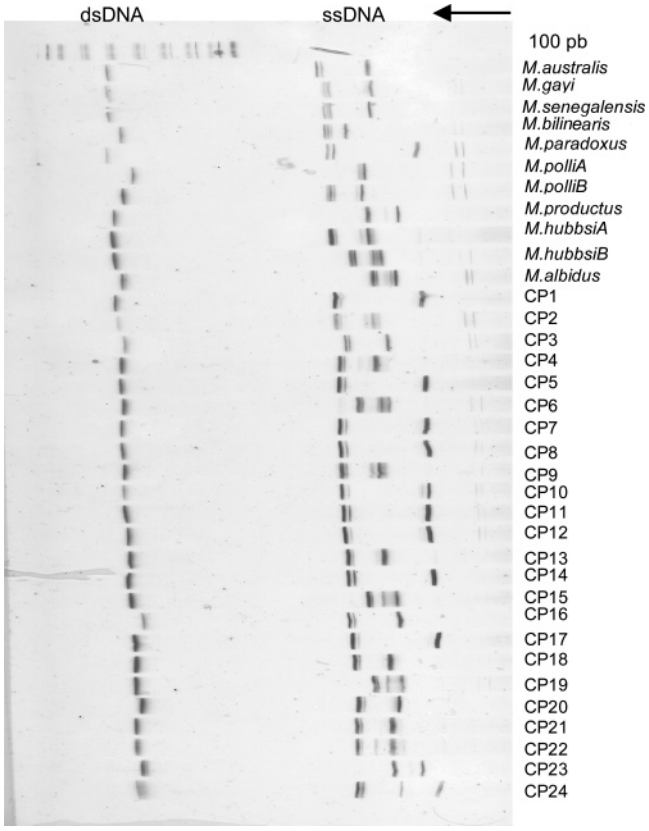

Figure 6. SSCP analysis of commercial samples using the Kocher fragment. The arrow indicates the migration direction.

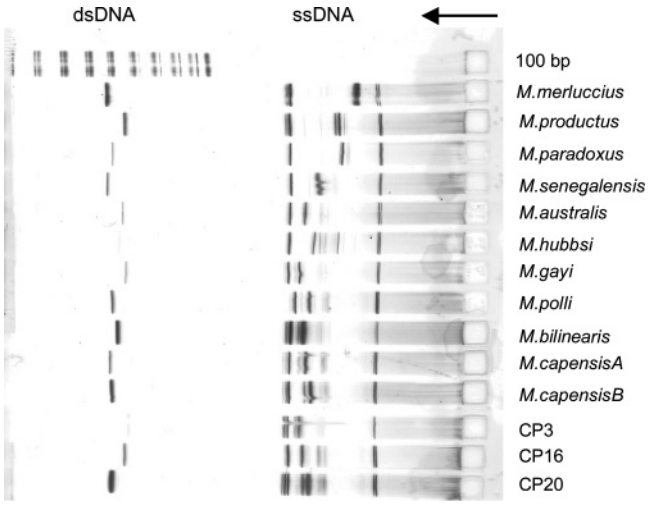

Figure 7. SSCP analysis of commercial samples using the SB fragment. The arrow indicates the migration direction.

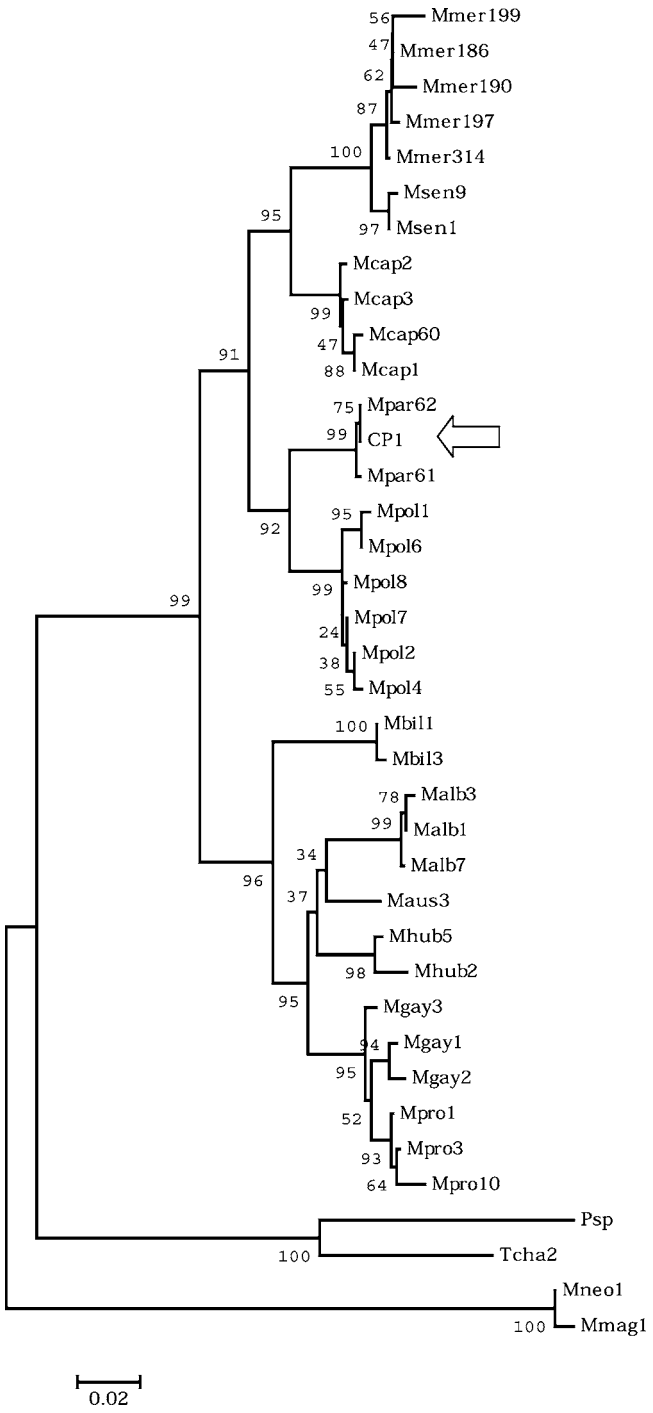

Figure 8. Phylogenetic tree showing the species identification by FINS of sample CP1. The arrow indicates commercial sample identification.

ing, especially when a large number of samples must be examined, and also expensive. Employing SSCP as a screening method for hake species identification will considerably decrease the time needed to identify a large number of samples. In some cases when SSCP patterns are doubtful, the sequencing analysis could be employed as a complementary method for SSCP.

Conclusion. The SSCP technique proposed in this work allows for the identification of 11 species of *Merluccius* genus. This technique has advantages over other published methods because only one PCR step is needed, which saves time and money. In this work, extremely closely related species can be distinguished from one another, and although the technique is very sensitive to the detection of base changes, intraspecific variation of patterns seems less problematic than that obtained by RFLP.

ACKNOWLEDGMENT

We thank Helena Pazó, Susana Otero, and Marta Pérez Testa for their technical assistance. We are also in debt with all of the companies and the research institutions that kindly supplied hake species.

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Received for review January 16, 2007. Revised manuscript received April 20, 2007. Accepted May 9, 2007. We are thankful for the financial support of the Ministerio de Educación y Ciencia of the Spanish government for Project AGL2004-06411-C02-01. A.S. thanks Diputación de Pontevedra for awarding her a doctoral fellowship.

JF070132C