RECENT ADVANCES IN THE STUDY OF
FISH EGGS AND LARVAE
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PCR-SSCP of the 16S rRNA gene, a simple methodology for species identification of fish eggs and larvae

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SUMMARY: Patterns of the 16S rRNA gene obtained in 8 and 12% acrylamide gels by the SSCP (Single Strand Conformation Polymorphism) method were different for various marine fish species (*Macrorhamphosus scolopax*, *Scomber scombrus*, *Lepidorhombus boscii*, *L. whiffiagonis*, *Trachurus trachurus*, *T. mediterraneus*, *Molva molva*, *Merluccius merluccius*). SSCP patterns of this gene were employed to successfully identify formaldehyde-fixed eggs of different species (*Merluccius merluccius*, *Scomber scombrus*, *Macrorhamphosus scolopax* and *L. whiffiagonis*) in plankton samples. The advantages of SSCPs in comparison with current genetic methods of egg identification are based on their technical simplicity and low price. The application of the PCR-SSCP methodology is proposed for routine genetic analyses in plankton surveys.

Keywords: fish species, genetic markers, ichthyoplankton, mitochondrial genes, PCR-SSCP.

RESUMEN: PCR-SSCP DEL GEN 16S RRNA, UN MARCADOR MOLECULAR PARA IDENTIFICAR HUEVOS Y LARVAS DE PECES. – Los patrones SSCP (Polimorfismo para la Conformación de Cadena Sencilla) obtenidos para el gen 16S rRNA en geles de acrilamida al 8% y al 12% fueron diferentes para diversas especies piscícolas marinas (*Macrorhamphosus scolopax*, *Scomber scombrus*, *Lepidorhombus boscii*, *L. whiffiagonis*, *Trachurus trachurus*, *T. mediterraneus*, *Molva molva*, *Merluccius merluccius*). Los patrones SSCP de este gen fueron empleados con éxito para la identificación de huevos de distintas especies (*Merluccius merluccius*, *Scomber scombrus*, *Macrorhamphosus scolopax* y *L. whiffiagonis*) fijados en formaldehído, obtenidos de muestras de plancton. Las ventajas de los SSCPs respecto a las metodologías genéticas existentes actualmente para identificación de huevos de peces en plancton se basan en su sencillez y bajo coste. Se propone la aplicación de metodología PCR-SSCP para el análisis genético rutinario de muestras de plancton.

Palabras clave: especies piscícolas, marcadores genéticos, ictioplancton, genes mitocondriales, PCR-SSCP.

INTRODUCTION

Egg identification in plankton samples is necessary for purposes of stock assessment (Pérez *et al*., 2005). At sea, plankton is sampled and normally preserved in formaldehyde to avoid shape and colour changes that may complicate the visual inspection of eggs to determine their developmental stage. The eggs of many marine fish species are morphologically very similar (Moser *et al*., 1984).

Species determination by visual inspection, generally based on diameter, colour, shape and oil globule, is thus very difficult. For example, eggs of European hake *Merluccius merluccius* and the megrims *Lepidorhombus whiffiagonis* and *L. boscii* overlap in size (egg diameter of 0.94-1.03 for *Merluccius merluccius* and 1.02-1.22 for both megrims), exhibit similar colour (transparent) and shape (spherical), and have one oil globule of 0.27 mm of diameter (on average). Misidentification of ichthyoplankton based on visual inspection has been recently reported (Pérez *et al*., 2005). Instead, species-specific genetic markers allow accurate identification of eggs. Initial visual sorting is necessary to separate fish eggs from the rest of the plankton specimens. Then, eggs can be separately inspected to determine their developmental stage and other characteristics of interest before squashing, DNA extraction and genetic species determination. DNA-based methods are the most useful tool for identifying marine organisms (Sweijd *et al*., 2000; Feral, 2002; Shao *et al*., 2002).

The first issue to be considered when one is developing species-specific markers is the type of marker to be employed. Target sequences are mitochondrial genes (Medeiros-Bergen *et al*., 1995; DeSalle and Birstein, 1996; Palumbi and Cipriano, 1998; Taylor *et al*., 2002) because their large number of copies per cell enables PCR amplification even in early developmental stages when the number of cells per egg is small. On the other hand, sequences must be conserved within species and variable between species. Highly conserved coding genes such as ribosomal DNA are thus the first choice.

There are different methods for visualising genotypes. Some automated or semi-automated protocols, such as Taq-Man, require sophisticated equipment and technological training of the experts in charge of typing (Taylor *et al*., 2002). Other cheap methods have been used to identify fish species, such as PCR followed by fragment size identification in agarose or acrylamide gels (Perez and Garcia-Vazquez, 2004), which can be applied by technicians without very specialised skills. Although the ideal method depends on the needs and available resources of the user, an easy and cheap methodology based on simple equipment is often needed for routine analyses.

The SSCP (single-stranded conformation polymorphism) method offers a sensitive but inexpensive and rapid method for determining differences in sequence in DNA samples. A major advantage of using this methodology is that no previous knowledge of the DNA sequence is needed, and different DNA sources from the same sample can be discriminated (Sunnucks *et al*., 2000). It has been widely applied in biomedical research (see a review in Hayashi and Yandell, 1993) and in population biology (Hedrick *et al*., 1999; Congdom *et al*., 2000; Small and Gosling, 2000; D'Urso *et al*., 2003). The range of topics studied employing SSCP to detect sequence variation is very wide, from intraspecific variation at population level (Taylor *et al*., 1999) to bacterial diversity (Schmalenberger and Tebbe, 2003). Species identification has been achieved using the SSCP method in aphids (Wilson *et al*., 1999; Sunnucks *et al*., 2000). As it is easy and inexpensive, this method can be considered for routine surveys of large sample sizes (Sunnucks *et al*., 2000).

This study aimed to analyse the usefulness of the SSCP method by employing the 16S rRNA gene as a genetic marker for species identification of marine fish eggs stored in 4% formaldehyde (the most common fixative employed for preservation of specimens in plankton surveys). The work was based on the discrimination between eggs of European hake (*Merluccius merluccius*) and megrims (*Lepidorhombus boscii* and *L. whiffiagonis*), currently possible only by PCR-fragment size determination in an automated sequencer (Perez *et al*., 2005). The advantages and disadvantages of these methods are discussed.

MATERIALS AND METHODS

Biological samples

To explore the potential utility of this method for specific identification of fish eggs from preserved plankton samples, 50 adult samples from eleven species of commercial interest were obtained from different Atlantic areas. These species were: *Lepidorhombus boscii*, *L. whiffiagonis*, *Merluccius merluccius*, *Macrorhamphosus scolopax, Scomber scombrus, Trachurus trachurus*, *Merlangius merlangus, Molva molva, Pollachius virens, Pollachius pollachius* and *Gadus morhua*. Tissue samples (gills or muscle) from adults caught in different areas of the distribution of these species were obtained and ethanol-preserved for further analysis.

A batch containing some formaldehyde-preserved eggs visually sorted from different plankton samples of the Bay of Biscay was prepared. The plankton samples were obtained employing bongo nets (60 cm diameter, 303 μm mesh) towed to a nominal depth of 200 metres, and retrieved obliquely. Fish eggs at each tow were manually sorted before the ship departed from the sampling station, then fixed in 4% buffered formaldehyde solution (pH = 7.5). The plankton sampling was carried out in the ICES VIIIc area (Bay of Biscay) in 2002. The eggs were preserved in the formalin solution for more than one year until January 2004, when laboratory analyses were carried out.

In order to compare this method with others currently available for fish egg identification (PCR amplification + fragment size determination in an automated sequencer; see Pérez *et al*., 2005), additional adult samples of *Merluccius merluccius*, *Lepidorhombus whiffiagonis* and *L. boscii* (Table 1, Fig. 1) were analysed by PCR-SSCP to test species-specificity of the patterns obtained. SSCP patterns of eggs fixed in ethanol were compared with those obtained from eggs of the same species fixed in formalin. Megrim (*Lepidorhombus whiffiagonis*) eggs were obtained by artificial fertilisation of mature males and females caught in the ICES VIa area in a commercial vessel, and fixed in ethanol (50% eggs) or 4% formaldehyde buffered with sodium tetraborate (50% eggs) 24 hours after fertilisation. We analysed 30 ethanol-fixed and 30 formaldehyde-fixed eggs one year after fixation.

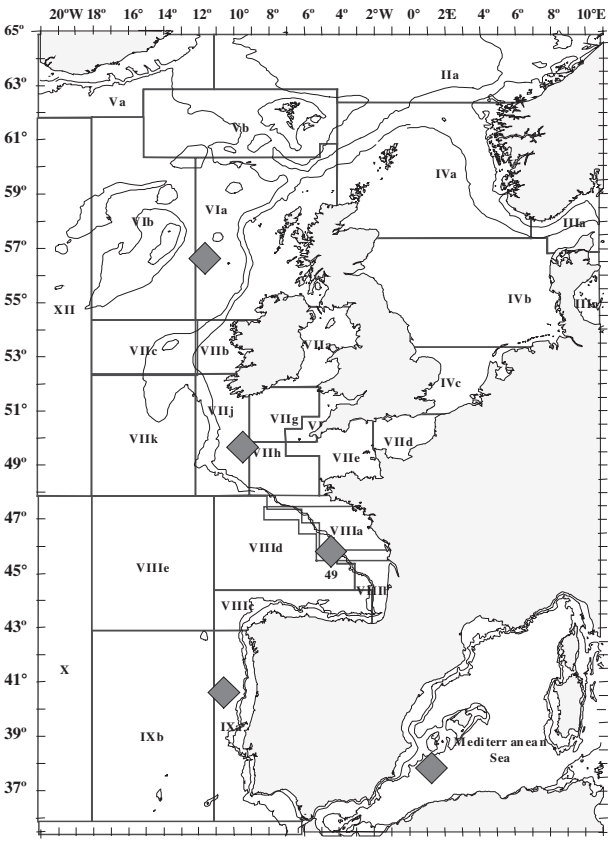

FIG. 1. – Map showing sampling areas (diamonds).

TABLE 1. – Number and origin of the adult individuals analysed

Species	Area	Number
Merluccius merluccius	ICES VI	20
	ICES VII	20
	ICES VIII	20
	ICES IX	20
	Aegean Sea	20
	TOTAL	100
Lepidorhombus boscii	ICES VII	20
	ICES VIII	20
	ICES IX	20
	Aegean Sea	20
	TOTAL	80
Lepidorhombus whiffiagonis	ICES VI	20
	ICES VII	20
	ICES VIII	20
	Spanish Mediterranean coast	20
	TOTAL	80

More details on plankton sampling and fertilisation experiments can be found in Pérez *et al*. (2005).

DNA extraction

DNA was extracted from ethanol-preserved adult samples following the Chelex-based protocol described by Estoup *et al*. (1996). From individual eggs, DNA was extracted using the QIAamp MiniKit DNA tissue Kit (QIAGEN). The formalin-fixed eggs were washed with PBS to remove the formalin prior to following the standard QIAGEN protocol. The extracted volume of eluted DNA was 200 µl for adults and eggs, performed in two 100 µl elution steps.

PCR-SSCP protocol

PCR amplification of the mitochondrial 16S rRNA gene segment (564-568 pb long) was performed using the primers H_{3080} (5'-CCGGTCTGAACTCAGATCACGT-3') and L_{2510} (5'-CGCCTGTTTATCAAAAACAT-3') described in Palumbi *et al*. (1991). The reaction was carried out in a total volume of 20 µl including 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 20 pmol of each primer, 20 ng of template

DNA and 1 U of DNA Taq polymerase (Gibco). The thermocycler conditions for the amplification were the following: an initial denaturing step at 95ºC for 5 min, then 30 cycles of 95ºC for 20 s, annealing at 48ºC for 20 s and 72ºC for 20 s, and one extension at 72ºC for 7 min.

10 µl of the PCR product were denatured at 95ºC for 5 min, then loaded in a 8% or 12% acrylamide gel (acrylamide/bisacrylamide 50:1) and run in a vertical electrophoresis apparatus at 80 V and 4ºC for 4 h. Then the gel was fixed in 10% ethanol for 8 min, oxidated in 1% HNO_3 for 3 min, washed twice in MilliQ water, stained in $AgNO_2$ (2.02 g/l) for 20 min, washed twice in MilliQ water again and revealed in 29.6 g/l Na_2Co_3 (anhydro) + 540 µl of 37% formaldehyde. Revealing was stopped with 10% acetic acid for 5 min, then the gel was washed in distilled water. SSCP variants were directly visualised in the gel. It is not useful to include standard size markers in the gel as references because in SSCP DNA chains are denaturalised and move differentially through the acrylamyde gel; differences in sequence, not in size, are detected.

In egg samples, PCR-SSCP was repeated at least twice in order to determine the reproducibility of the pattern obtained.

DNA purification and 16S rDNA sequencing

The amplified segment was sequenced for ten individuals of each SSCP variant found following the protocol described above. PCR products were visualised in 50 ml 1.5% agarose gels with 3 μl of 10 mg/ml ethidium bromide. 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 on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) with the BigDye 3.1 Terminator system in the Unit of Genetic Analysis of the University of Oviedo (Spain). Both strands (direct and reverse) of each DNA fragment were sequenced.

Sequence editing and analysis

Mitochondrial 16S rRNA genes were edited using the BioEdit Sequence Alignment Editor software (Hall, 1999). Sequences were aligned with the ClustalW application (Thompson *et al.*, 1994) included in BioEdit.

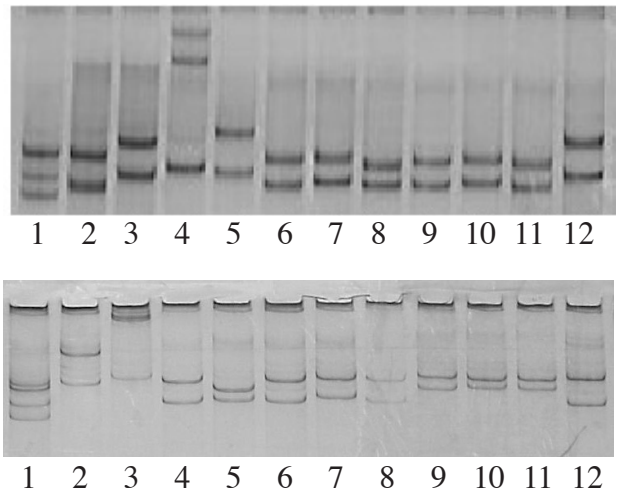

FIG. 2. – SSCP patterns obtained for 16S rRNA gene for different fish species, in gels with different acrylamide concentration. A) 8% acrylamide gel. From left to right: 1-11, control adults as following: 1, *Macrorhamphosus scolopax*, 2, *Scomber scombrus*, 3, *Trachurus trachurus*, 4, *Lepidorhombus boscii*, 5, *L. whiffiagonis*, 6, *Merluccius merluccius*, 7, *Merlangius merlangus*, 8, *Molva molva*, 9, *Pollachius virens*, 10, *Pollachius pollachius*, 11, *Gadus morhua*. 12, formaldehyde-fixed egg. The SSCP pattern at the 16S rRNA gene corresponds to the pattern of the control *Trachurus trachurus*. B) 12% acrylamide gel. From left to right: 1, *Macrorhamphosus scolopax*; 2, *Lepidorhombus whiffiagonis*; 3, *L. boscii*; 4, *Melanogrammus aeglefinus*; 5, *Molva molva*; 6, *Pollachius pollachius*; 7, *P. virens*; 8, *Gadus morhua*; 9, *Trachurus mediterraneus*; 10, *T. trachurus*; 11, egg of *T. trachurus*; 12, *Merluccius merluccius*.

RESULTS

Different patterns of bands were obtained for the eleven species considered in this study, allowing direct identification of some of them with the SSCP analysis of the 16S rRNA gene in both 8 and 12% acrylamide gels (Fig. 2A and B respectively). For example, in 8% acrylamide gels (Fig. 2A) *Macrorhamphosus scolopax* (snipe fish) exhibited a three-band pattern clearly distinguishable from the rest of the species. The same can be applied to *L. boscii*, *L. whiffiagonis*, *Merluccius merluccius*, *Scomber scombrus* (mackerel) and *Trachurus trachurus* (horse-mackerel). The rest of the Gadoid species (*Merlangius merlangus* whiting, *Molva molva* ling, *Pollachius pollachius* pollack, *P. virens* pollock, and *Gadus morhua* cod), however, exhibited less marked differences in SSCP bands. In 12% acrylamide gels it was also possible to distinguish *Molva molva* and *Pollachius virens* from other Gadidae species, as well as to differentiate *Trachurus mediterraneus* and *T. trachurus*.

Based on SSCP patterns, some eggs belonging to *L. whiffiagonis*, *Macrorhamphosus scolopax*,

TABLE 2. – Egg identification in formaldehyde-fixed plankton samples obtained from the Bay of Biscay.

N	Amplification	SSCP pattern
9	Yes	*Lepidorhombus whiffiagonis*
43	Yes	*Macrorhamphosus scolopax*
3	Yes	*Scomber scombrus*
2	Yes	*Merluccius merluccius*
9	Yes	Unknown
6	No	-

Scomber scombrus and *Trachurus trachurus* were identified in a batch prepared from plankton eggs sampled in the Bay of Biscay (Table 2). DNA amplification failed for six eggs (5.8%) and 9 eggs (13%) that exhibited unknown SSCP patterns.

In the extended study aimed to compare the SSCP method with that previously reported for egg identification of hake and megrims (PCR-fragment size determination), some intraspecific variation was found for megrims. The SSCP variants found for the 16S rRNA gene in adults of the three species considered are shown in Figure 3. A single pattern of two bands (A) was found for European hake *Merluccius merluccius*. Two variants were found for megrim *Lepidorhombus whiffiagonis*, one of two separate bands (B) and the other with a double and another single band (C), found in individuals from the Mediterranean Sea. Five variants (D, E, F, G and H) of different combinations of double and single bands were found for *Lepidorhombus boscii* in different areas of distribution of this species. The SSCP variants can be considered species-specific because all of them are particular to a single species. No one variant was shared by two species. The pattern A was exclusive of *Merluccius merluccius*, B and C of *Lepidorhombus whiffiagonis*, D, E, F, G and H of *L. boscii*. As the samples analysed covered the whole distribution of the species, the results indicate that the 16S rRNA gene SSCP can be employed as a species-specific molecular marker for identification of these species.

Sequences of the variants A (*Merluccius merluccius*), B (*Lepidorhombus whiffiagonis*) and D (*L. boscii*) are in Table 3. These sequences have been included in the GenBank (Accession numbers DQ304654, DQ304653 and DQ304652, respectively). For *Lepidorhombus whiffiagonis*, the differences between the variants B and C were due to an insertion of eight base pairs in position 350 in the variant C. For *Lepidorhombus boscii*, the differences between the SSCP variants were due to different combinations of the two substitutions and single-base insertions along the fragment.

PCR amplification of the 16S rRNA gene was successful for 93.3% of both ethanol and formaldehyde-preserved megrim eggs. The patterns of bands obtained in ethanol-preserved, formaldehyde-preserved and adult megrims *Lepidorhombus whiffiagonis* are shown in Figure 4. The eggs showed a type B variant, as expected from the origin of the spawning adults. The SSCP pattern obtained for eggs and adults was identical and very clear. The results were 100% reproducible in all cases. PCR-SSCP of the 16S rRNA gene can be employed for identification of hake and megrim eggs sampled in plankton surveys.

When the PCR-SSCP methodology is compared with that previously described for identification of formalin-fixed eggs in Pérez *et al*. (2005), some differences were found (Table 4). First, the equipment

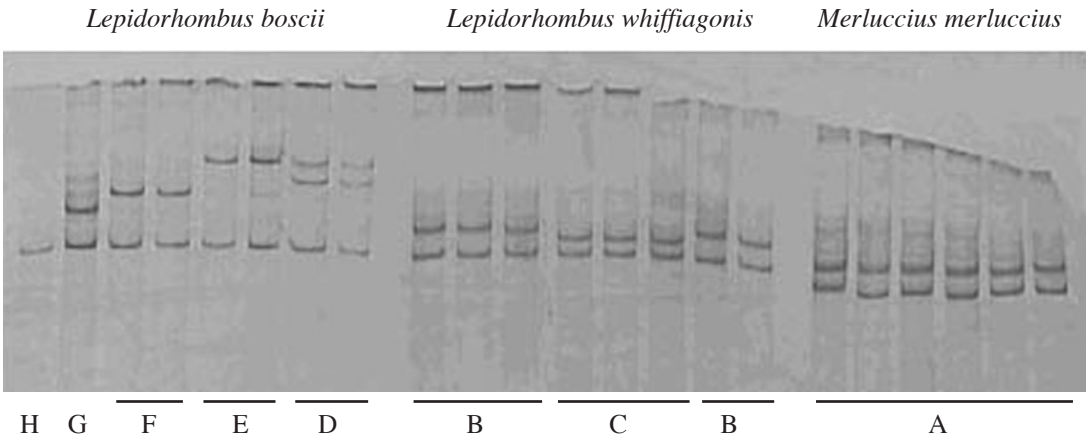

FIG. 3. – Acrylamide gel showing the SSCP variants found at the 16S rRNA gene for *Merluccius merluccius* (variant A), *Lepidorhombus whiffiagonis* (variants B and C) and *L. boscii* (variants D, E, F, G and H).

TABLE 3. – 16S rRNA gene sequences of *Lepidorhombus whiffiagonis* (SSCP variant B, Lw), *L. boscii* (SSCP variant D, Lb) and *Merluccius merluccius* (SSCP variant A, Mm), aligned with the program CLUSTAL. The consensus sequence is marked with asterisks.

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Lw  AGAGGTCCCGCCTGCCCAGTGACACCCAGTTCAACGGCCGCGGTATTTTGACCGTGCAAA 60
Lb  AGAGGTCCCGCCTGCCCAGTGACACCCAGTTCAACGGCCGCGGTATTTTGACCGTGCAAA 60
Mm  AGAGGTCCCGCCTGCCCTGTGACTATATGTTTAACGGCCGCGGTATCTTAACCGTGCGAA 60
    ****************** *****       *** ************ **  ******* **

Lw  GGTAGCGTAATCACTTGTCTTTTAAATGAAGACCTGTATGAATGGCATAACGAGGGCTTA 120
Lb  GGTAGCGTAATCACTTGTCTTTTAAATGAAGACCTGTATGAATGGCATAACGAGGGCTTA 120
Mm  GGTAGCGTAATCACTTGTCTTTTAAATGAAGACCTGTATGAATGGCATCACGAGGGCTTA 120
    ************************************************  **********

Lw  GCTGTCTCCTCTCTCCAGTCAATGAAGTTGATTTCCCCGTGCAGAAGCGGGGATAATTAC 180
Lb  ACTGTCTCCTCTCTCAAGTCAATGAAGTTGATCTCCCCGTGCAGAAGCGGGGATAATTAC 180
Mm  ACTGTCTCCCCTCTCCAGTCAATGAAATTGACCTCCCCGTGCAGAGGCGGGGATATCTTT 180
      ********  *****  **********  ****   ***********  *********   *

Lw  ATAAGACGAGAAGACCCTATGGAGCTTGAGACGGTAGGGCAGCCCATGTTAAACACCCCT 240
Lb  ATAAGACGAGAAGACCCTATGGAGCTTGAGACGATAGGGCAGCCCATGTTAAACACCCCC 240
Mm  ATAAGACGAGAAGACCCTGTGGAGCTTTAGACC-TAAAATAAACCACGTTTAATATACTA 239
    ******************** ******** ****   **      *   *** *** ** *   *

Lw  CTAT-CCCGGGCTAAACTAAATGGCCCCTGCCTTAA-TGTCTTCGGTTGGGGCGACCCTG 298
Lb  C-AC-ACCGGACCAAACTAAATGGCTCCTGCCTTAA-TGTCTTCGGTTGGGGCGACCCTG 297
Mm  AAGTTAATAGCAAAAACTTAATGGATATTTATTAAAGTGTCTTTGGTTGGGGCGACCGCG 299
              *    ***** *****     *    * **  ****** ************   *

Lw  GGG-AATACAAAACCCCCACGTGGAACAGGAGCACC---CCCCCCCACCAACTCCTCCAA 354
Lb  GGG-AATACAAAACCCCCACGTGGAATAGGAGCACCCGTCCCCCCCCCCCGACTCCTCCAA 356
Mm  GAGTAAAACACAACCTCCATGTGGA-TGGGGATACT----------ATCCCTAAAA 344
    * * **  *** **** *** *****    **    **                *   ***  **

Lw  ACTAGAGCCACACCTCAAATTAACAAAACTTTTGACCAAAA-TGATCCGGCAATGCCGAT 413
Lb  ACTAGAGCCACACCTCAAATTAACAAAACTTTTGACCAAAAATGATCCGGCAACGCCGAT 416
Mm  CTCAGAGCCTCCACTCCAAGCAACAGAAATTCTGACCTAC--TGATCCGGTATAACCGAT 402
      ****** *  *** **  **** ** ** ***** *    ******** *    *****

Lw  CAACGGACCAAGTTACCCTAGGG-ATAACAGCGCAATCCCCTTTT-AGAGCCCATATCGA 471
Lb  CAACGGACCAAGTTACCCTAGGGGATAACAGCGCAATCCCCTTTTTAGAGCCCATATCGA 476
Mm  TAACGAACCCAGTTACCCCAGGG-ATAACAGCGCAATCCCCTCTC-AGAGCTCATATCGA 460
     **** *** ******** **** ****************** *   ***** ********

Lw  CAAGGGGGTTTACGACCTCGATGTTGGATCA-GGACATCC--TAATGGTGCAGCCGCT-A 527
Lb  CAAGGGGGTTTACGACCTCGATGTTGGATCA-GGACATCC--TA-TGGTGCAGCC-CT-A 530
Mm  CGAGGGGGTTTACGACCTCGATGGTGGATCAAGGACCTTCCTTATGGGTGCAGCCGCTTA 520
    * ********************** ******* **** * *   **  ********* ** *

Lw  TTAAGGG-TTCGTTTGTTCA-CGATTAA-GTCCTAAGTGATGGG 568
Lb  T--AAGG-TTCGTTTGT-CA-CGATTAA-GTCT--AGTGTTGGG 566
Mm  TTGAGGGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGATCTG 564
    *   * **  *********  ** *******  ***     *** *   *
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TABLE 4. – Comparison of PCR-SSCP and PCR-fragment size determination methods for identification of formalin-fixed fish eggs. Costs: actual costs (approx.) in euros, at the University of Oviedo (Spain) in 2005. Time required from the end of the PCR until visualisation of the PCR product. Success: proportion of formalin-fixed eggs successfully typed, in percent. Sample sizes: 122 and 220 for PCR-SSCP and PCR-fragment size determination, respectively.

Feature	PCR-SSCP	PCR-fragment size determination
Equipment required	Dual gel vertical unit of electrophoresis + Power supplier	Automated sequencer (i.e. Genetic Analyser) + Computer with software (i.e. GenMapper)
Equipment cost	€1,200 + €1,000	€300,000 + €6,000
Maintenance costs of the equipment, per year	None	€6,000
Analysis cost (64 samples)	€60	€230.4
Time required (64 samples)	5 h	3 h 45 min (16 capillaries)
Success	95%	85% *
Technical expertise	Basic molecular biology	Specialised technician

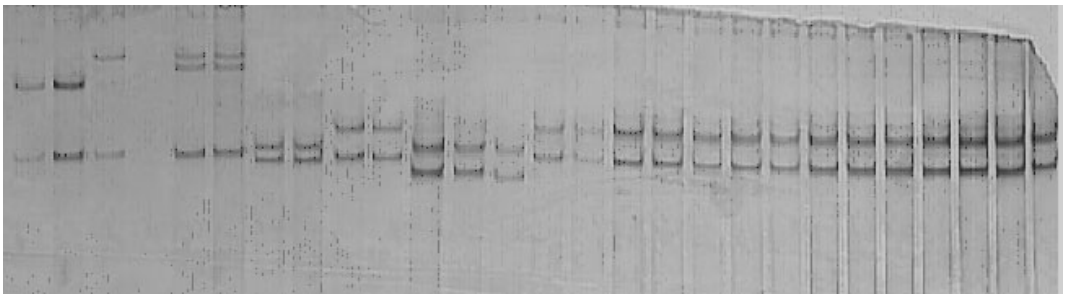

FIG. 4. – Acrylamide gel showing the SSCP-PCR pattern found for *Lepidorhombus whiffiagonis* eggs fixed in ethanol (13-19) or in 4% formaldehyde (20-26). Control adults: 1-5, *Lepidorhombus boscii* (variants D, E and F); 6-7, Mediterranean *L. whiffiagonis* (variant C); 8-9, Atlantic *L. whiffiagonis* (variant B); 10-12, *Merluccius merluccius* (variant A). Both ethanol-fixed and formaldehyde-fixed megrim eggs presented the variant B.

required for SSCP was considerably simpler and cheaper than that required for typing eggs employing PCR + fragment size determination in automated sequencer. Second, the technical expertise required for detecting SSCP in acrylamide gels consisted of basic skills in molecular biology (PCR, preparing and loading acrylamide gels, basic silver staining protocols), whereas an expert technician is required to manage an automated sequencing machine. Third, costs of PCR-SSCP were much lower for the above reasons. Higher success of genetic typing for formalin-fixed eggs was obtained by the SSCP method than by the PCR + fragment size determination protocol (95 and 85% respectively). The time required for completing each protocol was similar.

DISCUSSION

Species-specific 16S rRNA gene SSCP patterns were obtained for several fish species, allowing identification of the eggs of these species in plankton samples (Table 2, Fig. 2). *Macrorhamphosus scolopax*, *Lepidorhombus whiffiagonis*, *Trachurus trachurus*, *Scomber scombrus* and *Merluccius merluccius* eggs were easily identified in a sample of ichthyoplankton. However, this marker provided similar SSCP patterns for some Gadoid species; for example, *Merlangius merlangus* and *Gadus morhua* exhibited bands that were practically indistinguishable in 8% acrylamide gels (Fig. 2A). For some species this problem can be solved with some minor technical modifications. For example, *Pollachius pollachius* and *P. virens* provided almost identical SSCP patterns in 8% acrylamide gels (Fig. 2A) but were easily distinguishable in 12% acrylamide gels (Fig. 2B). On the other hand, some eggs obtained from plankton samples (Table 2) provided unknown SSCP patterns, indicating that they belonged to species not considered in the present work. Standardisation of the technique should include clear descriptions of SSCP patterns obtained for each commercial species at different acrylamide concentrations. It is also necessary to consider the need to keep standard DNA samples of the target species to be included as references in routine gels. However, a different species from those analysed here that is present in the plankton tows may have the same banding pattern as one in the analysis. This problem is impossible to solve, with this or any other method, unless all fish species present in a given marine region are analysed. Ideally, if all species are sampled it would be possible to find a molecular marker (or a combination of different markers) that is useful for identifying all species in this gene or in other genome regions with different degrees of conservation.

The SSCP patterns obtained for the three species with morphologically similar eggs (European hake and megrims) were species-specific. No one variant was shared by two species. The large number of adults analysed, 80-100, obtained from different marine areas of the distribution of each species, confirms the universal validity of the marker here described. Species-specific fragment size differences within the 16S rRNA gene had been already reported for these three species (Pérez *et al*., 2005). However, fragment size determination in degraded DNA samples, such as formaldehyde-fixed eggs, is not easy (Díaz-Viloria *et al*., 2005). In the case described by Pérez *et al*. (2005) it required fluorescence detection in an automated sequencer, because agarose gels were not sensitive enough for detecting amplification bands. The SSCP method offers some advantages with respect to fragment size determination (Table 4). Although a little more time-consum-

ing than automated fragment size identification, it is simpler and cheaper. It does not depend on sophisticated equipment and specialised expertise is not necessary for egg typing. This method is sensitive but inexpensive and rapid (Sunnucks *et al*., 2000), three characteristics that represent an improvement with respect to previously described genotyping methods. It could be considered for routine surveys of large sample sizes.

SSCP methodology has been applied for species identification in aphids (Wilson *et al*., 1999), but its utility in formaldehyde-preserved tissues is reported here for the first time. The eggs analysed in this study had been preserved in formalin for one year. The PCR-SSCP patterns of formaldehyde-fixed eggs were all reproducible, although some authors (O'Leary *et al*., 1994; de Giorgi *et al*., 1994; and Díaz-Viloria *et al*., 2005) claimed the reduction of reliability of PCR when tissues are preserved in formaldehyde. The whole 16S rRNA gene, more than 500 bp long, was amplified in more than 90% formaldehyde-fixed eggs. The great length of the amplified fragment may represent an advantage for employing this methodology in formalin-fixed tissues. Although the method can be considered robust enough for analysing fragments as large as 775 bp (Sunnucks *et al*., 2000), only 80% of single base pair differences can be resolved by the SSCP technique for 400 bp fragments (Girman, 1996). Thus, single-nucleotide changes eventually induced by formalin exposure would probably produce differences in SSCP patterns for short sequences but, as occurred in the present case, would remain undetected in longer fragments, preserving species-specificity.

Different SSCP patterns were found within *Lepidorhombus* species. The existence of intraspecific variants for *Lepidorhombus whiffiagonis* and *L. boscii* may be used for detecting genetic variation that can be applied for population studies in these two species, as described for other taxa (revision in Sunnucks *et al*., 2000). Moreover, 8-nucleotide-long insertion/deletion in Mediterranean *L. whiffiagonis* samples suggests the existence of a subspecies in this region (García-Vázquez *et al*., 2006). Further studies will be carried out employing this approach to study the population structure of these species, which is unknown at the present.

The SSCP method should be further developed for ichthyoplankton identification. For example, species-specific single-nucleotide variation, impossible to detect by this method in a sequence more than 500 bp long like the 16S rRNA gene, can be detected in shorter sequences (Sunnucks *et al*., 2000). If the method is considered for routine surveys, SSCP patterns of marker sequences should be obtained and characterised for all fish species whose eggs are present in plankton in a given marine area. With these relatively simple developments, the SSCP technique appears to be promising for ichthyoplankton analysis due to its simplicity, low cost and easy handling.

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