



Analytical Methods

Differentiation of Sparidae species by DNA sequence analysis, PCR-SSCP and IEF of sarcoplasmic proteins

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ABSTRACT

The increasing global trade of fishery and aquaculture products makes it necessary to develop methods for species identification in case of fish fillets or other highly processed seafood with external morphological characteristics (e.g. gills, fins) of the original fish being removed.

Species identification methods based on DNA- or protein-analysis, like DNA sequencing of PCR products, PCR-SSCP (polymerase chain reaction – single strand conformation polymorphism), and isoelectric focusing (IEF) of water-soluble proteins of fish fillet were applied successfully to distinguish between various sparid species including *Sparus aurata*, *Spondyliosoma cantharus*, *Boops boops*, *Acanthopagrus bifasciatus*, *Argyrops spinifer*, *Pagellus bogaraveo* and *Lithognathus mormyrus* of the family Sparidae available on European markets.

Indication of the presence of parvalbumins in light muscle of all of the studied species is presented by the location of heat-stable proteins in the anodic range of the IEF gels.

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1. Introduction

The world market of fish is steadily growing. More and more new marine and fresh water fish species from tropical and subtropical waters are found not only in local markets, but also markets in distant countries (FAO, 2010). The correct declaration and labelling of processed fish is a prerequisite of fair trade and prevention of fraud (Hanner, Becker, Ivanova, & Steinke, 2011). Unambiguous labelling of fish is hampered by the fact that often more than one common name is assigned to a given fish species on European, Australian or U.S. markets. This can be the generic term, e.g. *Pagrus* spp., or a common name like sea bream. Secondary, with the use of same name for different species, as allowed by German Regulations (BLE, 2011), seafood products may consist of more than one species. In both cases, a potential deception can arise, if products are of variable sensory quality, value or product formulation/ recipe.

Sparidae or sea breams are distributed worldwide from temperate to tropical waters; many of them are living in South African waters (Hanel & Tsigenopoulos, 2011). They are chiefly marine, usually inshore, but some periodically enter estuaries. Hermaphroditism, both protandrous and protogynous, is common in this family (Smith & Heemstra, 2003; Whitehead, Bauchot, Hureau, Nielsen, & Tortonese, 1986). There are about 112 species in 35 genera (Fischbase, 2010).

Most species belonging to the family Sparidae, and especially those species native to the North Eastern Atlantic and Mediterranean coastal waters, are of high commercial importance as food and for recreational fishery (De la Herrán, Ruiz Rejón, Ruiz Rejón, & Garrido-Ramos, 2001). *Sparus aurata* (gilthead sea bream), sold in Germany as Dorade royal, is the most used species of Sparidae in aquaculture worldwide followed by *Pagrus auratus* (silver sea bream). In 2008, aquaculture production peaked in Europe at ca. 95 000 tons with Greece producing more than half (FAO statistics, 2009). The high efficiency in production of *S. aurata* in the Mediterranean aquaculture has led to attempts to rear other sea breams and hybrids (Basurco, Lovatelli, & Garcia, 2011).

Spondyliosoma cantharus (black sea bream) and *Boops boops* (Bogue) have a higher importance on the Mediterranean fish markets in countries like France, Spain and Italy. In 2009, total production in Europe was nearly 64,000 tons for *S. cantharus* and more than 8500 tons for *B. boops* (FAO statistics, 2009).

Acanthopagrus bifasciatus (two-bar sea bream) is a tropical species caught in coral reefs (Smith & Heemstra, 2003). Some other sea breams of commercial interest and occasionally introduced to the Northern European market are *Argyrops spinifer* (king soldier bream) from the Eastern Mediterranean Sea and *Pagellus bogaraveo* (black spot sea bream) as an important food fish in coastal regions of the Mediterranean Sea and Eastern Atlantic Ocean. *Lithognathus mormyrus* (striped sea bream) is common in the Mediterranean area, but of minor commercial interest, mainly caught by anglers (Smith & Heemstra, 2003; Whitehead et al., 1986).

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Due to their morphological similarities, mainly their reddish colour and body shape, *Pagrus pagrus* (red porgy), *Pagrus caeruleostictus* (blue-spotted sea bream) and *P. auratus* (silver sea bream) are often mixed up and as a consequence sold under the same market name, primarily in Germany as Dorade rosé.

Due to the ecological distribution of members of Sparidae from Atlantic, Pacific and Indian oceans, there are differences in sensory properties and prices to be expected.

A variety of methods are used to identify commercially important fish species belonging to the family Sparidae. Eight species, which are or could be important in commercial Mediterranean aquaculture, were successfully discriminated with allozyme markers (Alarcón & Alvarez, 1999). Four of them are also the subject of this study: *S. aurata*, *P. bogaraveo*, *L. mormyrus* and *S. cantharus*.

In another study, application of polymerase chain reaction followed by a restriction fragment length polymorphism analysis (PCR-RFLP) was used to differentiate between *Dentex dentex* and *S. aurata*, as well as other species like *Dicentrarchus labrax* and *Umbrina cirrosa* (Cocolin, Ágago, Manzano, Lanari, & Comi, 2000).

As frozen fillets from related fish species are difficult for consumers to distinguish, a DNA-based method (DNA barcoding) was developed by Filonzi, Chiesa, Vaghi, and Nonnis Marzano (2010), which could protect consumers from a possible deception by substitution with lower-value fish. Incorrect labelling in 22% of samples was detected by DNA-sequencing of mitochondrial genes, cytochrome b and cytochrome oxidase subunit I (Filonzi et al., 2010).

Raw and heated fish muscle extracts analyzed by IEF (isoelectric focusing) often possess several protein bands in the anodic part of the gel (Girija & Rehbein, 1988). These proteins having pI values between 3.9 and 5.5 were identified as belonging to parvalbumins (Rehbein, Kündiger, Pineiro, & Perez-Martin, 2000). Parvalbumins are calcium-binding, acidic proteins with a low molecular mass (~11 kDa) existing in high concentration in light (white) muscle of fish (Schoenman, Chiaro, Jones, Bastin, & Coughlin, 2010). Due to their heat-stability, they are easily distinguished from other sarcoplasmic proteins in heated samples using SDS-PAGE or IEF.

Parvalbumin proteins can also be used for species identification of raw or heat-processed fish because they display species specific patterns in IEF (Arif, Jabeen, & Hasnain, 2007). In a study by Rehbein et al. (2000), the black seabream (*S. cantharus*) was easily

distinguished from other fish species like mullet, flounder, sole and cod by the patterns of acidic proteins in IEF.

The aims of the present study were (i) to develop a rapid low cost IEF or SSCP method for testing incoming goods and compliance with labelling regulations and (ii) establishing DNA sequences for tropical and subtropical Sparidae species using these methods. The development of new rapid, low-cost methods to identify species of the family Sparidae in various commercial products could be helpful for food control laboratories to detect mislabelling of seafood (Jacquet & Pauly, 2008).

2. Material and methods

2.1. Fish samples

Fish samples were purchased either from retailers and wholesale traders in Northern Germany or collected in the Bay of Biscay by the German research vessel Walther Herwig III. Fillets were received deep frozen with or without skin; gutted fish were received either fresh or deep frozen. In Table 1 authenticated samples are listed as identified by visual inspection of the whole animal (Carpenter & Niem, 2001; Smith & Heemstra, 2003; Whitehead et al., 1986) and/or by comparing the sequence of a defined DNA fragment with entries in GenBank using BLAST. In Table 3 analysed, but not authenticated commercial samples are listed.

2.2. Extraction and quantification of DNA

DNA was isolated from 100 mg of fish fillet using the detergent cetyltrimethylammonium bromide (CTAB) according to Rehbein (2005). The DNA content of extracts was estimated by measuring the DNA-dependent enhancement of fluorescence intensity of the dye Hoechst 33258 against calf thymus DNA as a standard (Downs & Wilfinger, 1983).

2.3. Polymerase chain reaction (PCR) followed by single strand conformation polymorphism analysis (SSCP)

For all amplifications DNA concentration in the PCR assay was adjusted to 1 ng DNA/μl; the primer concentration was 0.5 μmol/

Table 1
List of authentic samples (Date of BLAST: 2011-08-16).

Code	Origin	Product	Latin name (English name)	Authentication
1	Wholesale trader	Whole	<i>Argyrops spinifer</i> (King soldier bream)	vi [*] ; BLAST: 95% id to AF240717.1/AB458407.1 (<i>Argyrops spinifer/bleekeri</i>)
2	Wholesale trader	Whole	<i>Acanthopagrus bifasciatus</i> (Two bar sea bream)	vi ^{**} ; BLAST: 91% id to AM944826.2 (<i>Acanthopagrus taiwanensis</i>)
3	Wholesale trader	Whole	<i>Pagrus caeruleostictus</i> A (Blue spotted sea bream)	vi; BLAST: 100% id to DQ197975.1
4	Wholesale trader	Whole	<i>Pagrus caeruleostictus</i> B	vi
5	Research vessel	Whole	<i>Spondyliosoma cantharus</i> A ^{***}	vi; BLAST: 100% id to EF427607.1
6	Research vessel, Bay of Biscay, wild catch	Whole	<i>Spondyliosoma cantharus</i> B (Black sea bream)	vi
7	Research vessel, caught in the Bay of Biscay	Whole	<i>Spondyliosoma cantharus</i> C	vi
8	Research vessel, caught in the Bay of Biscay	Whole	<i>Boops boops</i> A (Bogue)	vi; BLAST: 99% id to FJ440629.1
9	Research vessel, caught in the Bay of Biscay	Whole	<i>Boops boops</i> B	vi
10	Research vessel, caught in the Bay of Biscay	Whole	<i>Pagellus bogaraveo</i> (Black spot sea bream)	vi; BLAST: 100% id to AB305023.1
11	Wholesale trader	Whole	<i>Sparus aurata</i> A	BLAST: 100% id to EU224082.1
12	Wholesale trader, aquaculture Greece	Whole	<i>Sparus aurata</i> B (Gilthead sea bream)	vi

^{*} vi = visual inspection of a whole animal.

^{**} visual identified species: not in GenBank.

^{***} different specimens of same species.

1. PCR was performed with reagents from Solis BioDyne (Tartu, Estonia) using HotFirePol DNA polymerase I (final activity 2.5 units), BD Buffer (5 µl/assay), dNTP mix (200 µmol/l final concentration), MgCl₂ (2.5 mmol/l final concentration); assay volume was 50 µl.

Amplification of a 464 bp fragment of the mitochondrial cytochrome b gene was performed with the primer pair: L14735: 5'AAAAACCAACCGTTGTTATTCAACTA and H15149ad: 5'GCICCTCA-RAATGAYATTGTCCTCA, which had been found to react with a large number of species from different fish families. (Wolf, Burgener, Hübner, & Lüthy, 2000).

With the following PCR conditions amplicons suited for sequencing were obtained for all species analysed: preheating 15 min/95 °C, 35 cycles of 40 s/94 °C, 80 s/50 °C, 80 s/72 °C, final heating for 7 min at 72 °C.

SSCP:PCR assay (6 µl) were diluted 1:4 with denaturing solution containing 0.1% Bromphenol Blue and Xylenecyanol FF (Fluka Chemie AG, Buchs) in a mixture of 4.75 ml formamide + 0.25 ml 0.2 M NaOH. The solution was heated for 5 min at 95 °C to get single-stranded DNA (ssDNA), then cooled in iced water and applied immediately to rehydrated polyacrylamide gel. DNA-HP Clean Gels 10% were rehydrated with Delect Gel Buffer

				40
<i>A. spinifer</i>	ATGAAATTTT	GGCTCCCTAC	TCGGCCTCTG	CCTAATCTCT
<i>A. bifasciatus</i>	GTGAAATTTT	GGATCCCTCC	TCGGTCTCTG	CCTAATTTCC
<i>P. caeruleostictus</i>	ATGAAACTTT	GGCTCCCTAC	TTGGTCTCTG	CCTAGTCTCC
<i>S. cantharus</i>	ATGAAATTTT	GGATCCCTAC	TTGGTCTTTG	CCTAATTTCC
<i>B. boops</i>	ATGAAATTTT	GGTTCCCTGC	TTGGCCTCTG	TCTTATTTCC
<i>P. bogaraveo</i>	ATGAAACTTT	GGATCCCTTC	TTGGTCTCTG	CCTAATCTCT
<i>S. auratus</i>	ATGAAATTTT	GGATCCCTCC	TCGGTCTCTG	TCTAATTTCT
Sample 16a	ATGAAATTTT	GGATCCCTGC	TCGGCCTTTG	TTTAATCTCC
Sample 16d	ATGAAATTTT	GGCTCCCTAC	TTGGTCTCTG	CCTAATTTCT
Sample 17	GTGAAATTTT	GGATCCCTAC	TTGGTCTCTG	CCT CG TCTCT
				80
<i>A. spinifer</i>	CAGCTTCTCA	CAGGACTCTT	CCTTGCTATG	CACTATACCT
<i>A. bifasciatus</i>	CAACTTCTCA	CAGGATTATT	TCTTGCTATA	CATTACACCT
<i>P. caeruleostictus</i>	CAACTCCTCA	CAGGACTATT	CCTTGCCATG	CATTACACCT
<i>S. cantharus</i>	CAACTCCTCA	CAGGACTCTT	CCTTGCCATA	CATTACACCT
<i>B. boops</i>	CAGCTCCTTA	CAGGGCTATT	CCTCGCCATG	CATTATACCT
<i>P. bogaraveo</i>	CAACTCCTTA	CAGGACTATT	TCTTGCTATA	CATTACACCT
<i>S. auratus</i>	CAGCTTCTGA	CAGGGCTATT	CCTCGCTATG	CACTACACTT
Sample 16a	CAACTCCTAA	CAGGATTATT	CCTTGCCATA	CACTACACCT
Sample 16d	CAACTTCTCA	CAGGACTGTT	CCTTGCCATA	CATTACACCT
Sample 17	CAACTTCTTA	CAGGATTATT	CCTTGCTATG	CATTATACCT
				120
<i>A. spinifer</i>	CCGATATTGC	CACAGCCTTC	TCCTCCGTCG	CCCACATCTG
<i>A. bifasciatus</i>	CCGATATCGC	TACAGCCTTT	TCCTCCGTAG	CCCATATCTG
<i>P. caeruleostictus</i>	CCGACATTGC	TACAGCCTTT	TCTTCTGTTG	CTCACATCTG
<i>S. cantharus</i>	CAGACATCGC	CACAGCCTTT	TCTTCTGTCG	CACACATCTG
<i>B. boops</i>	CCGATATCGC	CACAGCCTTC	TCTTCCGTTG	CCCACATCTG
<i>P. bogaraveo</i>	CCGATATTGC	CACAGCCTTT	TCCTCTGTCG	CCCACATCTG
<i>S. auratus</i>	CCGATATCGC	CACAGCCTTC	TCTTCCGTAG	CCCACATCTG
Sample 16a	CAGACATTGC	CACAGCCTTT	TCCTCCGTCG	CACATATCTG
Sample 16d	CAGACATCGC	CACAGCCTTT	TCTTCTGTTG	CCCACATTG
Sample 17	CCGATATCGC	CACAGCTTTC	TCCTCCGTAG	CCCACATCTG
				160
<i>A. spinifer</i>	CCGAGATGTA	AACTACGGCT	GACTCATCCG	TAATCTTCAC
<i>A. bifasciatus</i>	TCGAGATGTT	AATTATGGCT	GACTAATCCG	AAACTCCAC
<i>P. caeruleostictus</i>	CCGAGATGTA	AACTACGGCT	GACTTATCCG	TAATCTTCAT
<i>S. cantharus</i>	CCGAGATGTA	AATTACGGCT	GACTCATCCG	AAATCTTCAC
<i>B. boops</i>	CCGAGATGTA	AATTATGGCT	GACTCATCCG	AAACTACAC
<i>P. bogaraveo</i>	TCGAGACGTA	AACTACGGAT	GGCTAATCCG	AAATCTCCAC
<i>S. auratus</i>	CCGAGATGTA	AATTACGGAT	GGCTCATCCG	AAACTTCAC
Sample 16a	TCGAGACGTA	AACTACGGCT	GACTTATCCG	TAACCTCCAC
Sample 16d	TCGAGACGTA	AACTACGGCT	GACTTATCCG	CAACCTCCAT
Sample 17	TCGGGACGTA	AACTACGGCT	GACTTATCCG	CAACCTCCAC
				200
<i>A. spinifer</i>	GCCAATGGAG	CATCCTTCTT	CTTTATCTGC	ATTTACCTCC
<i>A. bifasciatus</i>	GCCAACGGAG	CATCTTTTCT	CTTCATCTGC	ATTTACCTTC
<i>P. caeruleostictus</i>	GCCAACGGAG	CATCCTTCTT	CTTCATCTGC	ATCTACCTTC
<i>S. cantharus</i>	GCCAACGGAG	CATCTTTCTT	TTTTATTTGC	ATTTACCTTC
<i>B. boops</i>	GCCAACGGAG	CATCTTTCTT	CTTCATCTGC	ATTTACCTTC
<i>P. bogaraveo</i>	GCTAATGGAG	CATCTTTCTT	CTTTATCTGT	ATTTACCTTC
<i>S. auratus</i>	GCCAACGGAG	CATCTTTCTT	TTTTATTTGT	ATTTACCTCC
Sample 16a	GCTAACGGAG	CATCCTTCTT	CTTTATTTGC	ATCTACCTCC
Sample 16d	GCCAACGGAG	CATCCTTCTT	TTTCATTTGC	ATCTATCTCC
Sample 17	GCAACGGAG	CATCAATTCT	TTTTATTTGT	ATTTACCTCC

Fig. 1. Sequence (200 bp fragment of the cytochrom b gene) alignment of fish samples belonging to the family Sparidae (*A. spinifer*, *A. bifasciatus*, *P. caeruleostictus*, *S. cantharus*, *B. boops*, *P. bogaraveo* and *S. aurata*) and commercial samples (labelled as *S. cantharus* and *L. mormyrus*). Differences to the sequence of *A. spinifer* are given in bold.

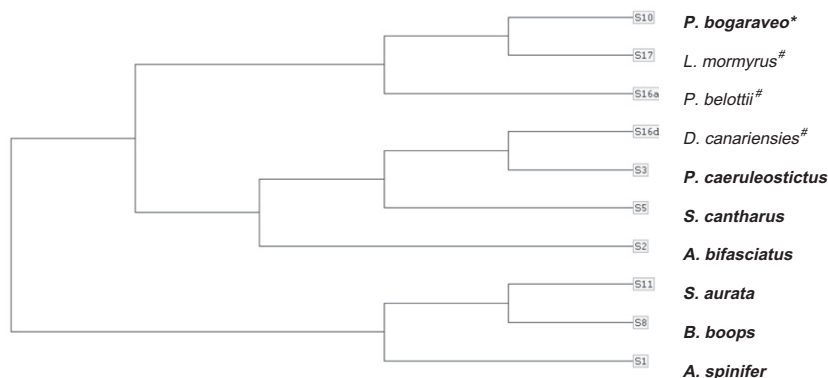


Fig. 2. Dendrogram of sparid samples (*A. spinifer*, *A. bifasciatus*, *P. caeruleostictus*, *S. cantharus*, *B. boops*, *P. bogaraveo* and *S. aurata*) including commercial samples (labelled as *S. cantharus* and *L. mormyrus*) calculated from 200 bp sequences of the cytochrome b gene by DNASIS. *Authentic samples in bold. #Nearest match obtained by BLAST.

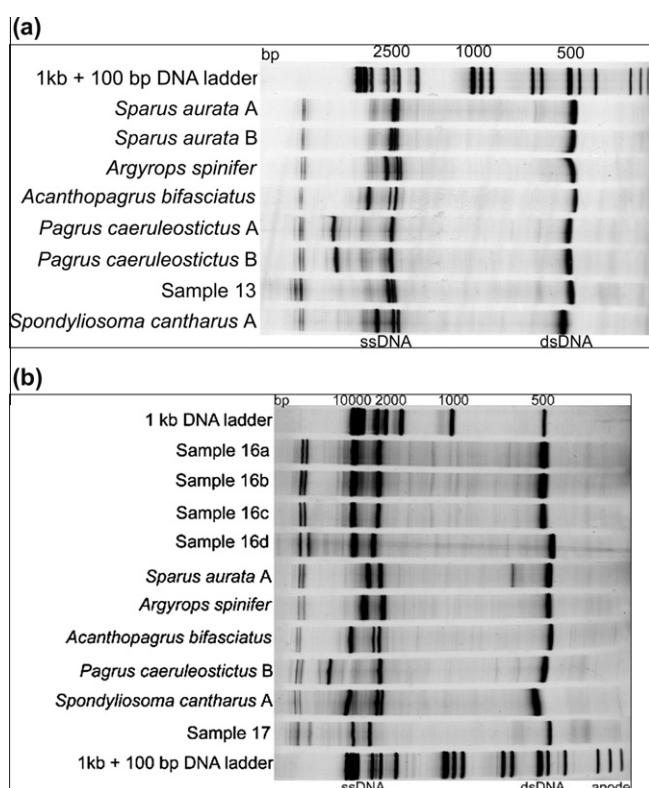


Fig. 3. PCR-SSCP (DNA-HP Clean Gel 10%; Delect Buffer). (a) Authentic fish species of the family Sparidae (*S. aurata*, *A. spinifer*, *A. bifasciatus*, *P. caeruleostictus* and *S. cantharus*) and sample 13, labelled as *S. aurata*. (b) Different products of sparid fish (*S. aurata*, *A. spinifer*, *A. bifasciatus*, *P. caeruleostictus*, *S. cantharus*) and commercial fish samples (16a–d, 17), labelled as *S. cantharus* and *L. mormyrus*.

(Gelcompany, Tübingen, Germany), electrode strips were soaked in Delect Buffer.

Electrophoresis took place in three steps in the cooled (10–15 °C) Multiphor II – chamber (GE Healthcare, Freiburg, Germany). Electrophoresis settings for a DNA-HP Clean full-size gel were: (1) 200 V, 20 mA and 10 W for 10 min, (2) 375 V, 30 mA and 20 W for 50 min, (3) 450 V, 30 mA and 20 W, for about 60 min.

Silver staining of the gel was done according to the protocol from Gelcompany, as described in Schiefenhövel and Rehbein (2010).

2.4. DNA sequencing

Purification of PCR products and determination of DNA sequences were described earlier in Schiefenhövel and Rehbein

Table 2

List of other authentic samples collected from research cruises, and commercial products from the German market (Date of BLAST: 2011-08-16).

Code	Origin	Product	Labelling (Latin name)	Result of BLAST
13	Wholesale trader	Fillet	<i>Sparus aurata</i>	n.d.
14	Research vessel, caught in the Bay of Biscay	Whole	<i>Spondyliosoma cantharus</i>	n.d.
15	Research vessel, caught in the Bay of Biscay	Whole	<i>Spondyliosoma cantharus</i>	n.d.
16a	Wholesale trader, caught in Middle East Atlantic	Fillet	<i>Spondyliosoma cantharus</i>	BLAST: 99% id to EF456013.1 (<i>Pagellus bellottii</i>)
16b	Wholesale trader, caught in Middle East Atlantic	Fillet	<i>Spondyliosoma cantharus</i>	n.d.
16c	Wholesale trader, caught in Middle East Atlantic	Fillet	<i>Spondyliosoma cantharus</i>	n.d.
16d	Wholesale trader, caught in Middle East Atlantic	Fillet	<i>Spondyliosoma cantharus</i>	BLAST: 96% id to DQ197940.1 (<i>Dentex canariensis</i>)
17	Wholesale trader	Whole	<i>Lithognathus mormyrus</i>	BLAST: 97% id to DQ197961.1
18	Wholesale trader	Whole	<i>Lithognathus mormyrus</i>	n.d.

n.d. = not determined, analysed by IEF only.

(2010). Similarities in nucleotide sequences were searched in GenBank (National Center for Biotechnology Information, NCBI) using the programme BLAST (Basic Local Alignment Search Tool; May 2010). Sequences of the cytochrome b gene of authentic fish samples were submitted to GenBank like *A. spinifer* (Acc.-No. HM352756), *A. bifasciatus* (Acc.-No. HM352757), *Pagrus caeruleostictus* (Acc.-No. HM352763), *S. cantharus* (Acc.-No. HM352762), *B. boops* (Acc.-No. HM352758) and *P. bogaraveo* (Acc.-No. HM352759).

The phylogenetic tree function of DNASIS® Max Version 2.0 (Hitachi) was used to establish a dendrogram.

2.5. Isoelectrical focussing (IEF)

Extraction of proteins from white muscle, heating and dialysis of extracts, and conditions of IEF were performed according to Schiefenhövel and Rehbein (2010). Protein bands were visualized with a Coomassie staining solution. The different pI values of fish proteins were calculated against the standard protein mixture Broad pI Kit 3.5–9.3 (GE Healthcare, Freiburg, Germany) or the

Serva IEF Marker 3.5–10.7 (Serva Electrophoresis, Heidelberg, Germany) by means of the computer program Quantity One (Bio-Rad, version 4.6.8).

2.6. Recording stains-all spectra of heat-stable proteins

Preparation of dialysed, heat stable proteins was performed as described in Schiefenhövel and Rehbein (2010). Stains-all spectra were measured according to a modified method from Campbell, MacLennan, and Jorgensen (1983).

3. Results and discussion

3.1. DNA-analysis

All sparid species examined in this study reacted with the universal primer pair L14735 and H15149ad of the cytochrome b gene yielding amplicons of ~464 bp to be used for sequencing and SSCP analysis. Unambiguous sequences were obtained for all reference or commercial samples; sequences of 6 authentic fish samples (see Section 2.4 in “Materials and methods”) were submitted to GenBank. The authenticity of reference samples was confirmed by DNA sequencing and BLAST (Table 1). The submission of sequences with unequivocal assignment to a given fish species is a prerequisite for successful use of GenBank by food control laboratories to identify samples (Rehbein, Müller-Hohe, & Hanel, 2009;).

Nucleotide sequences (200 bp) of seven authentic species (*A. spinifer*, *A. bifasciatus*, *P. caeruleostictus*, *S. cantharus*, *B. boops*, *P. bogaraveo*, *S. aurata*) and three commercial samples (sample 16a and d, labelled as *S. cantharus*, and sample 17, labelled as *Lithogantus mormyrus*) are compared in Fig. 1 and shown as a dendrogram (Fig. 2) calculated from the DNA sequences given in Fig. 1. The studied species are members of different genera, and displayed a number of sequence differences being sufficient for distinction between species, in spite of the short DNA segment used. By constructing appropriate primers, DNA segments of ~200 bp may be used to identify sparid fish not only in case of raw fillet, but also as heated products, like fried or cooked fillet or canned fish (Rasmussen & Morrissey, 2008).

The phylogenetic relationships between sparids and their subfamilies were first studied by Hanel and Sturmbauer (2000) based on 16S rRNA gene, who concluded the current taxonomy does not reflect the evolutionary history of the family. This finding was later confirmed by De la Herrán et al. (2001), Orrell, Carpenter, Musick, and Graves (2004) and Chiba, Iwatsuki, Yoshino, and Hanzawa (2009) using other markers. This view, especially the nonmonophyly of the described subfamilies Sparinae, Pagellinae, Denticinae and Boopsinae is corroborated by the dendrogram shown in Fig. 2, placing sparids from the same subfamily into different branches of the tree (e.g. the *Boopsinae* species *B. boops* and *S. cantharus*).

In SSCP, analysis five of the examined species (*A. spinifer*, *A. bifasciatus*, *P. caeruleostictus*, *S. cantharus*, *S. aurata*) showed distinctive patterns of single stranded DNA (Fig. 3). Intra-species variability of the DNA pattern was observed in case of *S. aurata* A (Fig. 3a and b). The figures are taken from two SSCP gels run with different times of electrophoresis. It has been demonstrated that the duration of electrophoresis can influence the movement of single stranded DNA through the polyacrylamide gel (Rehbein, 2005).

Analysis of the commercial samples 16a–d (labelled as *S. cantharus*) by SSCP revealed mislabelling of these products (Fig. 3b). Authentic A did not show the same SSCP profile like the commercial samples 16a–d; the heterogeneous patterns of this sample set did not match to the ssDNA pattern of any other Sparidae species (Fig. 3b). Sequencing of the amplicon of sample 16a and 16d and application of BLAST gave for sample 16a a 99% identity to *Pagellus*

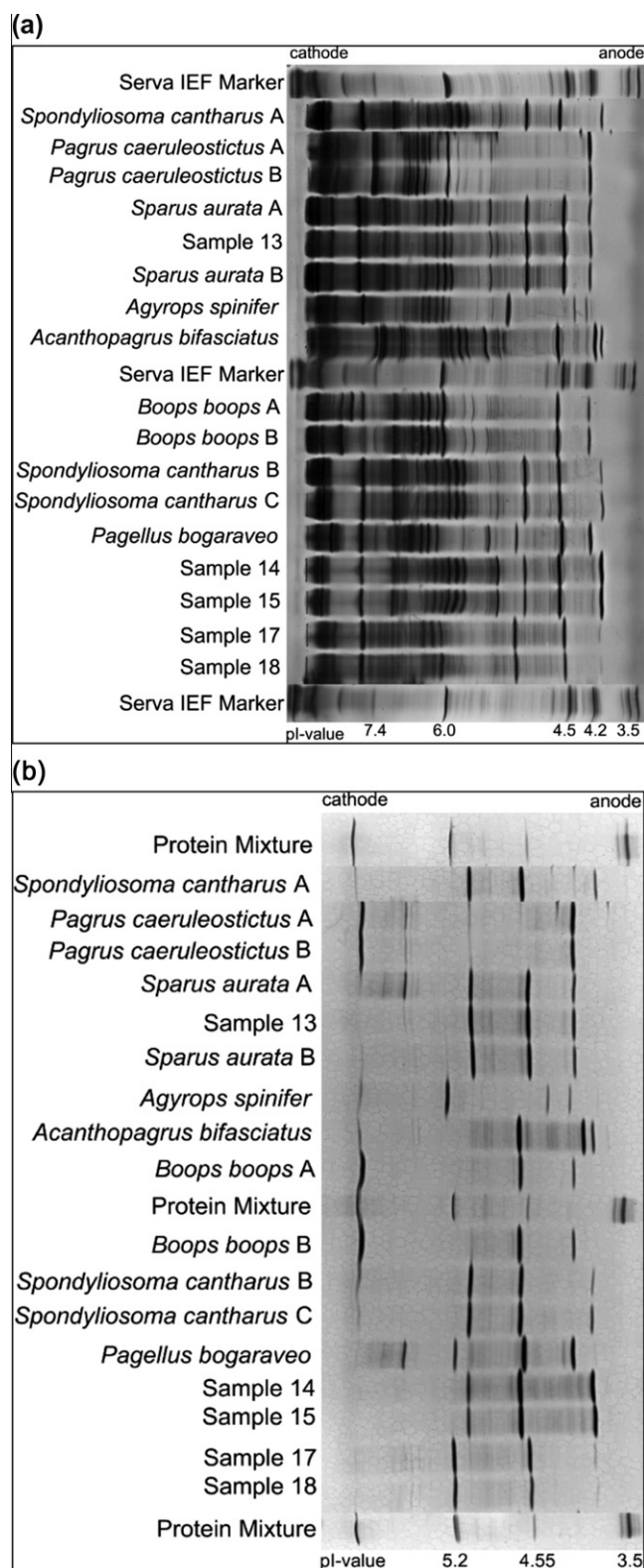


Fig. 4. (a) Protein patterns (IEF with Servalyt-Precote 3–10) of authentic sparid fish species (*S. cantharus*, *P. caeruleostictus*, *S. aurata*, *A. spinifer*, *A. bifasciatus*, *B. boops* and *P. bogaraveo*) and commercial samples (*S. aurata*, *S. cantharus* and *L. mormyrus*). (b) Pattern of heat stable proteins (IEF with Servalyt-Precote 3–6) of authentic sparid fish species (*S. cantharus*, *P. caeruleostictus*, *S. aurata*, *A. spinifer*, *A. bifasciatus*, *B. boops* and *P. bogaraveo*) and commercial samples (*S. aurata*, *S. cantharus* and *L. mormyrus*).

bellottii and for sample 16d a 96% identity to *Dentex canariensis* (Table 2). In addition, there is also an 11%, respectively 13%,

disagreement in nucleotide sequence for both samples in comparison with *S. cantharus* sequence (Fig. 1). From these results following conclusions can be drawn: (i) None of the samples 16 a–d, labelled as *S. cantharus*, did contain this species; (ii) SSCP analysis was successful in uncovering the false declaration (Fig. 3b), and (iii) sequencing allowed species identification of at least one of the samples (16a).

3.2. Protein-analysis: species identification

The seven species of the family Sparidae expressed species specific protein profiles, characterized by strong protein bands in the anodic part of the IEF gel (Fig. 4). With the exception of raw extracts of *B. boops* and *Pagrus caeruleostictus*, showing protein polymorphism in the cathodic part of the gel (Fig. 4), no intra-species variability of the protein patterns has been observed. The pI values have been compiled in Table 3 (Supplementary data).

S. aurata A and B, and commercial sample 13, labelled as *S. aurata*, showed the same protein profile (Fig. 4a), corroborating the result of SSCP analysis (Fig. 3a). *S. cantharus* A, B and C specimen displayed the same protein pattern, matching to the pattern of samples 14 and 15, labelled as *S. cantharus*, with 2 bands (pI 3.99 and 5.29) varying in intensity (Fig. 4a). However, the protein profile (data not shown) and pI values (Supplementary data: Table 3) of commercial sample 16a (identified as *P. belottii* by BLAST as described above) were found to be different to the data of authentic samples of *S. cantharus*, indicating mis-labelling of this sample; this result is in accordance with the findings from DNA analysis (see previous section).

Products A and B of species *B. boops* and *Pagrus caeruleostictus* denoted minor intra-species variability IEF of bands (Fig. 4a). On the other hand, the SSCP analysis showed homogeneity in bands for products of *Pagrus caeruleostictus* A and B (Fig. 3).

3.3. Protein-analysis: indications of the presence of parvalbumins

The above mentioned protein bands in the anodic part of the IEF gel were found to be heat-stable (Fig. 4b) and coloured blue to violet by reacting with the cationic carbocyanine dye, “stains-all”. The wavelengths of the absorption maxima for “stains all” treated heat stable proteins of a number of fish species have been compiled (table 4, Supplementary data). The maxima were located between 607 nm and 639 nm, as to be expected from blue colour of the solutions, indicating the presence of calcium-binding proteins (Campbell et al., 1983).

Due to their physico-chemical properties (heat stability, low pI) and reactivity against the cationic carbocyanine dye, “stains-all” the acidic proteins were assumed to belong to the class of parvalbumins (Arif et al., 2007; Schiefenhövel & Rehbein, 2010).

Proteins belonging to the family of parvalbumins are known to have a high allergenic potential (Kruse Fæste, 2010). They have been found in the light (white) rather than in dark (red) muscle of fish (Schoenman et al., 2010). Fish allergies seem to occur more often in populations having high fish consumption (Kruse Fæste, 2010). In general, people who are allergic to one fish species should not ingest any other, because cross allergies can occur (Arif et al., 2007). However, the allergenic potential of fish may vary according to the concentration of parvalbumin in the fillet (Focant, Vandewalle, & Huriaux, 2003; Schoenman et al., 2010).

Parvalbumin proteins can also be used for species identification in raw or heat-processed fish, because they display species specific patterns in the acidic range in isoelectric focusing (Arif et al., 2007). Also the results of this study showed, that the prominent acidic protein bands were sufficient to distinguish between seven sparid species analysed by IEF (Fig. 4b). The species showed pI-values of heat-stable, acid proteins between 3.8 and 5.8 (Supplementary

data: Table 4). In case of the black seabream (*S. cantharus*), the pI-values of heated and dialysed proteins correspond with those from a study by Rehbein et al. (2000).

4. Conclusions

PCR-based DNA analysis and protein isoelectric focusing have been found to be useful tools for identification and differentiation of species belonging to the family of Sparidae. Even closely related fish species could be distinguished through their unique protein, SSCP and sequence profile; exceptions have to be kept in mind, as some species displaying heterogeneity in SSCP and IEF analysis. In that case, more than one method may be necessary to differentiate between species. All of the fish species analysed expressed parvalbumins, giving them an allergic potential.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.10.057>.

References

- Alarcón, J. A., & Alvarez, M. C. (1999). Genetic identification of sparid species by isozyme markers: Application to interspecific hybrids. *Aquaculture*, 173, 95–103.
- Arif, S. H., Jabeen, M., & Hasnain, A.-U. (2007). Biochemical characterization and thermostable capacity of parvalbumins: The major fish-food allergens. *Journal of Food Biochemistry*, 31, 121–137.
- Basurco, B., Lovatelli, A., & Garcia, B. (2011). Current status of Sparidae aquaculture. In M. A. Pavlidis & C. C. Mylonas (Eds.), *Sparidae biology and aquaculture of gilthead sea bream and other species* (pp. 1–50). Wiley-Blackwell.
- BLE (Federal Agency for Agriculture and Food) (2011). *List of market names of products from fisheries and aquaculture*. <http://www.ble.de>.
- Campbell, K. P., MacLennan, D. H., & Jorgensen, A. O. (1983). Staining of the Ca²⁺-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye “Stains-all”. *The Journal of Biological Chemistry*, 258(25), 11267–11273.
- Carpenter, K.E., & Niem, V.H. (2001). FAO Species Identification Guide for Fishery Purpose. *The Living Marine Resources of the Western Central Pacific: Bony fishes part 3 (Menidae to Pomacentridae)* (Vol. 5). Rome: Food and Agriculture Organization of the United Nations.
- Chiba, S. N., Iwatsuki, Y., Yoshino, T., & Hanzawa, N. (2009). Comprehensive phylogeny of the family Sparidae (Perciformes: Teleostei) inferred from mitochondrial gene analysis. *Genes and Genetic Systems*, 84, 153–170.
- Cocolin, L., D'Agaro, E., Manzano, M., Lanari, D., & Comi, G. (2000). Rapid PCR-RFLP method for the identification of marine fish fillets (Seabass, Seabream, Umbrine, and Dentex). *Journal of Food Science*, 65, 1315–1317.
- De la Herrán, R., Ruiz Rejón, C., Ruiz Rejón, M., & Garrido-Ramos, M. A. (2001). The molecular phylogeny of the Sparidae (Pisces, Perciformes) based on two satellite DNA families. *Heredity*, 87, 691–697.
- Downs, T. R., & Wilfinger, W. W. (1983). Fluorimetric quantification of DNA in cells and tissues. *Analytical Biochemistry*, 131, 538–547.
- FAO statistics (2009). Available from <<http://www.fao.org/fishery/statistics/en>> Accessed December 2011.
- FAO (2010). *FACT SHEET: The international fish trade and world fisheries*. United Nations Food and Agriculture Organization. Available from <<http://www.fao.org>> Accessed November 2011.
- Fischbase (2010). Available from <<http://www.fishbase.org>> Accessed March 2010.

- Filonzi, L., Chiesa, S., Vaghi, M., & Nonnis Marzano, F. (2010). Molecular barcoding reveals mislabelling of commercial fish products in Italy. *Food Research International*, 43, 1383–1388.
- Focant, B., Vandewalle, P., & Huriaux, F. (2003). Expression of myofibrillar proteins and parvalbumin isoforms during the development of a flatfish, the common sole *Solea solea*: comparison with the turbot *Scophthalmus maximus*. *Comparative Biochemistry and Physiology*, 135B, 493–502.
- Girija, N., & Rehbein, H. (1988). Comparison of parvalbumin patterns from different fish species by isoelectric focusing of muscle extracts. *Comparative Biochemistry and Physiology*, 91B, 723–728.
- Hanel, R., & Sturmbauer, C. (2000). Multiple recurrent evolution of trophic types in Northeastern Atlantic and Mediterranean seabreams (Sparidae, Percoidae). *Journal of Molecular Evolution*, 50, 276–283.
- Hanel, R., & Tsigenopoulos, C. (2011). Phylogeny, evolution, and taxonomy of sparids with some notes on their ecology and biology. In M. A. Pavlidis & C. C. Mylonas (Eds.), *Sparidae biology and aquaculture of gilthead sea bream and other species* (pp. 51–73). Wiley-Blackwell.
- Hanner, R., Becker, S., Ivanova, N. V., & Steinke, D. (2011). FISH-BOL and seafood identification: geographically dispersed case studies reveal systemic market substitution across Canada. *Mitochondrial DNA*, 22(S1), 106–122.
- Jacquet, J. L., & Pauly, D. (2008). Trade secrets: Renaming and mislabelling of seafood. *Marine Policy*, 32, 309–318.
- Kruse Fæste, C. (2010). Fish allergen detection. In B. Popping, C. Diaz-Amigo, K. Hoenicke, & John Wiley & Sons (Eds.), *Molecular biological and immunological techniques and applications for food chemists* (pp. 407–422). Weinheim: Wiley-VCH.
- Orrell, T. M., Carpenter, K. E., Musick, J. A., & Graves, J. E. (2004). Phylogenetic and biogeographic analysis of the Sparidae (Perciformes: Percoidae) from cytochrome b sequences. *Copeia*, 2002(3), 618–631.
- Rasmussen, R. S., & Morrissey, M. T. (2008). DNA-based methods for the identification of commercial fish and seafood species. *Comprehensive Reviews Food Science Safety*, 7, 280–295.
- Rehbein, H. (2005). Identification of the fish species of raw or cold-smoked salmon and salmon caviar by single-strand conformation polymorphism (SSCP) analysis. *European Food Research and Technology*, 220, 625–632.
- Rehbein, H., Kündiger, R., Pineiro, C., & Perez-Martin, R. I. (2000). Fish muscle parvalbumins as marker proteins for native and urea isoelectric focusing. *Electrophoresis*, 21, 1458–1463.
- Rehbein, H., Müller-Hohe, E., & Hanel, R. (2009). False fish – A report of difficulties to identify a “sole”. *Informationen aus der Fischereiforschung*, 56, 35–40.
- Schiefenhövel, K., & Rehbein, H. (2010). Identification of barramundi (*Lates calcarifer*) and tilapia (*Oreochromis* spp.) fillets by DNA-and protein-analytical methods. *Journal of Consumer Protection and Food Safety*, 6, 203–214.
- Schoenman, E. R., Chiaro, J. A., Jones, A., Bastin, L. D., & Coughlin, D. J. (2010). A comparative analysis of parvalbumin expression in pinfish (*Lagodon rhomboides*) and toadfish (*Opsanus* sp.). *Comparative Biochemistry and Physiology*, 155A, 91–99.
- Smith, M. M., & Heemstra, P. C. (2003). *Smith's sea fishes*. Struik Publishers, Springer-Verlag.
- Whitehead, P. J. P., Bauchot, M.-L., Hureau, J.-C., Nielsen, J., & Tortonese, E. (1986). *Fishes of the North-eastern Atlantic and the Mediterranean* (Vol. II). United Nations Educational, Scientific and Cultural Organization (Unesco).
- Wolf, C., Burgener, M., Hübner, P., & Lüthy, J. (2000). PCR-RFLP analysis of mitochondrial DANN: differentiation of fish species. *Lebensmittel-Wissenschaft & Technologie*, 33, 144–150.

1 **Supplementary data**

2
3 Tab. 3: pI values of protein bands obtained by IEF (see Figure 4)

4

code	Species	pI-values: > 6	6 - 5	< 5	pI-values (heated and dialysed)
5	<i>Spondyliosoma cantharus</i> A	7.09 6.63	5.92 5.81 5.70	4.95 4.58 3.98	5.03 4.59 3.80
3	<i>Pagrus caeruleostictus</i> A	6.89 6.44 6.34 6.27 6.24 6.19	5.96	4.20	5.79 4.03
4	<i>Pagrus caeruleostictus</i> B	6.89 6.43 6.33 6.23	5.95	4.19	5.80 4.04
11	<i>Sparus aurata</i> A	7.15 6.77 6.61	6.08 5.98 5.90 5.38	4.93 4.52 4.18	5.51 5.03 4.54 4.02
12	<i>Sparus aurata</i> B	7.16 6.77 6.61	6.07 5.98 5.88 5.36	4.92 4.51 4.17	5.01 4.52 4.01
1	<i>Argyrops spinifer</i>	7.10 6.26 6.18 6.08 6.01 5.92	5.13	4.36 4.22	5.23 4.31 4.05
2	<i>Acanthopagrus bifasciatus</i>	6.88 6.79	5.89 5.77 5.67 5.42	4.56 4.04 3.92	4.62 3.91 3.81
8	<i>Boops boops</i> A	7.28 7.09 6.61 6.26 6.17 5.98		4.57 4.17	5.81 4.61 4.02
9	<i>Boops boops</i> B	7.10 6.62 6.26 6.18 5.99		4.58 4.18	5.82 4.62 4.02
6	<i>Spondyliosoma cantharus</i> B	7.09 6.62	5.92 5.81 5.71	4.95 4.56 3.95	5.05 4.61 3.82
7	<i>Spondyliosoma cantharus</i> C	7.08 6.62	5.92 5.81 5.72	4.95 4.56 3.93	5.06 4.62 3.83
10	<i>Pagellus bogaraveo</i>	7.10 6.41 6.28 6.19 6.09	5.40 5.06	4.54 4.20	5.53 5.19 4.60 4.05

Table 4: Maxima of absorption spectra ($OD_{\max}$) of “stains-all” treated thermostable proteins of sparid fishes

code	Species	Wavelength [nm]	$OD_{\max}$
1	<i>Argyrops spinifer</i>	624.0	0.87
2	<i>Acanthopagrus bifasciatus</i>	636.9	0.98
3	<i>Pagrus caeruleostictus</i> A	636.1	1.17
4	<i>Pagrus caeruleostictus</i> B	630.9	1.13
5	<i>Spondyliosoma cantharus</i> A	639.0	1.00
6	<i>Spondyliosoma cantharus</i> B	634.0	0.86
7	<i>Spondyliosoma cantharus</i> C	640.0	0.96
8	<i>Boops boops</i> A	633.0	1.01
9	<i>Boops boops</i> B	638.0	1.05
10	<i>Pagellus bogaraveo</i>	640.0	1.13
11	<i>Sparus aurata</i> A	630.9	1.05

