Methods of gadoid fish species identification in food and their economic impact in the Czech Republic: a review

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ABSTRACT: The purpose of the present study was to give an overview of imported and traded gadoid fish species (*Gadiformes*) in the Czech Republic and to describe available methods for their authentication. Due to the increasing interest of customers in the purchase of buy fish meat and other seafood animals, it is necessary to have available analytical methods with discriminating power of respective fish species. With regard to different values and prices of various fish species, these may be adultered. Until recently, electrophoretic, chromatographic and immunological methods based on the analysis of proteins extracted from fish musculature seemed to be promising. Using these methods, various fish species can be identified in fresh, chilled and frozen products. However, they often fail in heat treated products. Molecular biology methods based on DNA analysis are more reliable and suitable for the analysis of fish products that have been heat treated during the production process.

Keywords: cod fish; fish species identification; electrophoresis; PCR-RFLP; cytochrome *b* gene; food adulteration

List of abbreviations

AK = adenylate kinase; **ARGK** = arginine kinase; **ATP** = adenosine triphosphate; **CE** = capillary electrophoresis; **CK** = creatine kinase; **CR** = Czech Republic; **DGGE** = denaturating gradient gel electrophoresis; **DHA** = docosahexaen acid; **DNA** = deoxyribonucleotide acid; **EPA** = eicosapentaen acid; **EU** = European Union; **FINS** = forensically informative nucleotide sequencing; **G 3-PD** = glycerol 3-phosphate dehydrogenase; **HPLC** = high performance liquid chromatography; **IEF** = isoelectric focusing; **LDH** = lactate dehydrogenase; **MDH** = malate dehydrogenase; **MS** = mass spectrometry; **mt cyt *b*** = mitochondrial cytochrome *b* gene; **Mw** = molecular weight; **NDKA** = nucleoside diphosphate kinase A; **NTSs** = nontranscribed spacers; **PCR** = polymerase chain reaction; **PCR-RAPD** = polymerase chain reaction – random amplified polymorphic DNA; **PCR-RFLP** = polymerase chain reaction – restriction fragment length polymorphism; **PCR-SSCP** = polymerase chain reaction – single strand conformation polymorphism; **pI** = isoelectric point; **RP-HPLC** = reversed phased – high performance liquid chromatography; **SDS-PAGE** = sodium dodecyl sulphate – polyacrylamide gel electrophoresis; **urea-IEF** = urea isoelectric focusing; **2DE** = two dimensional electrophoresis

Contents

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1. Introduction

Due to an increasing range of fish species and a variety of fish products appearing in the market, it is inevitable to develop and innovate current methods used for fish species identification. Labelling of respective fish species is necessary from an aspect of prevention of adulteration because fish species of different values and prices may be fraudulently substituted. Gadoid fish are an important group of sea fish imported to the Czech Republic (CR). Fish of different meat values and commercial use are classified in this group. Three genera of gadoid fish are the most significant: *Gadus*, *Theragra* and *Merluccius*. The spectrum of gadoid fish products offering in the Czech market includes frozen whole fishes, frozen fillets or blocks of meat and other processed products (fish fingers, fish cakes, canned cod's liver, surimi, spreads, salt-preserved cod or cod in mayonnaise).

2. Fish product market in CR

2.1. Fish consumption in CR

Production and consumption of fish and fish products in CR does not represent a substantial proportion of meat production as it does in some other countries. Since 1992, a completely different assortment of sea and freshwater fish and fish products including fresh and frozen seafood has appeared in the CR market. In previous years, the market was above all supplied with carp products, fillets and canned fish. Consumption of fish products in CR is slowly increasing, although it is still low in comparison with other EU countries. According to the data presented by the Czech Statistical Office annual fish consumption in 1993–2003 in CR reached 4.5 to 5.4 kg per person, of which 0.9 to 1.1 kg were freshwater fish (Figure 1). The world and EU fish consumption is on average 16 and 11 kg/person per year, respectively.

2.2. Nutritional value of fish meat

A wide range of gadoid fish is used by the food industry (Table 1). Dietetic value of fish meat is high due to a favourable ratio of essential amino acids and lipid composition, above all the content of omega-3 fatty acids (eicosapentaene acid – EPA and docosahexaenoic acid – DHA). EPA and DHA are polyunsaturated fatty acids derived from alfa-linolenic acid that act preventatively against development of cardiovascular diseases.

Table 1. Gadoid fish usable in the food industry (Konecny and Pavlicek, 1997)

Species (Latin name)	Species (English name)	Geographic area	Meat characteristics
Gadus morhua	Atlantic cod, cod	North Atlantic and North seas of Europe	dry, white, high value meat, mostly fillets, livers for canning
Gadus macrocephalus	Pacific cod	Pacific Ocean from North America to Kamchatka	dry, white, tasty meat, mostly fillets
Gadus ogac	ogac cod, greenland cod	North-West Atlantic, Canada shore	dry, white, tasty meat
Boreogadus saida	polar cod	Arctic, Subarctic seas	tasty meat
Micromesistius poutassou	blue whiting, poutassoo	North-West Atlantic, Mediterranean and Black Seas	tasty, less value meat, fish fingers or frozen musculature
Melanogrammus aeglefinus	haddock, small haddock, gibber	West Atlantic	tasty, white, rather dry, high value meat, fillets or for smoking
Merlangius merlangus	whiting	West Europe, Mediterranean and Black Seas	white meat of specific taste, mostly salted and canned
Pollachius virens	lieu noir, coalfish, saithe	North Atlantic, USA and Canada East shore	rather dark meat, tasty, sold fresh, frozen and canned in oil
Pollachius pollachius	pollack	Baltic and Mediterranean Seas	tasty, high value smoking meat
Theragra chalcogramma	walley pollock, Alaska pollack	North Pacific Ocean	soft, tender, white, high value meat, fresh, frozen – in a form of fillet blocks of pure musculature
Trisopterus esmarkii	Norway pout	around Island	tender, white, high value meat
Trisopterus luscus	bib, whiting pour	Mediterranean Sea	tasty meat, for smoking and canning
Brosme brosme	tusk, torsk, cusk	Atlantic	tender, white, high value meat
Molva molva	ling	Norwegian and North Seas	tender, white, dry meat
Molva elongata	Mediterranean ling	Mediterranean Sea, the bay of Biscay	medium value meat
Molva dypterygia	blue ling, trade ling	Norwegian and North Seas	tasty meat
Phycis phycis	codling, forkbeard	Mediterranean Sea	tasty meat
Urophycis chuss	red hake, Squirrel hake	West Atlantic	tasty, white, soft meat, not very popular
Urophycis tenuis	white hake, mud hake, Boston ling	West Atlantic	tasty, white, tender, rather soft meat
Merluccius merluccius	hake, European hake	shelf waters of West Europe and Mediterranean Sea	tasty, white, tender, high value meat, sold fresh, frozen, in fillets, canned
Merluccius capensis	Cape hake, stock fish	shelf waters of South-West Africa	tasty, white, tender, rather dry meat, imported in a form of frozen fillets
Merluccius bilinearis	silver hake, Atlantic hake, offshore hake	east shores of North America and Caribbean Sea	tasty, tender, white, fresh, frozen, filleted, smoking and canning meat
Merluccius hubbsi	Argentine hake	shelf waters of Brazil, Argentina and Uruguay	tasty, tender, white meat
Merluccius productus	North Pacific hake	Pacific Ocean, from California to Alaska	tasty, off-white colour, tender meat for various purposes
Merluccius gayi	Pacific silver hake, Chilean hake	Pacific Ocean, from Ecuador to Chile	tasty, white, tender, rather soft meat for various purposes

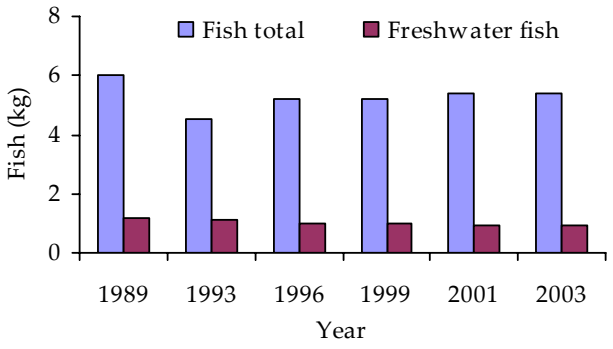

Figure 1. Fish consumption (kg/inhabitant/year; Czech Statistical Office)

Moreover, fish meat contains high concentrations of lipophilic vitamins (A, D and E), muscle fibres are tender, proportion of ligamentous tissue is low and the content of essential elements (I, Ca, P, K, F, Se, Zn, Fe and Cu) is high. Sea fish contain much higher iodine levels in comparison with freshwater fish: freshwater fish eel (*Anguilla*) – 4 µg/100 g; herring (*Clupea*) – 50 µg/100 g; cod (*Gadus*) – 200 µg/100 g. Protein content ranges between 15 and 20%, lipid content vary from less 2% in gadoid fish (*Gadiformes*), perch (*Perca*) and pike (*Esox*) to more than 10% in fish such as herring (*Clupea*), tuna (*Thunnus*), mackerel (*Scomber*) and eel (*Anguilla*). Codfish liver is a fat reservoir; its content is 40 to 65% (Mares, 2003).

2.3. Seafood importation into CR

Between January 1, 2006 and March 31, 2006 almost 3.5 mil tons of deep-frozen fish fillets and fresh sea fish was imported to the CR according to information obtained from the Customs Administration (Table 2). It follows from those data that fish of hake (family Merlucciidae), Alaska pollack (*Theragra chalcogramma*) and blue whiting (*Micromesistius poutassou*), were most often imported, in the form of fish fillets and chilled and frozen fish meat. Importation of sea fish, as well as other food industry commodities, is subject to customs control where among other security checks, documents and identity are checked to ascertain whether species, origin and labelling correspond to the covering documentation. With respect to the loss of the morphological characteristics of fish imported as fillets or blocks of compact meat, any species identification according to morphological signs is impossible. Due to this fact, high value fish species may be substituted with less value ones.

The globalization and opening of an international market, increasing requirements of customers and different values and prices of respective fish species may lead to falsification of covering documentation to foodstuffs, usually with the aim to achieve higher trading profit. This may result in serious allergic responses to a particular foodstuff component which was not mentioned on the product label. In case the fish have been caught in polluted water, the origin of fish may be falsified. Such fish often contain toxic substances, which may induce health problems in customers (Martinez and Friis, 2004).

3. Legislation concerning food adulteration, specifically adulteration of fish and fish products

3.1. EU legislation

Current legislation secures the rights of the customers to be informed about product content through a packet label. Anonymous (2000a) that entered into force in January, 2002 includes obligatory data for labelling of fish products, particularly the name of fish species (preferably both business and scientific names), geographical origin and technology of production (wild or farm kept fish). "Deception of customers" in the inland market is treated by the Anonymous (2005) on unfair trade practices.

3.2. CR legislation

When CR joined EU on May 1, 2004 legislation was harmonized and EU regulations were adopted by the Czech legislation. Fish species substitution is viewed as food adultery, and deception of customers was integrated in Anonymous (1997) as amended by later legislation. Basic conception concerning declaration of origin, quality and ingredients of foodstuffs and potential sanctions resulting from their omission is included.

The Act does not define the term "food adulteration". However it says, "it is prohibited to put foodstuffs with false labelling" into circulation. The meaning of the term is defined in the Anonymous (1992) as follows: "Nobody shall deceive customers, above all shall not show untrue, undocumented, incomplete, unclear or exaggerated data, or hold

Table 2. Fish imported into CR (Customs Administration of CR – January 1, 2006 to March 31, 2006)

Frozen fish	Amount (t)	Fish fillets and other fish meat fresh, chilled or frozen	Amount(t)
Salmo sp.	212.8	*Salmo* sp.	56.1
Oncorhynchus sp.	96.4	*Oncorhynchus* sp.	7.3
Other *Salmonidae*	21.8	*Anguilla sp.*	0.9
Clupea sp.	3.6	Other fresh water fishes	37.8
Scomber sp.	64.7	*Thunnus* sp.	2.0
Merluccius sp.	1.0	*Scomber* sp.	7.7
Other fresh water fishes (without *Carp* sp.)	2.0	*Merluccius hubbsi*	452.3
Trachurus trachurus	1.9	*Merluccius* sp.	189.5
Other marine fishes	5.5	*Mustelus* sp.	6.8
		Clupea sp.	85.2
		Theragra chalcogramma	858.5
		Other fishes	590.8
		Surimi	154.8
		Other fish meat	20.0
		Clupea sp. – slices	234.7
		Micromesistius poutassou	220.7
Total	409.7	Total	2 925.1

back data about real value of the product". Based on the Anonymous (2003b) on general safety of products "Distributors shall not distribute such products in the market net, if they – based on their information and professional knowledge – know or may presume that these do not meet requirements for product safety."

The following set of public decrees and other statutory instruments contain exact parameters characterising respective food industry commodities:

A. Anonymous (2001) concerning meat, meat products, fish, animals originating from aquaculture and aquaculture products, eggs and egg products. This presents classification of species, groups/subgroups of products and their designation. Besides species and group/subgroup designation, animal genus or species shall be given.

B. Anonymous (2000b) concerning frozen foodstuffs.

C. Anonymous (2003a) concerning veterinary requirements for animals originating from aquaculture and aquaculture products, products of fishing and live clams, and on veterinary conditions of their importation from third world countries.

4. Customer's protection and analytical detection methods of food adulteration

Every customer has the right to be truly informed about the identity of a purchased product. Above all, non-detailed and misleading labelling may cause customers' disorientation in the fish product market. Trade designation of a fish product does not often correspond to a scientific taxonomy system. Various equivalents of one fish species designation exist in one language, and translation to another language can make the meaning unclear.

Inspection Authorities of CR need fast and reliable methods that would allow analysis of large numbers of samples; these would be a means to prevent food adulteration and protect customers' rights. Prevention and check of fish product adulteration is complicated by extending assortment in the food processing industry

using fillets or minced fish meal as raw materials for fish products (Pineiro et al., 1999).

5. Overview of methods available for detection of gadoid fish and fish product adulteration

Due to the fact that most fish are imported in a form of pressed meat blocks or fillets, their identification according to external characteristics is impossible. Therefore, methods based on protein or nucleic acids analyses are used. These methods allow the following:

1. distinguish respective fish species
2. distinguish fish subspecies within one species
3. distinguish fish species in a mixed product
4. quantify a respective fish species in a product

5.1. Morphological identification

Methods of fish species identification based on external characteristics are applicable to whole or slightly processed fish. Species identification becomes much more difficult in fish that have undergone processing by the food industry and morphological criteria cannot be used (Sotelo et al., 1993; Aranishi, 2005; Dooley et al., 2005).

5.2. Protein analysis based methods

A lot of characteristics of a food industry product result from physical, chemical and functional properties of its constituent proteins, and their changes during technological processes (Recio et al., 2001). Physical chemical differences in proteins consisting in the sizes of their molecules and different charges may be assessed by electrophoretic ion migration in electrical field, method of isoelectric focusing and chromatography.

5.2.1. Electrophoretic methods

Electrophoretic methods are based on the ability of molecules to migrate according to their molecular weight (Mw) in the electric field due to the effect of electrostatic forces attracting them to reversely charged electrode. The migration is performed on agarose or polyacrylamide gel. Various modifications of electrophoretic methods are used depending on a type of the analysed product:

isoelectric focusing (IEF)
urea isoelectric focusing (urea-IEF)
sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)
two dimensional electrophoresis (2DE)
capillary electrophoresis (CE)

5.2.1.1. Isoelectric focusing (IEF)

The IEF method is based on isoelectric point (pI) differences of respective separated proteins. The principle of the method is the formation of a gradient on the gel. The moving protein molecules come to a site where pH corresponds with its pI, become neutral and their movement stops. IEF is an electrophoretic technique, which most likely possesses the highest ability to distinguish proteins. Compounds that differ from each other by 0.001 pH units can be separated by this method.

5.2.1.1.1. IEF and detection of non-heat treated fish musculature proteins

The fraction of sarcoplasmic proteins that represent 20 to 35% of fish muscle proteins are used, among others, for species identification of non-heat treated fish musculature by means of IEF. They include enzymes participating in metabolic processes (lactate dehydrogenase – LDH, glycerol 3-phosphate dehydrogenase – G 3-PD, arginin kinase – ARGK, creatin kinase – CK, adenylate kinase – AK, malate dehydrogenase – MDH, aldolase etc.) and other proteins (parvalbumin, myoalbumin, globulin). The proteins in the sarcoplasmic fraction are excellently suited to distinguishing fish species, as each species has a characteristic band pattern when separated by the isoelectric focusing method (Recio et al., 2001). Most of the sarcoplasmic proteins dissolve in aqueous solutions and undergo denaturation during heat processing. IEF of sarcoplasmic proteins has a higher discriminating power compared with classical electrophoresis (Rehbein et al., 1999).

Based on the differentiation of sarcoplasmic proteins using the IEF method, a library of commercial fish species classified in *Pleuronectiformes* and *Gadiformes* was formed (Tepedino et al., 2001). These two orders include fish species of variable commer-

cial values, which may be substituted for each other because they are imported filleted. Gadoid fish species were distinguished according to the presence of specific enzymes LDH, G 3-PD, ARGK, CK, AK and MDH in the fraction of sarcoplasmic proteins separated by IEF in the pH range of 3.5–9.5 (Tables 3 and 4). According to electrophoretic mobility of LDH and G 3-PD, majority of raw tested gadoid fish were distinguished, even the closely related species of the Merlucciidae family (Pineiro et al., 2000). A system based on a similar principle has been developed to distinguish other gadoid (Tables 3 and 4) and non-gadoid species by the IEF method of sarcoplasmic proteins (Ataman et al., 2006).

5.2.1.1.2. IEF and detection of heat treated fish musculature proteins

Musculature proteins undergo denaturation during heat treatment. Heat resistant sarcoplasmic proteins (parvalbumins) and some myofibrillar proteins (Sotelo et al., 1993; Rehbein et al., 1999; Pineiro et al., 2001) are significant markers for electrophoretic discrimination between heat treated fish species. Several isoforms of parvalbumins – acidic, heat stable, calcium binding and low Mw protein – exist. Parvalbumins of different fish species and isoforms of one species differ in pI and therefore they may be distinguished by IEF. Gadoid fish musculature contains large amounts of parvalbumins in comparison with e.g. salmonoid fish. However, the process of sample preparation demands addition of solutions that can increase solubility and differentiability of proteins.

Micromesistius poutassou (Tables 3 and 4) and other non-gadoid species have been identified in long-life canned products by the IEF method (Rehbein, 1990). Using IEF parvalbumins in an immobilized pH gradient, species of gadoid fish (Tables 3 and 4), halibut and salmonoid fish were identified (Esteve-Romero et al., 1996). Analysis of raw and cooked fish musculature allowed unequivocal species identification in all cases.

5.2.1.2. Urea-IEF and SDS-PAGE

Proteins in heat treated musculature can be detected by urea-IEF and SDS-PAGE. The difference consists in the process of sample preparation. Heat denatured proteins can be dissolved in solutions such as 2% sodium dodecyl sulphate (SDS) or 8M urea, and separated by urea-IEF or SDS-PAGE. Urea increases solubility of proteins and improves their differentiability (quality of differentiation and number of detectable proteins). Protein-bound SDS compensates for differences in charges; proteins move in gel only according to the Mw. The values of surface charges of resulting SDS-protein complexes are identical and their conformation gets unified insomuch that the relative Mw of protein is comparable with the size of its complex with SDS. Electrophoresis in solutions with SDS or urea is applicable to distinguish fish species after heat treatment; however, not in canned products (Mackie et al., 1999).

Low Mw proteins including myosins (14–23 kDa), troponins (19–30 kDa) and parvalbumins (12 kDa) are the most suitable candidates for the use of the urea-IEF and SDS-PAGE methods to distinguish respective fish species. Species identification of raw or heat treated gadoid fish by urea-IEF and SDS-PAGE (Etienne et al., 1999; Pineiro et al., 1999; Rehbein et al., 1999) are presented in Tables 3 and 4. An isoelectric calibration kit containing fish muscle parvalbumins was prepared and tested for its suitability for IEF in the presence of 8M urea. Comparison of pI's determined under native or denaturing (urea) conditions shows that the presence of urea resulted in a pI shift of parvalbumins to higher values. It may be explained by the influence of urea on the dissociation constants of the acidic and basic groups of proteins, and the denaturing effect of urea resulting in the conformation change of protein (Rehbein et al., 2000).

5.2.1.2.1. IEF and SDS-PAGE sensitivities for fish species identification

Comparison of accuracy and sensitivity of respective electrophoretic techniques was performed. The urea-IEF, SDS-PAGE and native IEF methods have been compared from an aspect of their ability to electrophoretically distinguish between *Gadus morhua, Theragra chalcogramma, Pollachius virens* and *Macruronus novaezelandiae* (Tables 3, 4) and other fish species from fish products (Etienne et al., 2001). All three methods distinguished fish species from formed products, however, only urea-IEF and SDS-PAGE were effective in high pressure treated products. It was not possible to distinguish *Gadus morhua* from *Theragra chalcogramma* by SDS-PAGE even though this method can distinguish be-

Table 3. Species identification of gadoid fish by means of protein analysis based methods

Species	Method						References
	IEF	urea-IEF	SDS-PAGE	2DE	chromato-graphic	immunolo-gical	
Merluccius merluccius	+[1,2]	+[3]	+[4,5]	+[6]	+[7]	nt	[1]Pineiro et al. (2000), [2]Esteve-Romero et al. (1996), [3]Etienne et al. (1999), [4]Rehbein et al. (1999), [5]Pineiro et al. (1999), [6]Pineiro et al. (1998), [7]Pineiro et al. (1997)
M. australis	+[1]	nt	nt	+[2,3]	nt	nt	[1]Pineiro et al. (2000), [2]Pineiro et al. (1998), [3]Pineiro et al. (2001)
M. hubbsi	+[1]	nt	nt	+[2]	nt	nt	[1]Pineiro et al. (2000), [2]Pineiro et al. (1998)
M. gayi	+	nt	nt	nt	nt	nt	Pineiro et al. (2000)
M. capensis	+[1]	nt	nt	+[2]	nt	nt	[1]Pineiro et al. (2000), [2]Pineiro et al. (1998)
Gadus morhua	+[1,2,3]	+[4,5,6,]	+[7,8]	+[9]	+[10]	+[11]	[1]Pineiro et al. (2000), [2]Etienne et al. (2001), [3]Esteve-Romero et al. (1996), [4]Rehbein et al. (1999), [5]Etienne et al. (1999), [6]Etienne et al. (2001), [7]Rehbein et al. (1999), [8]Pineiro et al. (1999), [9]Pineiro et al. (1998), [10]Pineiro et al. (1997), [11]Dominquez et al. (1997)
Pollachius pollachius	+[1]	+[2]	+[3]	+[4]	+[5]	nt	[1]Pineiro et al. (2000), [2]Etienne et al (1999), [3]Pineiro et al. (1999), [4]Pineiro et al. (1998), [5]Pineiro et al. (1997)
Micromesistius poutassou	+[1,2]	nt	+[3]	+[4]	nt	nt	[1]Pineiro et al. (2000), [2]Rehbein (1990), [3]Pineiro et al. (1999), [4]Pineiro et al. (1998)
Merlangius merlangus	+[1,2]	+[3]	+[4]	nt	+[5]	nt	[1]Ataman et al. (2006), [2]Esteve-Romero et al. (1996), [3]Etienne et al. (1999), [4]Rehbein et al. (1999), [5]Pineiro et al. (1997)
Pollachius virens	+[1,2]	+[3,4]	+[5,6]	nt	nt	nt	[1]Ataman et al. (2006), [2]Etienne et al. (2001), [3]Etienne et al. (1999), [4]Etienne et al. (2001), [5]Etienne et al. (2001), [6]Pineiro et al. (1999)
Trisopterus minutus	+	nt	nt	nt	nt	nt	Ataman et al. (2006)
Theragra chalcogramma	+[1,2]	+[3,4]	+[5]	nt	+[6]	+[7]	[1]Esteve-Romero et al. (1996), [2]Etienne et al.(2001), [3]Etienne et al. (1999), [4]Etienne et al. (2001), [5]Pineiro et al. (1999), [6]Pineiro et al. (1997), [7]Ochiai and Watabe (2003)
Melanogrammus aeglefinus	+[1]	+[2,3]	+[4,5]	nt	nt	nt	[1]Esteve-Romero et al. (1996), [2]Rehbein et al. (1999), [3]Etienne et al. (1999), [4]Rehbein et al. (1999), [5]Pineiro et al. (1999)
Gadus ogac	+	nt	nt	nt	nt	nt	Esteve-Romero et al. (1996)
Gadus macrocephalus	+	nt	nt	nt	nt	nt	Esteve-Romero et al. (1996)
Molva molva	nt	+[1,2]	+[3]	nt	nt	nt	[1]Rehbein et al. (1999), [2]Etienne et al. (1999), [3]Pineiro et al. (1999)
Macruronus novaezelandiae	+	+	+	nt	nt	nt	Etienne et al. (2001)

+ = identified, nt = not tested

tween the other gadoid fish species. If proteins are not denatured (formed products), native IEF can be used. Urea-IEF is preferred for differentiation between closely related fish species with a high content of parvalbumin in musculature (gadoid fish). On the other hand, urea-IEF is less specific than SDS-PAGE for differentiation between salmonoid fish and tuna fish, whose proteins show neutral or alkalic characteristics (Etienne et al., 2001).

Species identification in smoked fish products (salmonids, eels) using SDS-PAGE, urea-IEF and native IEF methods was investigated by Mackie et al. (2000). Although more protein zones were obtained with SDS-PAGE than with urea-IEF, there

Table 4. Protein analysis based methods used for species identification of gadoid fish

Method	Analyzed proteins	Raw/Heat treated	Species	References
IEF	sarcoplasmic proteins	raw	*Merluccius merluccius*, *M. australis*, *M. hubbsi*, *M. gayi*, *M. capensis*, *Gadus morhua*, *Pollachius pollachius*, *Micromesistius poutassou*	Pineiro et al. (2000)
	sarcoplasmic proteins	raw	*Pollachius virens*, *Merlangius merlangus*, *Trisopterus minutus*	Ataman et al. (2006)
	acid sarcoplasmic proteins	heat treated (121°C)	*Micromesistius poutassou*	Rehbein (1990)
	protein extract	raw	*Gadus morhua*, *Theragra chalcogramma*, *Pollachius virens*, *Macruronus novaezelandiae*	Etienne et al. (2001)
	parvalbumins	raw and heat treated (100°C, 5 min in water bath)	*Gadus morhua*, *Theragra chalcogramma*, *G. ogac*, *G. macrocephalus*, *Melanogrammus aeglefinus*, *Merlangius merlangus*, *Merluccius merluccius*	Esteve-Romero et al. (1996)
Urea-IEF	parvalbumins	heat treated (85°C, 2 min in the sample core)	*Gadus morhua*, *Melanogrammus aeglefinus*, *Molva molva*	Rehbein et al. (1999)
	parvalbumins	raw and heat treated (85°C in the sample core)	*Gadus morhua*, *Melanogrammus aeglefinus*, *Merlangius merlangus*, *Pollachius virens*, *Pollachius pollachius*, *Theragra chalcogramma*, *Molva molva*, *Merluccius merluccius*	Etienne et al. (1999)
	protein fraction	HP[1] treatment	*Pollachius virens*	Etienne et al. (2001)
	protein fraction	raw	*Gadus morhua*, *Theragra chalcogramma*, *Pollachius virens*, *Macruronus novaezelandiae*	Etienne et al. (2001)
SDS-PAGE	parvalbumins	heat treated (85°C, 2 min in the sample core)	*Gadus morhua*, *Melanogrammus aeglefinus*, *Merlangius merlangus*, *Merluccius merluccius*	Rehbein et al. (1999)
	protein extract	raw and heat treated (85°C in the sample core)	*Gadus morhua*, *Pollachius pollachius*, *Theragra chalcogramma*, *Molva molva*, *Mellanogrammus aeglefinus*, *Pollachius virens*, *Micromesistius poutassou*, *Merluccius merluccius*	Pineiro et al. (1999)
	protein extract	raw	*Macruronus novaezelandiae*, *Pollachius virens*, *Gadus morhua**, *Theragra chalcogramma**	Etienne et al. (2001)
	protein extract	HP[1] treatment	*Pollachius virens*	Etienne et al. (2001)
2DE	parvalbumins		*Gadus morhua*, *Pollachius pollachius*, *Micromesistius poutasou*, *Merluccius merluccius*, *M. australis*, *M. capensis*, *M. hubbsi*	Pineiro et al. (1998)
	parvalbumin fraction	raw	*Merluccius australis*	Pineiro et al. (2001)
Chromatographic	sarcoplasmic proteins	raw	*Pollachius pollachius*, *Merluccius merluccius*, *Gadus morhua*, *Merlangius merlangus*, *Theragra chalcogramma*	Pineiro et al. (1997)
Immunological	protein extract	heat treated (smoked)	*Gadus morhua*	Dominquez et al. (1997)
	protein extract	raw	*Theragra chalcogramma*	Ochiai and Watabe (2003)

[1]HP treatment (high pressure treatment) – 350 MPa, 15°C, 15 min; *not identified

were generally fewer zones that were species determinating. Authors preferred urea-IEF method for authentication of some salmonoid fish species (*Salmo* and *Oncorhynchus*) in contrast to Etienne et al. (2001). SDS-PAGE ability to distinguish other fish species (*S. alpinus* and *O. mykiss*) was though

higher. The choice of a method depends on a type of an analysed product, particularly on parvalbumin content in musculature. However, these methods are time consuming and technical equipment requiring, which are their drawbacks (Rehbein et al., 1999).

5.2.1.3. Two dimensional electrophoresis (2DE)

Electrophoretic methods may be combined to reach better separation of proteins. The combination of IEF and SDS-PAGE is possible. This method consists in the separation of molecules according to pI and subsequently according to the size of molecules. 2DE of parvalbumins present in white musculature of gadoid fish was used for their identification (Tables 3 and 4). Detailed characterization of protein pI and Mw allowed differentiation between respective species including very closely related members of Merlucciidae. Species-specific parvalbumins (Mw < 13 kDa, pI < 4.6) have been observed in majority of species. *M. merluccius* shared one parvalbumin with *M. australis* and *M. hubbsi*, and two parvalbumins with *M. capensis* (Pineiro et al., 1998).

Pineiro et al. (2001) concentrated on the authentication of five species of hakes *M. merluccius, M. australis, M. hubbsi, M. gayi* and *M. capensis* based on differentiation between species-specific sarcoplasmic polypeptides using the 2DE method and subsequent mass spectrometry (MS) of the isolated proteins. Two groups of polypeptides separated by 2DE have been selected (group of polypeptides with pI 5.0–5.5; Mw 17 kDa and parvalbumin fraction of proteins with pI < 4.5; Mw < 12 kDa) for MS analysis. The group of polypeptides allowed classification of hake species in two categories:

1. *M. merluccius* and *M. capensis*
2. *M. australis, M. hubbsi* and *M. gayi*

In contrast, parvalbumin fraction allowed identification of *M. australis* species only (Table 3). Subsequent analysis of the group of 17 kDa polypeptides in *M. merluccius* species showed a high homology of these polypeptides with rat enzyme nucleoside diphosphate kinase A (NDKA), necessary for the synthesis of nucleoside triphosphates.

Accordingly, further research will be focused on the determination of amino acid composition of the hake homologue of NDKA as a potential compound for identification of fish species of family Merlucciidae (Pineiro et al., 2001). Protein solubility, prevention of their degradation and modification by endogenous proteolytic activity, and removal of interfering substances (if necessary) are necessary prerequisites that basically affect the quality of 2DE results.

5.2.1.4. Capillary electrophoresis (CE)

The CE method is based on differences in electrophoretic mobilities of analysed substances inside a very thin silica capillary column at a high electric tension. Application of various modifications of CE to animal protein analysis including identification of non-gadoid species by the analysis of sarcoplasmic proteins in fish musculature was reviewed by Recio et al. (2001).

5.2.2. Chromatographic methods

Chromatographic methods are separation techniques, based on different in affinity of an analyte to the mobile and stationary phases. The principle of the method is based on different hydrophobic qualities of proteins associated with amino acid composition. HPLC was described as a method suitable for fish species identification (Osman et al., 1987; Armstrong et al., 1992) also after cold or frozen storage. The HPLC method is not time demanding and can be fully automated.

Reversed phase – HPLC (RP-HPLC) was suggested for the identification of raw or frozen gadoid fish species (Tables 3 and 4). The method is based on separation of sarcoplasmic proteins and allows identification of all five gadoid fish species (Pineiro et al., 1997). The RP-HPLC method can also reveal significant differences in sarcoplasmic proteins usable for the identification of other fish species of families Salmonidae, Cyprinidae and Percidae (Knuutinen and Harjula, 1998).

The fact that a high number of samples are damaged during their preparation due to degradation or agglutination of sarcoplasmic proteins absorbing the UV beams is a drawback of this method. Their use is limited to fresh (unprocessed) fish. Chromatographic methods have been suggested for species quantification, particularly with regard to a high number of detector types developed for HPLC (Sotelo et al., 1993).

5.2.3. Immunological methods

Significant protein characteristic employed in the immunological analyses is their ability to react with specific antibodies. The advantages of immunological methods are simplicity, accuracy, and the ability to analyse high numbers of samples simultaneously. On the contrary, their drawback is potential cross-reaction between proteins of closely related fish species (Wolf et al., 2000). These methods are less applicable for an assessment of broad range of fish species, because it is necessary to prepare high numbers of specific antibodies. Various qualitative methods can be used for the detection of antigen-antibody reactions such as precipitation, immunodiffusion, immunoelectrophoresis or ELISA, which can also be used for quantitative assessment. Due to the fact that heat treatment may affect protein immunogenity, antibodies have been developed that can be used for fish species identification in cooked products (Sotelo et al., 1993). Using immunological methods, fish species identification in cooked and dried fish products including among others Alaska pollack (*Theragra chalcogramma*) was investigated (Ochiai and Watabe, 2003). Anti-myosin light chain rabbit antiserum and horseradish peroxidase conjugated anti-rabbit immunoglobulin was applied on protein bands obtained by means of SDS-PAGE. Most fish species were identified by means of fingerprinting of immunostained patterns, despite protein staining patterns were not sufficiently distinct for species identification. Dominquez et al. (1997) developed an immunodotting technique for differentiation between smoked fillets of Atlantic cod (*Gadus morhua*) and Common eel (*Anguilla anguilla*). Antiserum raised to codfish proteins did not show any cross reactivity with the eel proteins (Tables 3 and 4).

A variety of methods based on the protein analysis have been used in fish species identification. However, these methods often have a limited applicability for the highly processed fish products due to the protein denaturation. Methods based on the analysis of thermostable proteins (Asensio et al., 2003) or the use of specific chemical substances (urea, SDS), which make the denaturated proteins more distinguishable, provide much better solution.

5.3. PCR based methods

Development of molecular biology methods, especially polymerase chain reaction (PCR) and its modifications, based on amplification of specific DNA segments, contributed significantly to the detection of food adulteration (Lockley and Bardsley, 2000). Genomic or mitochondrial DNA can be used for amplification. However, mitochondrial DNA is more often used due to the following advantages:

1. A higher DNA amount in the extract and a higher number of copies compared to genomic DNA.
2. Its resistance is higher in comparison with heat treatment disintegration due to its spherical arrangement (Bossier, 1999).
3. Mitochondrial genome is maternally inherited, and then sequence ambiguities from heterozygous genotypes are theoretically avoided (Aranishi et al., 2005).

5.3.1. The use of species-specific primers

Species-specific primers only amplify DNA of a respective species under strict reaction conditions. This method represents a rapid means for identification; moreover, simultaneous analysis of multiple fish species is possible. However, it is necessary to know the analysed sequence, which is a disadvantage of the method. The fish species identification using species-specific primers was applied in some non-gadoid species (Cespedes et al., 1999; Asensio et al., 2001; Sezaki et al., 2005; Aspden et al., 2006).

5.3.2. The use of *5S rRNA* gene

PCR using *5S rRNA* gene as a genetic marker is based on an amplification of the nontranscribed spacers (NTSs) within this gene. Fragment size of the cluster differs between species. This approach was applied for the identification of hake species *Merluccius paradox*, *M. senegalensis*, *M. australis*, *M. gayi*, *M. bilinearis, M. merluccius, M. capensis* and *Macruronus magellanicus*. Its use allows also fish identification in heat treated products such as fish fingers. All species were possible to distinguish among each other except of *M. merluccius* and *M. capensis* that were identified by the subsequent RFLP analysis of mt cyt *b* gene (Perez and Garcia-Vazquez, 2004). The spacer region between the *5S rRNA* differs in size also between another cod species and can therefore be used for the differentiation of several gadoid species namely *Gadus morhua*, *Merlangius merlangus*, *Micromesistius*

poutassou, *Pollachius pollachius*, *Trisopterus luscus*, *Molva dipterygia*, *Molva molva* and *Merluccius merluccius*. The *5S rRNA* genes will amplify even in degraded tissue, such as canned fish (Moran and Garcia-Vazquez, 2006).

5.3.3. Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP)

The method includes amplification of a known DNA sequence using two specific primers, subsequent digestion of an amplicon with restriction endonucleases and separation and comparison of DNA restriction fragments. The disadvantage of RFLP analysis of PCR product is that incomplete digestion may occasionally occur and intraspecific variation could delete or create additional restriction sites (Lockley and Bardsley, 2000). An important factor of fish species identification using the RFLP method is the number of fish species included in the analysis. E.g., using restriction enzyme *Dde*I allows differentiation between *Gadus morhua* and *Gadus macrocephalus/ogac* and *Polachius pollachius*. If another species is included in the analysis, such as *Molva molva*, it may show a similar profile as *Gadus macrocephalus/ogac* (Calo-Mata et al., 2003).

5.3.3.1. PCR-RFLP analysis of mitochondrial cytochrome *b* gene

PCR-RFLP analysis of an amplified product of mitochondrial cytochrome *b* (mt cyt *b*) gene was developed for differentiation between nine fish species of family Merluciidae (Table 5) and other non-gadoid species. Restriction enzymes *Aci*I, *Dde*I, *Hae*III, *Hinc*II, *Hinf*I, *Msp*I and *Nla*III were used for the examined species. RFLP was also used for the analysis of mixtures of two or three fish species (five samples were prepared by combination of PCR products of respective fish species and five samples by combination of their tissues). All combined samples containing two fish species were identified. Authentic and combined samples were subsequently heat treated and analysed. Except for *Lepidorhombus boscii* all fish species in all heat treated samples were identified (Hold et al., 2001).

PCR-RFLP of mt cyt *b* gene encoding fragment was optimized for identification of three closely related gadoid fish species in fish products (Table 5). Restriction enzymes *Eco*32I and *Eco*105I split the PCR product to three fragments in *T. chalcogramma* and to two fragments in *G. macrocephalus*; it was not possible to split the PCR product with these restriction enzymes in *G. morhua* (Aranishi et al., 2005).

PCR-RFLP of mt cyt *b* gene was used for differentiation between other gadoid fish (Table 5). Restriction enzyme *Dde*I allowed identification of all species except for *T. chalcogramma* and *G. macrocephalus*. Nevertheless, combination of other restriction enzymes (*Nla*III and *Hae*III) allowed differentiation between these two species (Dooley et al., 2005). Akasaki et al. (2006) used the combination of restriction enzymes *Alu*I, *Fok*I, *Mbo*I and *Mse*I for differentiation between gadoid fish (Table 5). Another combination of restriction endonucleases (*Alu*I and *Hsp*92) was applied for the identification of *Merlangius merlangus* (Hisar et al., 2006).

By amplification of a specific segment of the mitochondrial genome (tRNA glu/cytochrome *b*) and subsequent use of restriction enzymes *Alu*I, *Dde*I, *Hae*III, *Hinc*II, *Hinf*I, *Mbo*II, *Nla*III and *Taq*I three gadoid fish species (Table 5) and other significant food industry fish species were distinguished. In most cases, two or three restriction enzymes were sufficient for species identification (Wolf et al., 2000).

5.3.4. Polymerase chain reaction – single strand conformation polymorphism (PCR-SSCP)

Using the PCR-SSCP method it is possible to distinguish alleles differing in one base. SSCP analysis is more demanding, because it does not record differences in sizes, but in secondary structures on gel. The course of the reaction depends on conditions of analysis, above all temperature. If stringent conditions are not observed, reproducibility of the method decreases. Application of PCR-SSCP to species identification, namely *Gadus morhua* in canned cod liver was described by Rehbein et al. (1997). Short segments (123–358 bp) of mt cyt *b* gene were amplified by the PCR and analysed by SSCP to get species-specific patterns of single-stranded DNA. SSCP analysis of the PCR product (148 bp) of mt cyt *b* gene for identification of tuna species was used in other fish and other animal spe-

Table 5. Gadoid fish species identification of by means of the PCR-RFLP method

Species	Gene	Enzymes	References
Gadus morhua *Pollachius virens* *Melanogrammus aeglefinus* *Merluccius hubbsi* *M. polli* *M. gayi* *M. australis* *M. bilinearis* *M. albidus* *M. productus* *M. merluccius* *M. senegalensis*	mt glu/cyt *b*	*Alu*I, *Dde*I, *Hae*III, *Hinc*II, *Hinf*I, *Mbo*II, *Nla*III, *Taq*I	Wolf et al. (2000)
	mt cyt *b*	*Aci*I, *Dde*I, *Hae*III, *Hinc*II, *Hinf*I, *Msp*I, *Nla*III	Hold et al. (2001)
Merluccius albidus *M. australis* *M. bilinearis* *M. capensis* *M. paradoxus* *M. gayi* *M. hubbsi* *M. merluccius* *M. polli* *M. productus* *M. senegalensis*	mt control region sequences	*Apo*I, *Dde*I, *Dra*III, *Mbo*II	Quinteiro et al. (2001)
Theragra chalcogramma *Micromesistius poutassou* *Gadus morhua* *Molva molva* *Pollachius pollachius* *Trisopterus luscus*	mt cyt *b*	*Dde*I, *Hinc*II, *Nla*III	Calo-Mata et al. (2003)
Theragra chalcogramma *Gadus macrocephalus* *Gadus morhua*	mt cyt *b*	*Eco*32I, *Eco*105I	Aranishi et al. (2005)
Molva molva *Pollachius virens* *Melanogrammus aeglefinus* *Brosme brosme* *Gadus morhua** *Gadus ogac** *Gadus macrocephalus** *Theragra chalcogramma**	mt cyt *b*	*Nla*III, *Rsa*I	Comi et al. (2005)
Gadus morhua *Gadus macrocephalus* *Pollachius virens* *Melanogrammus aeglefinus* *Merluccius merluccius* *Merluccius paradoxus* *Merlangius merlangus* *Theragra chalcogramma*	mt cyt *b*	*Dde*I, *Nla*III, *Hae*III	Dooley et al. (2005)
Gadus macrocephalus *Theragra chalcogramma* *Eleginus gracilis* *Arctogadus glacialis* *Micromesistius australis* *Merluccius gayi* *Merluccius australis* *Laemonema longipes* *Macruronus novaezelandiae*	mt cyt *b*	*Alu*I, *Fok*I, *Mbo*I, *Mse*I	Akasaki et al. (2006)
Merlangius merlangus	mt cyt *b*	*Alu*I, *Hsp*92	Hisar et al. (2006)

*not identified

cies. *Theragra chalcogramma* and *Melanogrammus aeglefinus* were distinguishable by this method. The method can be also used for identification of *Theragra chalcogramma* in surimi based products (Weder et al., 2001).

Identification of fish of family Salmonidae (raw and cold-smoked salmon and salmon roe) by the SSCP analysis of the PCR product of mt cyt *b* gene and two genes in chromosomes (*parvalbumin* gene and *growth hormone* gene) was studied by Rehbein (2005). All investigated fish species were identified by analysis of both mt cyt *b* gene and *growth hormone* gene. However, two char species could not be distinguished by the analysis of *parvalbumin* gene.

5.3.5. Polymerase chain reaction – random amplified polymorphic DNA (PCR-RAPD)

The RAPD is a type of PCR reaction, where the segments of DNA that are amplified are unknown (random). This method relies on the large and intact DNA template sequence, so that the ability to identify degraded DNA in the sample is limited. The primers chosen should give clear patterns with inter-species differences and should produce one, few or none individual-dependent polymorphic bands (Martinez et al., 2001). The RAPD has the lower discriminating power than targeted species-specific DNA methods. Still, this method has also been used in gadoid fish species identification. Scheider et al. (1997) presented the use of the RAPD fingerprinting for the identification of twenty nine fish species including *Pollachius* sp., *Merluccius* sp., *Theragra chalcogramma*, *Gadus morhua* and *Melanogrammus aeglefinus.* Using the combination of four unspecific primers, specific RAPD patterns for each fish species were determined not only in raw, but also in frozen, heated or autoclaved samples. For the fish species identification of the mixture of two or more fish species, the authors recommended the use of the species-specific primers. Martinez et al. (2001) carried out the comparison of SDS-PAGE and RAPD for the several fish species determination and *Gadus morhua* was included in this study. RAPD analysis was considered to be more discriminating technique, with the efficiency to identify fish species in processed (cooked, fried, gravad and smoked) fish samples.

5.3.6. Microsatellites

The microsatellites are polymorphic loci presented in the nuclear DNA that consist of tandem repeats of two, three or four nucleotides. The microsatellites are characterized by high mutability compared to other regions of DNA. They have been proved to be appropriate molecular markers for population analyses and evolutionary studies. D'Amato et al. (1999) have described microsatellite markers for *Macruronus magellanicus,* which can also amplify other gadoid fish. *Macruronus novaezelandiae* showed the highest level of polymorphism among the tested gadoids. Charrier et al. (2006) have investigated the genetic structure of a *Pollachius pollachius* population based on microsatellite markers. This system detected genetic differences originated from the geographic heterogeneity of fish species. Similarly, the genetic structure of *Micromesistius poutassou* (Ryan et al., 2005) or of *Gadus morhua* (Pampoulie et al., 2006) was examined. Isolation and characterization of eight microsatellite loci for *Urophycis tenuis* was used for the population genetic studies (Seibert and Ruzzante; 2006). Polymorphic microsatellite loci were also characterized and used for further studies of the population structure analysis of *Trisopterus minutus* (Zhang et al., 2001), *Melanogrammus aeglefinus* (O'Reilly et al., 2002), and *Gadus macrocephalus* (Canino et al., 2005).

Furthermore, the microsatellites present promising molecular markers for the detection of fishing fraud (Primmer et al., 2000; Chistiakov et al., 2006). The use of the heterologous microsatellites for the development of new markers for the codfish species identification was first described by Castillo et al. (2003). They have applied PCR method with two microsatellite loci as genetic markers for the identification of three hake species *M. merluccius*, *M. bilinearis* and *M. hubbsi.* The microsatellite-based technique is fast, and contrary to PCR-RFLP technique involves only one-step procedure for species-specific identification. Due to the fact that microsatellites are extremely polymorphic, this technique is more suitable for the study of the genetic diversity within populations of a single species or the determination of the individual genetic profile.

5.3.7. Lab-On-A-Chip technology

The Lab-On-A-Chip technology is an alternative approach for the visualization of the PCR prod-

ucts by the CE on a card-sized device. Dooley et al. (2005) have described replacing the gel-electrophoretic step in the PCR end-point detection, by employing this system. DNA fragments were detected using laser-induced fluorescence, which enables accurate sizing and quantification of DNA fragments. This presents a significant advantage over gel-based electrophoresis in terms of speed, simplicity and safety. This approach allowed identification of 5% fish species admixed into a product containing two fish species. The CE sensitivity and quantification ability is higher, the expenses are lower and less material and time is required in comparison with real-time PCR (Dooley et al., 2005).

5.3.8. DNA microarray

Another advanced approach in the field of DNA marker technologies for the animal species identification was introduced. DNA microarray (DNA chip) is rapid and provides simultaneous DNA screening of hundreds of species at once. The chip is a glass microscope slide with spots of probes oligonucleotides that are complementary to the specific target DNA sequence. The targets hybridise with the captured oligonucleotides on the chip and the fluorescent label, which is attached to the target during the PCR, is detected. The oligonucleotide microarray analysis of the PCR product from the mt cyt *b* gene was applied to identify different animal species in food samples (Peter et al., 2004). This was for the first time when detailed data about the use of the DNA microarray for the animal species differentiation in food was reported. The application of DNA chips for the identification of marine organisms is a very new and innovative field of research that just started to be developed.

5.3.9. Real-time PCR

The real-time PCR allows for the detection of PCR product during the early phases of the reaction. This ability of measuring the reaction kinetics of PCR provides a distinct advantage over traditional PCR detection. The real-time PCR method was developed for detection and quantification of *Melanogrammus aeglefinus*. Primers and TaqMan probe using the *transferrin* gene sequence were suggested. Presence of *M. aeglefinus* in concentrations of up to 7% in raw or slightly heat treated products could be detected. The method was also used in heat and vacuum treated foodstuffs, however, without quantification (Hird et al., 2005).

Taylor et al. (2002) identified three commercially significant gadoid species (*Gadus morhua, Melanogrammus aeglefinus* and *Merlangius merlangus*) using the multiplex (TaqMan) PCR method based on variation in a 103-bp region of the *ATP synthase subunit six* (*ATPase6*) and *eight* (*ATPase8*) genes.

5.3.10. Sequencing

Sequencing (determination of nucleotide sequence in one of the DNA chains) of the PCR product (especially FINS – forensically informative nucleotide sequencing) allows obtaining the most complete information including fish species identification. However, the method is time and technical equipment demanding. Pepe et al. (2005) identified fish of families Gadidae and Merlucciidae in 18 differently processed fish products by sequencing the PCR product of mt cyt *b* gene. The purpose of the PCR product sequencing is to confirm the results obtained by other PCR methods (Wolf et al., 2000; Calo-Mata et al., 2003; Perez and Garcia-Vazquez, 2004; Aranishi et al., 2005; Comi et al., 2005; Dooley et al., 2005; Rehbein, 2005; Akasaki et al., 2006).

5.3.11. Comparison of efficacy of PCR based methods

Comparison of FINS and PCR-RFLP methods was performed with the aim to distinguish and identify 17 commercially significant gadoid fish species. Sequencing and RFLP analysis of cyt *b*-PCR fragment using restriction enzymes *Dde*I, *Hinc*II and *Nla*III allowed differentiation between 15 gadoid fish species except for *Gadus ogac* and *Gadus macrocephalus*. The RFLP method is faster and less expensive than the FINS method (Calo-Mata et al., 2003). Fish of family Merlucciidae were identified by sequencing and PCR-RFLP analysis of mitochondrial DNA *control region sequences* (Table 5). Identification of *Merluccius* sp. was allowed by four restriction enzymes *Apo*I, *Dde*I, *Dra*III and *Mbo*II (Quinteiro et al., 2001). In another study, cyt *b* gene fragment was analysed by the RFLP method, single strand conformation polymorphism (SSCP), denaturating gradient gel electrophoresis (DGGE)

Table 6. Application of the RFLP, SSCP, DGGE and sequencing methods for species identification of gadoid fish (Comi et al., 2005)

Species	RFLP (*Nla*III, *Rsa*I)	SSCP	DGGE	Sequencing
Gadus morhua	–	–	+[a]	+
Gadus macrocephalus	–	+	+	+
Gadus ogac	–	–	+	+
Molva molva	+	+	+	+
Melanogrammus aeglefinus	+	–	+	+
Brosme brosme	+	–	+	+
Pollachius virens	+	+	+[a]	+
Theragra chalogramma	–	+	+	+

+ = identified, – = not identified
[a]*G. morhua* profile is comparable to *P. virens*

and direct sequencing (Table 6). Among the examined fish, samples of the following were included: *Gadus morhua, G. macrocephalus, G. ogac, Molva molva, Melanogrammus aeglefinus, Brosme brosme, Pollachius virens* and *Theragra chalcogramma*. From the selected restriction enzymes, only *Nla*III and *Rsa*I distinguished between the investigated species except for genus *Gadus* and *T. chalcogramma*. The SSCP analysis distinguished *G. macrocephalus* and *T. chalcogramma*, but *G. morhua* and *G. ogac* were not identified. The DGGE method distinguished almost all investigated species, however, *G. morhua* and *P. virens* showed similar profiles on gel. Visual identification was confirmed by cloning and both strand sequencing of the PCR products obtained from the species considered. Sequences were obtained for all the samples (Comi et al., 2005).

DNA analysis based methods started to be used for a wide range of fish including closely related species within the same family or genus. These techniques are more suitable compared with protein analysis based techniques, because they are in large measure independent on tissue types, age or technology of sample processing (Bossier, 1999; Lockley and Bardsley, 2000; Aranishi, 2005; Dooley et al., 2005). Moreover, DNA is present in all tissue types and is highly variable in the encoding sequence. Due to the fact that DNA molecules are less affected by food processing technologies (including heat treatment) than proteins, DNA analysis seems to be a promising method for fish species identification (Sotelo et al., 1993; Aranishi et al., 2005).

Information on the effects of various heat treatments of foodstuffs on DNA quality is generally scarce (Bossier, 1999). During the sterilization process, DNA may split into fragments of various sizes. Nevertheless, methods of molecular biology are most accurate and most reliable for gadoid fish species identification.

6. Conclusions

With regard to the amount of sea fish imported into CR and problems with taxonomy and different values of respective fish species in the customs taxation, substitution of species of high commercial value for species of less commercial value is possible. Accordingly, it is necessary to develop protocols for the identification of respective fish species from products processed under various conditions. Inspection Authorities need fast and reliable methods to analyse large series of samples. They might thus prevent food adulteration, protect the customers' rights or document and penalize cases of food adulteration.

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