

Application of DNA-based Techniques for Intraspecies Differentiation of Hake Fish

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Abstract

Fish species identification is important as the interest of consumers in sea-fish meat is increasing. The aim of this study was to determine hake species distribution on the Czech market by the PCR-RFLP and sequencing of mt cyt *b*, and to develop and optimise an alternative system for determination of hake species by sequencing and/or PCR-RFLP using *Pan I* sequence. Among 20 samples of hake obtained on the Czech market three species were identified: North Pacific hake (*Merluccius productus*), Argentine hake (*Merluccius hubbsi*) and South Pacific hake (*Merluccius gayi*). The approaches tested in our study represent a significant tool either for the differentiation of hake species from other gadoid species (PCR) or for intraspecies identification of different hake species (PCR-RFLP, sequencing). This knowledge can be applied in detection of fish species substitution within the consumers' rights protection.

Merluccius, PCR-RFLP technique, pantophysin I gene, cytochrome b gene, genomic DNA

Hake belong to an important group of gadoid fish (order *Gadiformes*, family *Merlucciidae*) imported to the Czech Republic in large numbers. Most often fish are imported as fillets, compressed blocks of meat or even processed products, which makes species identification based on morphological features impossible. Therefore, developing methods for species identification is crucial for the prevention of species substitution. Substitution of hake species for other gadoid species and other hake species is often noted. This is the reason for ensuring authenticity of the product, and preventing adulteration and improper labelling.

Methods based on protein analysis present an important tool for hake species identification. The use of isoelectric focusing (IEF) was described for *Merluccius merluccius*, *M. australis*, *M. hubbsi*, *M. gayi* and *M. capensis* identification (Pineiro et al. 2000). Another method, i.e. two-dimensional electrophoresis (2DE) was used for the detection of *Merluccius merluccius*, *M. australis*, *M. hubbsi* and *M. capensis* (Pinerio et al. 1998; Pineiro et al. 2001). For the identification of *Merluccius merluccius* urea-IEF (Etienne et al. 1999), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Rehbein et al. 1999; Pineiro et al. 1999) and chromatography (Pineiro et al. 1997) were employed. However, all protein-detection methods are very laborious, time consuming and require a well defined standard for comparison with unknown samples.

On the other hand, the introduction of molecular genetic methods, mainly PCR and its modifications (PCR-RFLP) or sequencing provides a major improvement in fish species differentiation. The use of PCR-RFLP which consists of PCR amplification of the mitochondrial cytochrome *b* (mt cyt *b*) gene following enzymatic restriction was described for the identification of a broad spectrum of *Merluccius* sp. (Hold et al. 2001; Quinterio et al. 2001). For hake species differentiation another genetic marker, the *5S rRNA* gene was used in PCR based on amplification of the non-transcribed spacers (NTSs) within this gene (Perez and Garcia-Vazquez 2004). The PCR method based on the partial *pantophysin I* (*Pan I*) genomic sequences was developed for the identification of six gadoid fish species (Hubalkova et al. 2008).

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The objective of this work was (1) to determine hake species distribution on the Czech market following our previous study (Hubalkova et al. 2008), by PCR-RFLP and sequencing of mt cyt *b*, and (2) to develop and optimise an alternative system for the determination of hake species by sequencing and/or PCR-RFLP using *Pan I* sequence.

Materials and Methods

Samples of hake and DNA analysis

Twenty samples of frozen fish products (declared as codfish) were obtained on the Czech market. The samples were either fillets or compressed blocks of meat. All samples were stored at -20°C until DNA preparation. DNA was isolated using DNeasy Tissue Kit (Qiagen, Hilden, Germany) as described in Hubalkova et al. (2008).

Conventional PCR analysis of *pantophysin I* (*Pan I*) gene

The conventional PCR method was carried out according to Hubalkova et al. (2008) as a monoplex PCR reaction using hake-specific primer pairs based on the partial *Pan I* genomic sequence amplification. The PCR reaction was performed in a final volume of 20 μl using the ThermoCycler Machine DYAD (Bio-Rad, Munich, Germany). The reaction mix contained 10 μl of PPP Master Mix (Top Bio, Prague, Czech Republic), 10 pmol of each primer set, and 4 μl of the DNA template. The amplification was carried out with an initial denaturing step at 95°C for 3 min, followed by 35 cycles and each cycle with denaturation at 95°C for 10 s, annealing at 65°C for 20 s, extension at 72°C for 2 min, and then a final extension at 72°C for 5 min.

PCR-RFLP analysis of mt cyt *b* gene

On account of the results of the conventional PCR method, the PCR-RFLP technique based on the mt cyt *b* gene was chosen for closer intraspecies (*Merluccius* spp.) determination. The PCR and restriction cleavage with *HaeIII*, *HinfI* and *AclI* were carried out as described previously (Hold et al. 2001).

Sequencing of PCR products from *Pan I* and mt cyt *b* genes

From each sample, two independent PCR reactions of *Pan I* and mt cyt *b* genes were carried out to eliminate *Taq* polymerase errors in the evaluation of the sequences. Obtained amplicons were cloned into the vector pDrive Cloning Vector (Qiagen, Hilden, Germany) and inserted into *Escherichia coli* Top10F' competent cells and screened by the blue-white test. Randomly chosen white colonies were checked by PCR for the presence of the insert using specific and M13 universal primers (Hubalkova et al. 2008). Positive clones were purified by QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and the PCR products were sent for sequencing to a commercial facility (MWG Biotech, Ebersberg, Germany).

PCR-RFLP analysis of *Pan I* gene

Based on the analysis of restriction sites by WebCutter (<http://rna.lundberg.gu.se/cutter2/index.html>) of partial *Pan I* sequences of *Merluccius* spp. gained by sequencing, restriction enzyme *HincII* (New England Biolabs, Ipswich, USA) was selected for possible differentiation of *Merluccius* spp. PCR products of *Pan I* partial sequence were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and 14 μl of each PCR product was digested with 5 units of enzyme in a final volume of 20 μl at 37°C overnight.

Results and Discussion

Conventional PCR analysis of *Pan I* gene

With the conventional PCR technique using the specific primer pairs derived from the partial genomic sequence of the *Pan I* gene all 20 investigated samples have amplified the same length of the PCR product of 201–202 bp. Obtained PCR products were cloned and sequenced to reveal the potential intraspecies variability among particular hake sequences.

Determination of *Merluccius* spp. by mt cyt *b* PCR-RFLP

For closer intraspecies differentiation among *Merluccius* spp., the PCR-RFLP analysis of the mt cyt *b* gene (464 bp) was chosen (Hold et al. 2001). Using a combination of the restriction enzymes *HaeIII*, *HinfI* and *AclI* (Table 1, Plate XVII, Fig. 1), we identified North Pacific hake (*Merluccius productus*), Argentine hake (*Merluccius hubbsi*) and South Pacific hake (*Merluccius gayi*). Nevertheless, the restriction patterns obtained after digesting the 464 bp amplicon of the samples 10–20 were quite weak (Table 2), so that the identification of these species was not absolutely confirmative using this method. On that account we aimed to devise an alternative system more suitable for hake species differentiation.

Table 1. The RFLP profiles (bp) from cytochrome *b* sequence (464 bp) and *pantophysin* I sequence (202 bp)

Species	Cytochrome <i>b</i> gene (464 bp)			<i>Pantophysin</i> I gene (202 bp)
	<i>Hinf</i> I	<i>Hae</i> III	<i>Aci</i> I	<i>Hinc</i> II
<i>M. gayi</i>	U	39, 109, 315	42, 51, 160, 210	83, 118
<i>M. productus</i>	81, 382	39, 109, 315	42, 51, 160, 210	83, 118
<i>M. hubbsi</i>	81, 382	39, 109, 126, 189	42, 43, 51, 76, 84, 167	U

U - unaffected

Table 2. Intraspecies identification of 20 hake species using different molecular biological methods

Sample No.	Conventional PCR method (<i>Pan</i> I gene – 202 bp)	PCR-RFLP analysis (mt <i>cyt b</i> gene); REs: <i>Hinf</i> I, <i>Hae</i> III, <i>Aci</i> I (Hold et al. 2001)	Sequencing of mt <i>cyt b</i> gene	PCR-RFLP analysis (<i>Pan</i> I gene); RE: <i>Hinc</i> II
1	<i>Merluccius</i> sp.	<i>M. gayi</i>	<i>M. gayi</i>	<i>M. gayi</i> / <i>M. productus</i>
2	<i>Merluccius</i> sp.	<i>M. productus</i>	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
3	<i>Merluccius</i> sp.	<i>M. productus</i>	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
4	<i>Merluccius</i> sp.	<i>M. productus</i>	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
5	<i>Merluccius</i> sp.	<i>M. hubbsi</i>	<i>M. hubbsi</i>	<i>M. hubbsi</i>
6	<i>Merluccius</i> sp.	<i>M. productus</i>	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
7	<i>Merluccius</i> sp.	<i>M. hubbsi</i>	<i>M. hubbsi</i>	<i>M. hubbsi</i>
8	<i>Merluccius</i> sp.	<i>M. gayi</i>	<i>M. gayi</i>	<i>M. gayi</i> / <i>M. productus</i>
9	<i>Merluccius</i> sp.	<i>M. gayi</i>	<i>M. gayi</i>	<i>M. gayi</i> / <i>M. productus</i>
10	<i>Merluccius</i> sp.	<i>M. hubbsi</i> *	<i>M. hubbsi</i>	<i>M. hubbsi</i>
11	<i>Merluccius</i> sp.	<i>M. productus</i> *	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
12	<i>Merluccius</i> sp.	<i>M. productus</i> *	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
13	<i>Merluccius</i> sp.	<i>M. hubbsi</i> *	<i>M. hubbsi</i>	<i>M. hubbsi</i> *
14	<i>Merluccius</i> sp.	<i>M. hubbsi</i> *	<i>M. hubbsi</i>	<i>M. hubbsi</i>
15	<i>Merluccius</i> sp.	<i>M. productus</i> *	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
16	<i>Merluccius</i> sp.	<i>M. hubbsi</i> *	<i>M. hubbsi</i>	<i>M. hubbsi</i>
17	<i>Merluccius</i> sp.	<i>M. hubbsi</i> / <i>M. productus</i> *	<i>M. hubbsi</i>	<i>M. hubbsi</i>
18	<i>Merluccius</i> sp.	<i>M. hubbsi</i> *	<i>M. hubbsi</i>	<i>M. hubbsi</i>
19	<i>Merluccius</i> sp.	<i>M. gayi</i> / <i>M. hubbsi</i> *	<i>M. productus</i>	<i>M. gayi</i> / <i>M. productus</i>
20	<i>Merluccius</i> sp.	<i>M. hubbsi</i> *	<i>M. hubbsi</i>	<i>M. hubbsi</i>

The RFLP profile not clearly identifiable

Sequencing

The corresponding PCR products obtained from the mt *cyt b* gene of all 20 samples were also sequenced. Nucleotide sequences were compared and aligned with sequences of the mt *cyt b* gene published in the GenBank. The samples that were not unambiguously identified by the PCR-RFLP technique, were determined (Table 2) by sequencing of the mt *cyt b* gene. Sequencing confirmed the results of PCR-RFLP analysis in all but two samples (17 and 19). According to these results, the sequences of 20 samples of the *Pan* I gene were aligned, and three sequences of the *Pan* I gene for North Pacific hake (*Merluccius productus*), Argentine hake (*Merluccius hubbsi*) and South Pacific hake (*Merluccius gayi*) were deposited in the GenBank (Acc. Nos. EU301630, EU301631, EU301632). Alignment of sequences showed that the similarity among them varies from 97.0 to 99.5% (Fig. 2).

PCR-RFLP analysis of *Pan* I gene

In order to develop an alternative technique for *Merluccius* spp. differentiation, we employed PCR-RFLP analysis of the *Pan* I partial genomic sequence. *In silico* analysis

	5 15 25 35 45 55
<i>M. productus</i>	CCCGTTTAGG TTTGTGGTTG CGTCATTCCCT CTCACTGTCT CGAATAACTC GTGGCTGGTT
<i>M. hubbsi</i>
<i>M. gayi</i>

	65 75 85 95 105 115
<i>M. productus</i>	GATTTTTTTT TTTTAATGGA GGACTTGTGA CAATGTTATT GTTTCACCTA CTCAGGTTGA
<i>M. hubbsi</i>A.....
<i>M. gayi</i>

	125 135 145 155 165 175
<i>M. productus</i>	CGCAGCATCC ATTCAATTC CCTGACTGTC AGAATGGCAC CACCAGTATT TACCTTATGG
<i>M. hubbsi</i>	T.....AG.....T.....
<i>M. gayi</i>A.....

	185 195
<i>M. productus</i>	GGAACCACAG TTCGTCTGCA CA
<i>M. hubbsi</i>
<i>M. gayi</i>

Fig. 2. Partial *Pan I* sequences alignment of *Merluccius* spp.

revealed that it will probably not be possible to distinguish between *M. gayi* and *M. productus* because there is a SNP in position 166 in *M. productus* where G could be replaced by A (Fig. 2). This also causes amino acid substitution (Asparagine to Serine). No other possible targets for restriction analysis were found. By the PCR-RFLP of the *Pan I* gene we were able to reliably determine *M. hubbsi* only. For these purposes, the restriction enzyme *HincII*, that can distinguish *M. hubbsi* (unaffected by *HincII*) from *M. gayi* and *M. productus* (both cleaved with *HincII* into two fragments in position of site 118) was generated (Table 1). These results were compared with the results of the PCR-RFLP and sequencing of the mt *cyt b* gene whereas hake intraspecies identification using *HincII* cleavage of the *Pan I* gene was confirmed (Table 2). Concerning the discrepancy between the results of PCR-RFLP and sequencing of the mt *cyt b* gene of samples 17 and 19, the PCR-RFLP results of the *Pan I* gene of samples 17 and 19 were consistent with the results of mt *cyt b* gene sequencing. The RFLP profiles of the mt *cyt b* gene of the samples 17 and 19 did not fully correspond with the restriction patterns (Table 2) as described by Hold et al. (2001). Visual interpretation of the signals could simulate the presence of a mixture of two *Merluccius* species; however, this was disproved by the sequencing of mt *cyt b* gene.

The aim of this study was to illustrate the usefulness of different methods of molecular biology for hake species discrimination. For the differentiation of hake species from other gadoid species the conventional PCR method based on the amplification of a partial genomic sequence of the *Pan I* gene can be used (Hubalkova et al. 2008). Nevertheless, for intraspecies identification of hake species the application of other methods (e.g. the PCR-RFLP technique) is advisable. The PCR-RFLP of the mt *cyt b* gene may be used for intraspecies identification of hake species (Hold et al. 2001), although our results were not always of predicative significance. The innovative contribution of this study lies in the application of the partial *Pan I* sequences of three hake species (Fig. 1) as an alternative tool for detection of the above mentioned species in fish meat and fillets. PCR-RFLP of the *Pan I* gene using *HincII* can differentiate *M. hubbsi* from *M. productus* and *M. gayi*. The most direct means of obtaining information from PCR products is by direct nucleotide sequencing employing universal primers (Kocher et al. 1989). Sequencing

offers unambiguous identification of individuals of a single species of hake. Considering the higher cost and time consumption, sequencing is not suitable for routine analytical purposes. All the above described and approved techniques represent an important tool for disclosing fish food adulteration in terms of species substitution detection. Among other things, the study also gives an overview of the spectrum of hake species offered on the Czech market.

Využití metod založených na analýze DNA pro mezidruhové rozlišení štikozubce

Druhová identifikace ryb nabývá na významu s ohledem na stoupající zájem spotřebitelů o rybí maso. Cílem studie, která navazuje na předchozí práci popisující techniky PCR-RFLP a sekvenování mitochondriálního genu cytochrom *b*, bylo zjistit druhové zastoupení štikozubců na českém trhu a vyvinout alternativní systém pro druhovou identifikaci štikozubců pomocí sekvenování *a*/nebo PCR-RFLP v oblasti genu *Pantophysin I*. Mezi 20 vyšetřenými vzorky štikozubců byly zjištěny tři druhy: štikozubec pacifický (*Merluccius productus*), štikozubec argentinský (*Merluccius hubbsi*) a štikozubec chilský (*Merluccius gayi*). Testované postupy představují významný nástroj pro odlišení štikozubců od ostatních druhů treskovitých ryb (PCR) nebo pro mezidruhové rozlišení jednotlivých štikozubců (PCR-RFLP, sekvenování). Metody vyvinuté pro odhalování druhových záměn ryb mohou být využity zejména v oblasti ochrany práv spotřebitelů.

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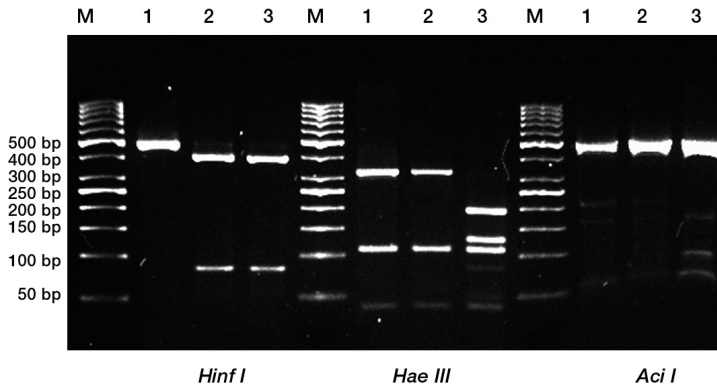


Fig. 1. RFLP profiles from mt *cyt b* gene (464 bp) fragmented with enzymes *HinfI*, *HaeIII* and *AclI* (1 - *M. gayi*, 2 - *M. productus*, 3 - *M. hubbsi*); 50 bp marker (M)