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IDENTIFICATION OF THE COMMON CARP (*CYPRINUS CARPIO*) SPECIES USING REAL-TIME PCR METHODS

S u m m a r y

Before being put out onto the market many fish species sold around the world need to be processed, which may result in the subsequent removal of characteristics used for their classification (head, fins, internal organs). The biochemical characterization of fish species could be achieved using proteins or DNA sequences as species-specific markers. However, since different fish products undergo different processes, the method of analysis has to be chosen according to the modifications undergone by fish constituents during processing.

As DNA molecules are more resistant than proteins to various processes (including thermal treatment), DNA analysis appears to be a promising method for fish species identification. For the species identification of the Common Carp (*Cyprinus carpio*) among 15 different freshwater fish species a specific predesigned molecular - genetic marker of Common Carp (Cyprinus carpio) was used, which comes from the mtDNA control D - loop area. Next we analyzed the presence of mtDNA in DNA isolates of the 15 kinds of freshwater fish and compared them with the Common Carp markers by using the following two PCR identification methods. The isolates were diluted to 10 % concentration, using the TaqMan Real-Time PCR method and the SYBR® Green Real-Time PCR method.

The results of using the optimized the SYBR® Green Real-Time PCR method for species identification of the Common Carp (*C. carpio*) pointed to its suitability. We were able to create an analysis of the monitored standard curve which represented the PCR positive control (C. carpio), containing the characteristic melting peak (up to the melting point 80.72 °C). A single peak indicated a single product (*C. carpio*) which can be verified upon characterization of the PCR product by agarose gel electrophoresis.

The TaqMan Real-Time PCR method with a TaqMan probe is a very sensitive and reliable method of authentication used on food of animal origin. The suitability of this method, which we used for species identification of the Common Carp (*C. carpio*), was confirmed. Thanks to using this method, already in the 17th cycle of the PCR amplification procedure, the presence of the Common Carp gene (*C. carpio*) was detected in the positive control and not detected in the rest of the fish samples.

Key words: species identification, Common Carp (Cyprinus carpio), Real-Time PCR method

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Introduction

Over the last years there has been a tremendous growth in fish consumption due to changes in consumer attitudes towards health and nutrition [4].

Depending on their source (i.e., natural or processed), the quality and price of these products, even from the same fish species, vary with the production location (i.e., domestic or imported). Labeling regulations of commercial fishery products also differ among countries. Consequently, techniques are needed for authentication of commercial fishery products in order to guarantee accurate labeling and to prevent unfair competition [19]. The increase in international trade and global seafood consumption, along with fluctuations in the supply and demand of different fish and seafood species, have resulted in intentional product mislabeling. The effects of species substitution are far-reaching and include economic fraud, health hazards, and illegal trade of protected species [24].

The international fish market, estimated by the FAO at around 60 billion tons/year, may imply more than 20,000 species of fish [21]. For instance, about 420 species of fish are sold on the German market alone [10]. Market globalization, large numbers of both exploited species and processed fish products explained why the substitution of a less valuable species for a valuable one (representing a commercial fraud), may be a common phenomenon difficult to detect. For instance, a study on food fish in the United States revealed that three-quarters of fish sold as the threatened ‘‘red snapper’’ *Lutjanus campechanus* were mislabeled and belonged to other species [18]. Likewise, Pepe et al. [20] found that 84.2 % (16/19) of surimi-based fish products sold as *Theragra chalcogramma* were actually prepared from species different from the one declared [21].

Fish species identification is traditionally based on external morphological features. Yet, in many cases fish, and especially at their different developmental stages are difficult to identify by morphological features characteristic. DNA-based identification methods offer an analytically powerful addition or even an alternative to immunological techniques such as immunodiffusion and ELISA [28].

Allozyme analysis has traditionally been used to authenticate the species of commercial fish sharing a few morphological features. However, recently DNA analysis has been developing rapidly as an alternative, since it is common and highly sensitive. In particular, analysis of mitochondrial DNA (mtDNA), which has species-specific features in the base sequences and a high genetic stability due to maternal inheritance, has been used widely in the study of phylogeny and population structure of fish and in the authentication of fish products. Complete mtDNA sequences of many fish species are already available in DNA databases, i.e., GenBank, EMBL, DDBJ and MitBASE [7, 14, 17, 31]. Thus, mtDNA analysis is useful for authentication of ingredients of commercial fishery products. The mtDNA is of maternal inheritance and has no re-

combination in all vertebrates, so that the sequence of mtDNA is more conservative [3, 23]. The mtDNA lies in the inner membrane of mitochondria and is easily affected by oxidative phosphorylation. The rate of base substitution on mtDNA is higher than that on genomic DNA [16, 27].

Genetic identification of species is based on the principle of DNA polymorphism, or genetic variations that take place as a result of naturally occurring mutations in the genetic code [11]. To detect species-specific genetic polymorphism, DNA is first extracted from the target organism and then the DNA fragment(s) of interest is amplified using PCR. The resulting PCR amplicons are then analyzed to reveal the characteristic polymorphism under study [24].

The present study was undertaken to develop a simple, rapid and accurate method based on the Real-Time PCR analysis of the specific pre-designed molecular - genetic marker which comes from the mtDNA control D - loop area, for identification of the Common Carp (*Cyprinus carpio*) among different fish species.

Material and Methods

Fish Samples

Frozen samples of 15 fish species (table 1) were obtained from and authenticated by the Department of Poultry Husbandry and Small Livestock at the Slovak University of Agriculture in Nitra.

DNA extraction

Fish samples of 300 mg were minced using a sterile surgical blade and transferred into a 2 mL Eppendorf tube. DNA was extracted from 300 mg of homogenized portions using the protocol of NucleoSpin® Food Isolation Kit (Macherey-Nagel) for isolation of genomic DNA from food. DNA isolates were then diluted to 10 % concentration.

Primers

SYBR® Green Real-Time PCR method

The specific marker for species identification of the Common Carp (*Cyprinus Carpio*) was amplified using the following primers:
Carp1-F (5´-TGGCATCTGGTTCCTATTTCA-3´),
Carp1-R (5´-CCAAAGGGGGCACTATGTAA-3´) designed by Židek and Golian, 2008 [32].

Table 1

Names and categories of fish samples studied.
Nazwy gatunkowe i kategorie badanych próbek ryb.

Number of samples Numer próbki	Common name Nazwy zwyczajowa	Scientific Name Genus / Species Nazwy gatunkowe Rodzaj/Gatunek
1.	Brown trout / Pstrąg potokowy	*Salmo trutta fario*
2.	Alpine bullhead / Głowacz pręgopłetwy	*Cottus poecilopus*
3.	Grayling / Lipień pospolity	*Thymallus thymallus*
4.	Rainbow trout / Pstrąg tęczowy	*Oncorhynchus mykiss*
5.	Black bullhead / Sumik czarny	*Ameiurus melas*
6.	European eel / Węgorz europejski	*Anguilla anguilla*
7.	Northern pike / Szczupak pospolity	*Esox lucius*
8.	Wels catfish / Sum pospolity	*Silurus glanis*
9.	Sterlet / Czeczuga	*Acipenser ruthenus*
10.	European perch / Okoń europejski	*Perca fluviatilis*
11.	Freshwater bream / Leszcz	*Abramis brama*
12.	European chub / Kleń europejski	*Leuciscus cephalus*
13.	Common barbel / Brzanka pospolita	*Barbus barbus*
14.	Common nase / Świnka zwyczajna	*Chondrostoma nasus*
15.	Rainbow trout / Pstrąg tęczowy	*Oncorhynchus mykiss*
16.	Common Carp / Karp zwyczajny	*Cyprinus carpio*

TaqMan Real-Time PCR method

The designed primers and TaqMan probe are specific for all lines of the specific pre-designed molecular - genetic marker which comes from the mtDNA control D - loop area, for identification of the Common Carp (*Cyprinus carpio*). Primer pairs and TaqMan probe were prepared in final form by Roche, Slovakia.

Sequences of primers and TaqMan probes were as follows:
D – Loop F (5´-CATCTGGTTCCTATTTCAGGGA-3´),
D – Loop R (5´-GGCACTATGTAAGGATAAGTTGAACT-3´),
TM_{LNA} (LC640-TGCACTTGAGATAAAAGTATGTAA+T+T+CT-BBQ).

PCR amplification

SYBR® Green Real-Time PCR method

The thermal cycling profile was as follows: pre-incubation of the samples at 95 °C for 2 min. It was subsequently repeated by 50 cycles with a temperature profile: denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for

1 min 20 s with a final extension for 10 min at 72 °C. The melting curve of PCR products was launched by heating samples to 95 °C and immediate cooling to 65 °C for 15 seconds. The sample was heated at a rate of 0.1 °C per second and the fluorescence was measured after each temperature change of one tenth degree. PCR amplification was carried out in the capillary cycler ´LightCycler® 1.5´ using LightCycler software (version 4.5).

TaqMan Real-Time PCR method

The LightCycler® TaqMan® Master kit (Roche) was used. The thermal cycling profile was as follows: pre-incubation of the sample at 95 °C for 10 min. It was subsequently repeated by 45 cycles with a temperature profile: denaturation at 95 °C for 10 s, annealing at 63 °C for 30 s and extension for 20 s at 72 °C, followed by the measurement of fluorescence. The final cooling was at 30 s at 40 °C. PCR amplification was carried out in the capillary cycler ´LightCycler® 1.5´ using LightCycler software (version 4.5).

Results and Discussion

SYBR® Green Real-Time PCR method

As shown in Fig. 1 (fish samples 3, 6, 7, 12, 16; 17- H_2O), the earliest possible increase in the fluorescence and the creation of a specific curve with its exponential and linear phases was possible in the positive control (Sample "16" - *C. carpio*) only. In other fish samples a minimal increase in fluorescence was detected (Table 1- see samples).

Based on the results we can create the standard curve (Fig. 2). The standard curve representing the PCR positive control (Sample "16" - C. carpio) contains the characteristic peak (formed at the melting point 80.72 °C), which shows the right use of the specific primer pair. For other curves of the PCR products of other fish samples (samples "1" to "15", and sample "17" the negative control - H_2O), non-specific peaks were observed (Table 1 - see samples).

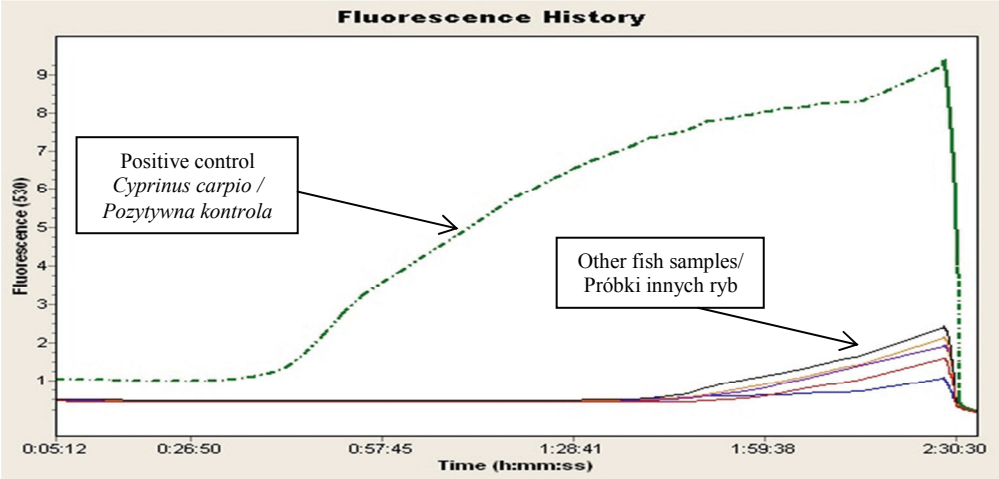

Fig. 1. The curves of the fluorescence during the PCR amplification for individual fish samples by using SYBR® Green Real-Time PCR method.

Rys. 1. Krzywe fluorescencyjne uzyskane w trakcie amplifikacji PCR dla poszczególnych próbek ryb przy użyciu metody SYBR® Green Real-Time PCR.

Table 2

Crossing thresholds of fish samples studied after PCR amplification by using SYBR® Green Real-Time PCR method.

Piki temperatury charakterystyczne dla badanych gatunków ryb metodą amplifikacji PCR przy użyciu techniki SYBR® Green Real-Time PCR.

Number of samples Numer próbki	Scientific Name Genus / Species Nazwy gatunkowe Rodzaj/Gatunek	C_t - (Crossing threshold) Pik maksymalny topnienia
15	*Cyprinus carpio* (positive control)	11,33
3	*Thymallus thymallus*	27,66
12	*Leuciscus cephalus*	31,36
7	*Esox lucius*	32,93
6	*Anguilla anguilla*	33,87

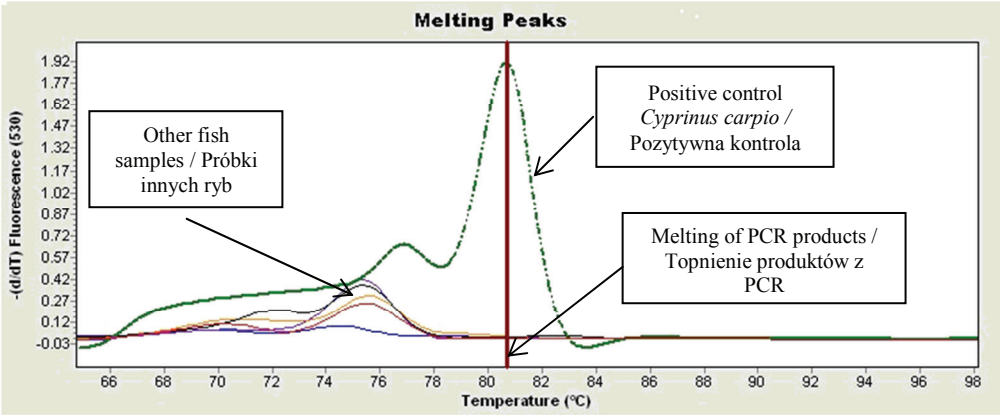

Fig. 2. The curves of the melting PCR products in individual fish samples generated by the SYBR® Green Real-Time PCR method.

Rys. 2. Krzywe topnienia produktów PCR poszczególnych próbek ryb otrzymane za pomocą metody SYBR® Green Real-Time PCR.

TaqMan Real-Time PCR method

As shown in Fig. 3, the earliest possible increase in fluorescence intensity and above the measurable nonspecific background in the positive control (Sample "16" - *C. carpio*) was in the 17th cycle of the PCR amplification. The fluorescence intensity corresponds to the shape of the curve, which passes from exponential phase to the linear growth phase. For Samples: "3" (*T. thymallus*), "4" (*O. mykiss*), "6" (*A. anguilla*), "7" (*E. lucius*) and "11" (*A. brama*) the nonspecific background levels were visible to about the 40th cycle of the PCR amplification. This is already the late phase of the PCR amplification in which we can not clearly demonstrate the specificity and affinity of the studied fish species with the positive control (*C. carpio*). For Samples "1" (*S. trutta fario*), "2" (*C. poecilopus*) "5" (*A. melas)*, "8" (*S. glanis*), "9" (*A. ruthenus*), "10" (*P. fluviatilis*), "12" (*L. cephalus*), "13" (*B. barbus*), "14" (*C. nasus*), "15" (*O. mykiss*), and "17" (H_2O - negative control) no changes in fluorescence intensity were observed, so those samples did not create the characteristic curve whether in their exponential or linear phase, i. e. there were excess levels of nonspecific background (Table 1- see samples).

The specificity and sensitivity of this technique, combined with its high speed, robustness, reliability, and the possibility of automation, contribute to the adequacy of the method for quantifying fish species in fishery products. Thus, for instance, Sotelo et al. [26] used the TaqMan assay for the identification and quantification of Cod (*Gadus morhua)*. Trotta et al. [30] used the Real-Time PCR for the identification of fish fillets from Grouper (Genera *Epinephelus* and *Mycteroperca*) and common substitute species. Hird et al. [5] used it for the detection and quantification of Haddock (*Mela-*

nogrammus aeglefinus). The presence of this fish in concentrations of up to 7 % in raw or slightly heat treated products could be detected. In another work, López and Pardo [13] applied the TaqMan Real-Time PCR technology for the identification and quantification of albacore and Yellow fin tuna (*Thunnus albacares*).

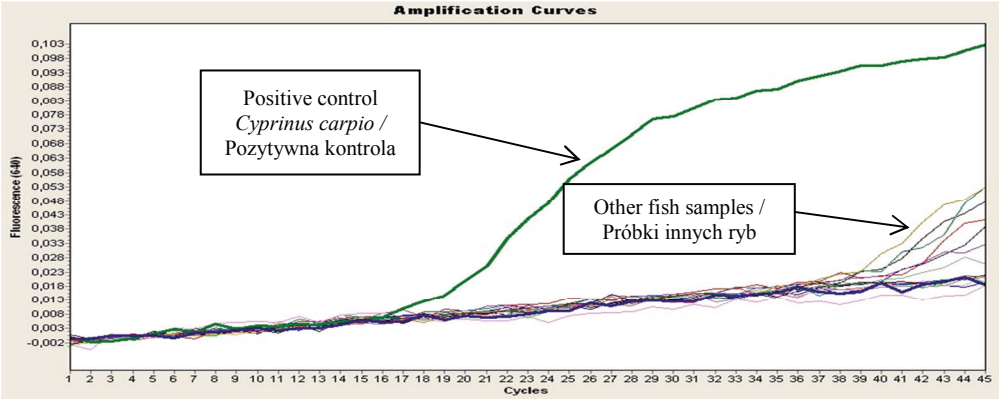

Fig. 3. The curves of the fluorescence during the PCR cycles in individual fish samples by using TaqMan Real-Time PCR method.

Rys. 3. Krzywe fluorescencyjne otrzymane z analizy PCR, za pomocą TaqMan Real-Time PCR, indywidualnych próbek ryb.

The development of analytical methods for fish species identification may help to detect and avoid willful, as well as unintentional substitution of different fish species and thus enforce labeling regulations [2, 12, 15, 29]. Numerous methods have been developed to authenticate various fish species in a wide range of food products, including soup and dried fins [6], surimi [20], fish roe [9], spicy roe [1], fish tails [25], canned sardine and sardine-type products [8], and canned tuna [22]. The development of these molecular methods helps not only to protect both consumers and producers from frauds, but may also help to protect fish species from over-exploitation or illegal trafficking [29].

Conclusions

1. The results of using the optimized SYBR® Green Real-Time PCR method for species identification of the Common Carp (*Cyprinus carpio*) prove its suitability. We were able to create an analysis of the monitored melting curves i.e. the curve representing the PCR positive control (*Cyprinus carpio*) contains the characteristic peak (up to the melting point 80.72 °C), which shows us the right use of the specific primer pair. Our results confirmed the suitability of this method for the identification of the species of the Common Carp (*Cyprinus carpio*).

2. The TaqMan Real-Time PCR method, using a TaqMan probe, is a very sensitive and reliable way of authentication of food of animal origin. Our results confirmed the suitability of this method for species identification of the Common Carp (*Cyprinus carpio*).

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IDENTIFIKACJA GATUNKU KARP ZWYCZAJNY (CYPRINUS CARPIO) PRZY UŻYCIU REAL-TIME PCR

S t r e s z c z e n i e

Przed wprowadzeniem do obrotu ryby są wstępnie przetwarzane, co może spowodować usunięcie tych części anatomicznych (tj. głowy, płetwy, organy wewnętrzne), na podstawie których identyfikuje się gatunek. Do identyfikacji ryb można wtedy zastosować charakterystykę biochemiczną, którą dla danego gatunku mogą być specyficzne białka lub sekwencja DNA – specyficzne markery danego gatunku ryb. Jednak ryby podlegają różnym procesom przetwórczym, w związku z czym musi być opracowana taka metoda ich biochemicznej identyfikacji, która byłaby zgodna ze zmianami składników tkanki ryb, zachodzącymi podczas tego przetwarzania.

Cząsteczki DNA są stosunkowo odporne na czynniki przetwórcze (włącznie z obróbką termiczną), dlatego analiza sekwencji DNA może być przydatną metodą do identyfikacji gatunkowej ryb. W celu gatunkowej identyfikacji karpia zwyczajnego, spośród 15 innych gatunków ryb słodkowodnych, opracowano specyficzny molekularno-genetyczny marker karpia zwyczajnego, który pochodził z mDNA (z obszaru pętli kontrolnej D). Następnie analizowano izobaty mDNA z 15 różnych gatunków ryb słodkowodnych, porównując je do markera karpia za pomocą dwóch metod identyfikacji PCR. Izolaty były rozcieńczane do 10 % stężenia w obu stosowanych metodach oznaczeń tj. TaqMan Real-Time PCR i SYBR® Green Real-Time PCR.

Na podstawie wyników badań karpia zwyczajnego (*Cyprinus carpio*), uzyskanych zoptymalizowaną metodą SYBR® Green Real-Time PCR, wykazano jej przydatność do identyfikacji gatunkowej. Monitorowano krzywą standardową topnienia PCR (z maksimum w temp. 80,72 °C) świadczącą o pozytywnej weryfikację *Cyprinus carpio* oraz krzywe topnienia pozostałych próbek ryb. Maksima topnienia poszczególnych (15) próbek ryb były następnie weryfikowane metodą elektroforezy żelowej na agarozie. Druga z zastosowanych metod TaqMan Real-Time PCR, z wykorzystaniem próbnika TaqMan, jest bardzo dokładną i wrażliwą metodą identyfikacji (potwierdzania autentyczności) karpia zwyczajnego (*Cyprinus carpio*). Dzięki zastosowanie metody amplifikacji już w 17. cyklu potwierdzono obecność, poprzez PCR, genu *Cyprinus carpio* w próbce kontrolnej i brak obecności tego genu w pozostałych próbkach ryb.

Słowa kluczowe: identyfikacja gatunku, karp zwyczajny, metoda PCR