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Eel species identification by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP)

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Summary

Eels offered on the Polish market are not only imported mainly from China but also from domestic catches. It is known that Chinese breeders are buying most of their montče eels from Europe, so it is highly probable that „Chinese" eels are Anguilla anguilla, but also Anguilla japonica. There is no data available concerning ratio between these two species on the Polish market. Morphological methods applied to establish this ratio are not reliable enough. Therefore the aim of the presently reported study was to differentiate the eel species using molecular methods. A total of 31 freshwater eels were collected from a local importer (21 samples) and from Lake Miedwie near Szczecin (10 samples). At the beginning of the eel identification process morphometric measurements have been performed. In attempting to distinguish A. japonica and A. anguilla PCR products of partial 16S rRNA gene, a PCR-RFLP procedure was applied, which is mainly base on nucleotide differences between species sequences. In this method the ApaI restriction enzyme was used to conduct the digestion of the PCR product. Primers named Ang211F and Ang211R were designed for the amplification the 211 bp of 16S rRNA sequence of both eel species. Electrophoretic pattern of PCR products from A. japonica and A. anguilla did not indicate any difference in length. As a result, ApaI produced fragments of 135 and 76 bp only for A. japonica, while the A. anguilla sequence was not digested with its length of 211 bp. Products of ApaI digestion of partial 16S rRNA gene of A. japonica and A. anguilla are suitable genetic markers to distinguish both eel species.

Keywords: fish, Anguilla anguilla, Anguilla japonica, eel, mtDNA, 16S rRNA

During the last decades a considerable decrease in the number of European eel species population has been observed and this has raised global discussion. The main topics are concerned with protection procedures and future plans to remit stable levels of population. Because of the low level of recruitment (approx. 0.1%) conservation plans will be introduced (21). Asian countries such as Japan and China have for a long time frequently eaten eel-based meals, but most European people do not like this snake-like fish. Most eel farms are located in Europe, so eel could quite easily be found in their restaurants. For example, much like carps in Poland, eels have their special place on the table in Spain. The highest amounts of eels presently are produced not in China but in Japan. In 1967 Japanese eel farmers introduced the much cheaper *Anguilla anguilla* eel-fry to replace *A. japonica*, but species mismatch and Novel Catfish Kidney Disease caused numerous death of eel-fry. After 1970's in Mainland China eels farms have gradually been established because imported eel-fry was from France and also supported by some culture solutions from Denmark. This helped in the success of a great number of farms. In Japan, the production of glass Japanese eel *A. japonica* in captivity succeeded in 2003. The Japanese have recently succeeded in producing the hybrid larvae which are a cross between European eel (male) and Japanese eel (female). The obtained larvae are growing normally and some of them will undergo metamorphosis into glass eel (16). A successful production of European eel larvae was performed in Denmark in 2007 (27). Finally, the great output and low production costs gave China first place in World eel production (11). Among others, European fishery market industry branches depend on glass eel fry costs, which is necessary for culture as well as for introduction (21). Moreover, the price has risen from $350 US to as much as $1000 for one kilogram of eel fry (20). Some or-

ganizations as the Swedish Environment Protection Agency (SEPA) have decided to add European eel to the second enclosure of the CITES Convention.

In connection with the statements of SEPA, the Polish fish market has gradually witnessed the appearance of eels from an Asiatic aquaculture (China, Indonesia), that belong to species such as *A. australis*, short-finned eel, and *A. japonica*, the Japanese eel. These species are supposed to augment the shortcomings of the European eel. Both species and their products have been present in the Far East for a long time but have different commercial values (23). In Polish importers of fish and fishery products it is possible to find two species: the European eel and Japanese eel. Imported transports are likely a mix of both species but in transport documents only one is usually declared, e.g. *A. japonica*. According to FAO data, the annual eel production in world aquaculture in 2005 reached 242 067 tons, with 233 045 tons of Japanese eel (4, 5). In 1998 in 10 European countries 182 eel cultures produced 10 thousand tons of all *Anguilla* species (7).

Eel species identification based on morphological differences (*A. anguilla* and *A. japonica*) might be misleading for proper differentiation of imported fishes, so it is essential to prepare a simple and fast method to identify imported eels. Furthermore, it is also crucial for further technological processes and consumption. European eels cultured in Asia are less immune to parasites in comparison to Japanese eels. Moreover, Japanese eels might also have residues of pharmaceuticals used to decrease mortality.

Based on a combination of morphological and molecular traits, 15 species have been officially recognized within the genus *Anguilla* (15). These species are rather uniform in their ecology and morphology, and all have a snake-like form, with very few species specific characteristics (26). Searching for genetic identities of four species of eels in the genus *Anguilla* (*A. reinhardti*, *A. rostrata*, *A. anguilla*, *A. japonica*) was examined by Lehmann et. al (14). From RAPD data, after phylogram analysis they suggested that *A. japonica* separated from the hypothetical ancestor of the Atlantic species, which split recently into *A. anguilla* and *A. rostrata*. Both *Anguilla* species identification by PCR-RFLP method was establish by Sezaki et al. (23). A new method for the identification of Japanese eel using real-time PCR was performed by Watanabe et al. (cit. 23).

Eel species are very similar to each other in this genus but in Japan the demand for Japanese eel is higher (Japanese eels are more expensive) than for Australian shortfin and longfin eels because of the taste and also on account of body composition (Department of Primary Industries Queensland 2001). Moreover, Asian farms are increasingly buying European glass eels to satisfy the Japanese market.

Taking also into account the health condition of consumers (higher levels of bromides with reduced flammability – Greenpeace) and properly described systematic position of fishes by importers is important for further technological processes. Data concerning brominated flame retardants is scarce because analytical methods are currently not well developed. Nevertheless, collected information showed that these chemicals may have a detrimental effect on humans and wildlife (22). Moreover, trends in glass eel recruitment to the European continent show steep declines from the 1980s. The possible causes include contamination with toxic PCBs, which are released from fat stores during their long-distance migration and interfere with reproduction (6, 9, 18, 19), infection with the swim bladder parasite *Anguillicola crassus* (24, 25), oceanographic and climatic changes (13), over-fishing and blockage of migration routes (6).

Infection with the parasite *Anguillicola crassus* has become a real threat since at the beginning of 1991 these roundworms were found in swim bladders (17). This parasite negatively affects the ability of eels to migrate to the Sargasso Sea (25). Moreover, two decade ago this parasite has been unintentionally brought to Europe from Asia with small eels for introduction (12).

This research has focused on the appropriate characterization of eels imported to Poland and species from the environment by means of genetic methods.

Material and methods

A total of 31 freshwater eels were collected from a local importer (21 samples) and from Lake Miedwie near Szczecin (10 samples). At the beginning of the eel identification process morphometric measurements have been performed, such as the pre-dorsal fin length (pD), pre-anal fin length (pA) and number of teeth on the upper jaw and vomer. A small piece of muscle (30 g) from each of the eels was minced and placed in 1.5 ml Safe-Lock micro test tubes (Eppendorf Inc.) and used for DNA extraction. We carried out this step according to peqGOLD Tissue Mini Kit (peq-lab, Biotechnologie GmbH) and after this all samples were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. A set of primers to amplify part of 16S ribosomal RNA gene was designed according to sequence data obtained from the GenBank database for *A. japonica* and *A. anguilla* (AB021748 and AB021749, respectively). All sequence comparisons were performed using BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast) in order to design primers, which was conducted by Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3). Part of the 16S rDNA was amplified by polymerase chain reaction (PCR) and the use of a pair of designed primers, Ang211F and Ang211R. The PCR amplification procedures were as follows: pre-denaturation at 94°C for 2 minutes, 30 cycles of denaturation (94°C for 30 seconds), annealing (61°C for 30 seconds) and extension (72°C for 30 seconds) and final extension at 72°C for 7 minutes. Each of the reacting 25 µl mixtures contain 2.5 µl MgCl$_2$ (25 mM), 5.0 µl 5X Colorless GoTaq Flexi Buffer, 1.6 PCR Nucleotide Mix (10 mM each), 0.5 µl primer (100 µM each),

1 U GoTaq Flexi DNA Polymerase (Promega, USA), and 1 µl template. After the addition of water to the final concentration and centrifugation, it was amplified in Mastercycler Gradient (Ependorf Inc.) amplifier. RFLP analysis was performed by digestion of amplified products using a digestion mix as follows: buffer 1.5 µl, 0.3 µl ApaI enzyme and 3.2 µl pure H_2O. Subsequently, 5 µl of digestion mix was then added to the PCR product and placed in Eppendorf thermocycler at 37°C for 3 hours. After that time it was subjected to electrophoresis 1.5% agarose gel electrophoresis and stained by ethidium bromide.

Results and discussion

Morphometric measurements have revealed that a preorbital length (prO) that ranged from 17.77 to 20.13% of total length is the only measurement that gives satisfactory results, but it is quite difficult to apply. A pair of primers named Ang211F and Ang211R (tab. 1) were designed for amplification 211 bp of 16S rRNA sequence of both eel species. The electrophoretic pattern of PCR products from *A. japonica* and *A. anguilla* did not show any difference in length. This means that the nonspecific-primer pair was designed properly, moreover the PCR does not produce any other unspecific products (fig. 1). In attempting to distinguish *A. japonica* and *A. anguilla* PCR products of partial 16S rRNA gene, the PCR-RFLP procedure was applied, which mainly bases on nucleotide differences between species sequences. In this method *ApaI* restriction enzyme was used to carry through digestion of PCR product. Only in an amplified sequence of *A. japonica* a restriction site for *ApaI* was found and gel electrophoresis showed a characteristic pattern (fig. 2). As a result, *ApaI* produced fragments of 135 and 76 bp only for *A. japonica*, while *A. anguilla* sequence was not digested with its length of 211 bp. Since about 1970 an extreme decrease in the amount of glass eel fry on the fish market has been observed. This data not only concern European eels (*A. anguilla*), but also Japanese eels (*A. japonica*) (1). The economic importance of this species is significant, because European consumers use approximately twenty five thousand tons every year (27). Worldwide production of eel reached 100-110 thousand tons in 1987, which gave an income estimated at 2-2.2 billion euro in that year (10).

On account of the yearly decrease in amounts of eel on the European market, strict species conservation (Council of the European Union 2006) is intensively discussed. In accordance with the earliest EU directive (Nr 13139/05 Peche 203 – KOM (2005) 427), it is crucial and urgent to introduce plans of eel species protection and catching in international open waters such as: lagoons, coastal waters, estuaries and inland waters of all organized countries (3). Taking into account the great decrease of eel fry controlled near estuaries opening into the Atlantic Ocean (France, England) scientists are looking for solutions to this pro-

Tab. 1. Designed primers according to 16S rRNA sequences data from GenBank for *A. japonica* and *A. anguilla*

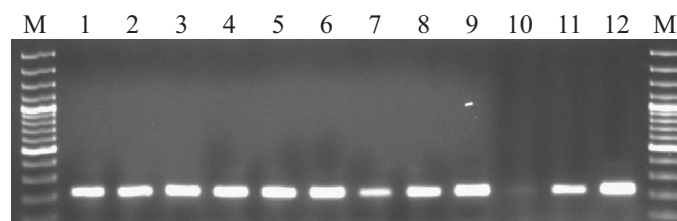| Ang211F	5'CTCCCCACCCCCTAAAAATA3'
Ang211R	5'TTGGGGTTAACGGTTTGTGGT3'

Fig. 1. Electrophoretic analysis of the PCR products from the part of 16S ribosomal RNA gene on 1.5% agarose gel: M – DNA Ladder Plus 100bp; 1-12 lanes 211bp PCR product of unknown and known eel species
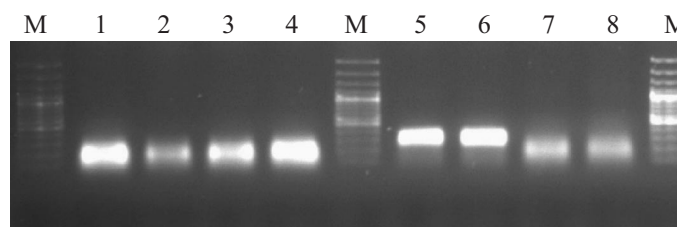

Fig. 2. Restriction fragment length polymorphism pattern of PCR product by using *ApaI* restriction enzyme on 1.5% agarose gel: M – DNA Ladder Plus 100bp; lanes 1, 2, 3, 4, 7, 8 – *A. japonica*; lanes 5, 6 – *A. anguilla*

blem. The market value of this species is significant because one kilogram of eel is more or less 25 EUR/kg and might be higher because the price for kilogram of glass eel fry is still rising.

It is highly probable that the lower level of eel population is caused by extreme changes in river-beds that hinder easy movement along the river. Furthermore, eel pathogens like herpes viruses which may lead to great losses. Lower level of eel population might be also be caused by changes in environment. Researchers hae determined that oceans that warm up as a result of climate change have modified its sea currents which might obstruct eel routes to spawning sites located in the Sargasso Sea (29). One of the greatest threats for eels is contamination with toxic PCB that are released from their fat during long migrations and later affect the procreation process (cit. 8).

Looking for markers that provide unambiguous identification of different eel species is a task that has been a concern for a long time now. Small morphological differences are usually useless for species identification, since they are not always present. Looking into a DNA sequence is a unique solution to find a fast and reliable method that might be utilized to identify unknown species. Allozymes, mtDNA, genomic DNA are genetic markers frequently used in differentiation species (8). Some results show that mtDNA is not an ideal genetic marker because circular mitochondrial

DNA has a high level of homogeneity among European eels. In the gene bank short sequences of nuclear and mitochondrial DNA are submitted that are useful in eel differentiation from distant geographic areas.

Based on the above data it is recommended that research conducted on genetic markers such as EST's, SNP's and microsatellite markers that are suitable for the identification of wild and cultured populations be developed. Precise verification of imported fish transports proposed in this research is essential because of two main reasons: first – controlling meat quality is highly important for human health; second – proper evaluation of their market value. Thus, to sum up, introducing strict genetic control of not only fresh but also frozen or processed fish should be taken into consideration as soon as possible. National organizations GS1, TraceFish project members and national working groups have prepared strict rules for tracking fish and fish products (2). These principles concern not only cultured and captured fish but also processed fish products. The European Commission, Parliament and European Council have introduced rules for fish labeling (EC) 2065/2001, a Product Safety Law concerning food safety and General Food Law for tracking and origin of the food. Tracking fish and fish products is much easier to conduct by means of molecular markers because these products are quite often thermally processed. Moreover, further laws in EU member states are still being prepared and will be introduced to control this type of trade (1).

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