

Rapid identification of European (*Anguilla anguilla*) and North American eel (*Anguilla rostrata*) by polymerase chain reaction.

Schnelle Identifikation des Europäischen (*Anguilla anguilla*) und Nordamerikanischen Aals (*Anguilla rostrata*) mit Hilfe der Polymerasekettenreaktion.

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Abstract

A rapid and cost effective DNA test is described to identify European eel (*Anguilla anguilla*) and North American eel (*Anguilla rostrata*). By means of polymerase chain reaction (PCR) technique parts of the mitochondrial cytochrome *b* gene are amplified with species specific primers which are designed to produce PCR fragments of different characteristic sizes for European and American eel. The size differences can easily be made visible by agarose gel electrophoresis.

Kurzfassung

Es wird ein schneller und kostengünstiger DNA-Test zur Identifikation des Europäischen (*Anguilla anguilla*) und Nordamerikanischen Aals (*Anguilla rostrata*) beschrieben. Mit Hilfe der Polymerasekettenreaktion(PCR)-Technik werden Teile des mitochondrialen Cytochrom-*b*-Gens mit artspezifischen Primern amplifiziert, die so gewählt sind, dass die entstehenden PCR-Produkte charakteristische Größen für den Europäischen bzw. Amerikanischen Aal aufweisen. Die Größenunterschiede können anschließend leicht auf einem Agarosegel sichtbar gemacht werden.

Introduction

The population of the European eel (*Anguilla anguilla*) is sharply declining and the recruitment is nowadays at a level of 1 % of the level reached in the 60s. In Germany efforts are made to restock natural waters like the River Elbe with fingerlings in order to support descending silver eels hopefully migrating to the Sargasso Sea and enhancing the spawning stock capacity in the future.

In European aquaculture, mainly the European eel is used for production. As the price for glass eels has risen up to 1000 Euro per kilogram, American glass eel (*Anguilla rostrata*), available for a much lower price, is increasingly imported and used as stocking material in aquaculture. At fingerling size, it is almost impossible to distinguish between American and European eel. As long as those eels are used for the production of fish used exclusively for human consumption there is no desperate need to ensure species identity. However, if aquacultured eels are to be used as fingerlings for restocking purposes in European waters it must be guaranteed that the species is *A. anguilla*. So the rapid

identification of those two eel species is an important issue concerning the restocking of natural populations. The consequences of massive stocking with *A. rostrata* in European waters can only be hypothesised. Competition with local eels in fresh and coastal waters for food and habitat is possible as well massive introgression if homing to the Sargasso Sea is successful.

There have been different approaches for species discrimination in eel as e. g. protein based methods like isoelectric focusing (Rehbein 1998), but also DNA based methods like sequencing of PCR fragments (Bastrop et al. 2000), restriction fragment length polymorphisms (RFLPs) of PCR products (Hwang et al. 2004; Bastrop et al. 2000), single strand conformation polymorphisms (SSCP) (Rehbein 1998), and species specific primer PCRs (Itoi et al. 2005).

In this study the mitochondrial cytochrome *b* gene was chosen as a PCR target. As a mitochondrial gene, it exists in a high number of copies within the cell. So it is easy to amplify mitochondrial DNA fragments from all kind of different tissues. Also the nucleotide

sequence is conserved within species so that there is not much heterogeneity to be expected within a species and the chance of no amplification in the PCR due to primer mismatching is low.

Material and Methods

We aligned the cytochrome *b* sequences retrieved from Genbank for *Anguilla anguilla* (AB021776, AF006715) *Anguilla rostrata* (AF006716, AF006717, AB021767) and *Anguilla japonica* (AF006702, AF006703, AB021772, AF479272) to design specific primer pairs for European and American eel amplifying parts of the cytochrome *b* region.

Using specially designed primers in a 4-primer mixture the resulting PCR products for *A. anguilla* and *A. rostrata* show different sizes, which can easily be distinguished by agarose gel electrophoresis: a 789 bp fragment for the European eel amplified with primer pair 2AngUniCytbF and 5AngAngCytbR and a 589 bp fragment amplified by 1AngRosCytbF and 6AngRosCytbR for the American eel. A second amplification product for *A. rostrata* amplified from primers 2AngUniCytbF and 6AngRosCytbR with a length of 869 bp is possible as 2AngUniCytbF is not specific for *A. anguilla* but also binds in the cytochrome *b* gene of *A. rostrata*.

Primers used:

1AngRosCytbF:
5'-CAAAGAAACATGAAACATTGGAGTC-3'
6AngRosCytbR:
5'-TTGGTACTACTATTAGAACTAGAATA-3'
2AngUniCytbF:
5'-ATGCCCTAGTGGATCTACCAAC-3'
5AngAngCytbR:
5'-GCGTAGGCAAATAGAAAATACCAC-3'

Template DNA was extracted from gill tissue preserved in ethanol using 10 % Chelex100 solution ac-

cording to standard protocols. The PCR reaction was carried out in a volume of 10 µl using 2.5 mM MgCl₂, 2 mM dNTPs, 1x PCR-Buffer (MWG®), 0.4 U Taq-Polymerase and 25 pmol of each Primer. Initial denaturation was 3 min, followed by 10 cycles of touch-down PCR with 40 sec denaturation at 94 °C, 72 °C annealing (–1°C each cycle) and 40 sec extension at 72 °C, followed by 25 cycles at an annealing temperature of 61 °C and a final extension of 5 minutes at 72 °C. 5 µl of the PCR reaction was put on 1% agarose gel together with size standards.

Anguilla rostrata samples from North America and *Anguilla anguilla* samples from the River Elbe were used as references in the test. The PCR test was applied to 200 samples from different aquaculture facilities to identify the species.

To validate the test and to confirm the identity of the PCR products the amplified fragments from the samples of *A. anguilla* from the Elbe River and *A. rostrata* from North America were cloned into plasmid vectors and sequenced.

Results

The results of the PCR reactions on a 1 % agarose gel are shown in Figure 1. Lanes 1 and 22 contain a 1 kb size standard. For the control DNAs of *A. rostrata* (lanes 12 to 15) and *A. anguilla* (lanes 16 to 19) PCR products are visible in the size range of the expected length of 789 bp for *A. anguilla* and 589 bp for *A. rostrata* respectively.

The negative control with distilled water instead of a DNA template did not amplify any DNA (lane 20). The analysed samples obtained from eel aquaculture facilities (lanes 2 to 11; only 10 out of 200 samples shown in Figure 1) show PCR products in a size range corresponding to the *A. anguilla* control reactions (compare lanes 16 to 19) except from one sam-

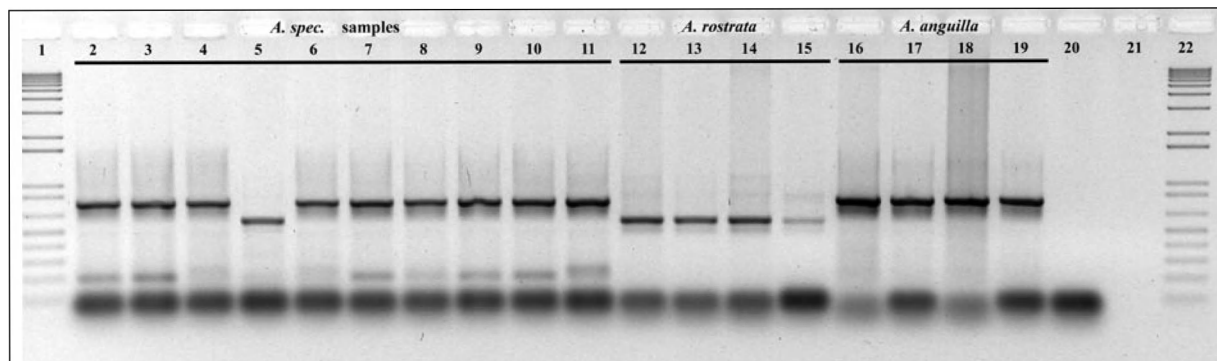


Figure 1: Agarose gel electrophoreses of species test PCR. Lanes 1 and 22 = 1 kb size standard; lanes 2 to 11 = tested DNA samples; lanes 12 to 15 = positive *A. rostrata* controls; lane 16 to 19 = positive *A. anguilla* controls. lane 20 = negative control; lane 21 = empty.

Agarosegelelektrophorese der Arten-Test-PCR. Spuren 1 und 22 = 1-kb-Längenstandard; Spuren 2 bis 11 = getestete DNA-Proben; Spuren 12 bis 15 = positive *A.-rostrata*-Kontrollen; Spuren 16 bis 19 = positive *A.-anguilla*-Kontrollen; Spur 20 = Negativ-Kontrolle; Spur 21 = leer.

ple (lane 5) that shows a PCR fragment corresponding in size to the *A. rostrata* control (compare lanes 12 to 15). Additional faint bands are visible in the reactions in lanes 2 to 11 at a size of approximately 200 bp. Under the described PCR conditions there is only very low amplification of the additional fragment (869 bp) expected for *A. rostrata* samples by amplification of primer 2AngUniCytbF and 1AngRosCytbR (lanes 12 to 15).

Cloning and sequencing of the amplified PCR fragments of expected size from the control reactions for *A. anguilla* (lanes 16 to 19) and *A. rostrata* (lanes 12 to 15) confirmed the PCR product as the desired part of the mitochondrial cytochrome *b* gene. To gain further evidence that the one sample showing a PCR fragment corresponding in size to *A. rostrata* (lane 5) actually is *A. rostrata*, the fragment was also cloned and sequenced together with the PCR product of lane 2, displaying the size of the *A. anguilla* controls. The resulting sequence data clearly identified the sample on lane 5 as *A. rostrata* and the one on lane 1 as *A. anguilla*.

Discussion

The presented test is fast, cost effective and reliable to discriminate between the European eel *A. anguilla* and the American eel *A. rostrata*. The time to perform the entire test, from tissue to agarose gel photography, is approximately 4.5 h, depending on the numbers of samples analysed simultaneously. The cost for DNA extraction from tissue and the ingredients of the PCR reaction are low.

The additional bands visible in Figure 1 do not interfere negatively with the accuracy of the test, neither does the additional band amplified faintly for *A. rostrata*. However, to ensure the reliability of the test, it is important to always include positive controls for *A. anguilla* and *A. rostrata* to the samples analysed.

Using an approach with a mixture of two primer pairs always results in a PCR product no matter which of the two species is used as template DNA. Identification

by positive signals for the two species ensures that a failure in the PCR due to e. g. inappropriate reaction conditions is clearly indicated by no amplification. If the PCR is working properly there is always an amplification and a PCR product corresponding to the expected length of 789 bp for the European eel and 589 bp for the American eel respectively.

To detect other *Anguilla* species than the described two the test could be modified by adding specific primers.

Considering the fact, that *A. rostrata* was found in samples from aquacultured eel offered for restocking, it is highly recommended to ensure species identity of a sub-sample by performing DNA tests like the test presented here.

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