SIST

SLOVENSKI STANDARD
kSIST-TS FprCEN/TS 17303:2018

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Živila - Črtno kodiranje DNA rib in ribjih proizvodov s pomočjo mitohondrijskih genskih segmentov citokrom b in citokrom c oksidaze I

Foodstuffs - DNA barcoding of fish and fish products using defined mitochondrial cytochrome b and cytochrome c oxidase I gene segments

Lebensmittel - DNA-Barcoding von Fisch und Fischprodukten anhand definierter mitochondrialer Cytochrom b- und Cytochrom c-Oxidase I-Genabschnitte

Produits alimentaires - Codes-barres d'ADN de poissons et de produits à base de poissons à l'aide de segments de gènes mitochondriaux du cytochrome b et cyctochrome c oxydase I

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ICS:

67.120.30	Ribe in ribji proizvodi	Fish and fishery products

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This draft Technical Specification is submitted to CEN members for Vote. It has been drawn up by the Technical Committee CEN/TC 275.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (FprCEN/TS 17303:2018) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This document is currently submitted to the vote.

Introduction

Food safety is a key aspect in terms of consumer protection. In the last three decades, globalization has taken place in the trade of food. Fish trade channels are becoming steadily longer and more complicated so that sophisticated traceability tools are needed to ensure food safety. Correct food labelling is a prerequisite to ensure safe fish products and fair trade as well as to minimize illegal, unreported and unregulated (IUU) fishing. In particular, the fact that fish is increasingly being processed in export countries makes the identification of species by morphological characteristics impossible.

The development of harmonized and standardized protocols for the authentication of fish products is necessary to establish reliable methods for the detection of potential food fraud.

1 Scope

This document describes a procedure for the identification of single fish and fish filets to the level of genus or species.

The identification of fish species is carried out by PCR amplification of either a segment of the mitochondrial cytochrome b gene (*cytb*) [1] or the cytochrome c oxidase I gene (*cox1, syn COI*) [2], [3] or both, followed by sequencing of the PCR products and subsequent sequence comparison with entries in databases [4], [5]. The methodology allows the identification of a large number of commercially important fish species.

The decision whether the *cytb* or *cox1* gene segment or both are used for fish identification depends on the declared fish species, the applicability of the PCR method for the fish species and the availability of comparative sequences in the public databases.

This method has been successfully validated on raw fish fillets, however, laboratory experience is available that it can also be applied to processed, e.g. cold smoked, hot smoked, salted, frozen, cooked, fried, deep-fried samples.

This document is usually unsuitable for the analysis of highly processed foods, e.g. tins of fish, with highly degraded DNA where the fragment lengths are not sufficient for amplification of the targets. Furthermore, it is not applicable for complex fish products containing mixtures of two or more fish species.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 24276, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

ISO 16577, *Molecular biomarker analysis — Terms and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
alignment
process or result of matching up the nucleotide residues of two or more biological sequences to achieve maximal levels of identity

[SOURCE: BLAST Glossary]

3.2
BLAST
(The Basic Local Alignment Search Tool) [4]
sequence comparison algorithm optimized for speed used to search sequence databases for optimal local alignments to a query

Note 1 to entry: It directly approximates alignments that optimize a measure of local similarity, the maximum signal pair (MST) score.

3.3
BOLD
(Barcode of Life Data Systems) [5]
informatics workbench aiding the acquisition, storage, analysis, and publication of DNA barcode records

Note 1 to entry: By assembling molecular, morphological, and distributional data, it bridges a traditional bioinformatics chasm. BOLD is freely available to any researcher with interests in DNA barcoding. By providing specialized services, it aids the assembly of records that meet the standards needed to gain BARCODE designation in the global sequence databases. Because of its web-based delivery and flexible data security model, it is also well positioned to support projects that involve broad research alliances.

[SOURCE: BOLDSYSTEMS About Us]

3.4
FASTA format
text-based format for representing either nucleotide sequences or amino acid sequences, which begins with a single-line description, followed by lines of sequence data

Note 1 to entry: The description line (defline) is distinguished from the sequence data by a greater-than (“>”) symbol at the beginning.

[SOURCE: BLAST topics, modified]

EXAMPLE: An example sequence in FASTA format is shown below:

> Sample_04_*cytb*
ATGGCCAGCCTCCGAAAAACTCATCCCCTTCTAAAGATTGCTAATGATGCATTAGTAGACCTTCCTGCCCCCTCTAACCTCT
CAACATTATGAAACTTCGGGTCTCTCCTAGGCCTCTGCTTAGCCGCCCAAATCTTAACAGGACTATTTCTAGCGATACATT
ATACCGCAAACGTCGAGATAGCTTTCTCATCCGTCGTACACATCTGCCGCGACGTAAATTACGGATGACTAATCCGCAACA
TACACGCCAACGGCGCTTCTTTCTTCTTCATCTGCCTCTACCTACACATTGCACGAGGCCTATATTACGGCTCCTACTTATT
CATAGAGACCTGAAACATTGGAGTTGTACTATTCCTTTTAGTAATAATGACCGCCTTCGTAGGCTACGTCCTCCCT

3.5
FishBase
global biodiversity online platform on finfishes providing a wide range of information on all species currently known in the world

3.6
GenBank
comprehensive public database of e. g. genetic sequences [6]

Note 1 to entry: GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Nucleotide Archive (ENA), and GenBank at National Center for Biotechnology Information (NCBI). These three organizations exchange data on a daily basis.

3.7
identity
extent to which two (nucleotide or amino acid) sequences have the same residues at the same positions in an alignment, often expressed as a percentage [Source: BLAST Glossary]

Note 1 to entry: In the database BOLD, the term similarity is used instead of identity.

3.8
introgressed DNA
DNA sequence (allele) from one taxonomic entity (species) incorporated in the gene pool of another, divergent entity (species)

Note 1 to entry: Introgression has usually happened via hybridization and backcrossing of individuals belonging to different species.

3.9
NCBI (National Center for Biotechnology Information)
institution which houses molecular biology databases (e.g. GenBank) and provides the BLAST suite

3.10
nucleotide collection (nr/nt)
non-redundant database consisting of GenBank sequences, in which identical sequences have been merged into one entry

3.11
query
sequence (or other type of search term) to which all of the entries in a data base are to be compared

[SOURCE: BLAST Glossary].

3.12
query coverage
percentage of query covered by alignment to the data base sequence

[SOURCE: BLAST help]

4 Principle

DNA is extracted from fish and fish products applying a suitable method. Segments of approximately 460 base pairs of the *cytb* gene and/or approximately 650 base pairs of the *cox1* gene are amplified by PCR. In the further course, the nucleotide sequence of the PCR product is determined by a suitable DNA sequencing method (e.g. Sanger sequencing). The sequence is evaluated by comparison to sequence entries in databases, thus allowing the assignment to a fish species or genus according to the degree of identity with stored sequences.

5 Reagents and materials

5.1 General

During the analysis, unless otherwise stated, use only reagents of recognized molecular biology grade and distilled or demineralized water or water of equivalent purity, according to ISO 24276. Regarding laboratory organization, see ISO 24276.

5.2 PCR reagents

5.2.1 Thermostable DNA polymerase (for hot start PCR) [1)]

5.2.2 PCR reaction buffer (including $MgCl_2$ or with separate $MgCl_2$ solution) [1)]

5.2.3 Deoxynucleoside triphosphate mix (dATP, dCTP, dGTP and dTTP) [2)]

5.2.4 Oligonucleotides (see Tables 1 and 2)

Table 1 — Oligonucleotides for amplification of the *cytb* gene region [1]

Name	DNA Sequence of oligonucleotide
L14735	5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3'
H15149ad	5'-GCI CCT CAR AAT GAY ATT TGT CCT CA-3'

Table 2 — Oligonucleotides for amplification of the *cox1* gene region [2], [3]

Name	DNA Sequence of oligonucleotide
VF2_t1	5'-***TGT AAA ACG ACG GCC AGT*** CAA CCA ACC ACA AAG ACA TTG GCA C-3'
FishF2_t1	5'-***TGT AAA ACG ACG GCC AGT*** CGA CTA ATC ATA AAG ATA TCG GCA C-3'
FishR2_t1	5'-***CAG GAA ACA GCT ATG AC***A CTT CAG GGT GAC CGA AGA ATC AGA A-3'
FR1d_t1	5'-***CAG GAA ACA GCT ATG AC***A CCT CAG GGT GTC CGA ARA AYC ARA A-3'
M13 tails of the primers are highlighted in bold and italic.	

5.2.5 Agarose

5.2.6 Suitable DNA length standard for assessing the amplification product length

5.2.7 Sequencing primers [3]

Table 3 — Sequencing primers for *cox1* PCR products

Name	DNA Sequence of oligonucleotide
M13F (−21)	5'-TGT AAA ACG ACG GCC AGT-3'
M13R (−27)	5'-CAG GAA ACA GCT ATG AC-3'

1) During the collaborative study the Maxima® Hot Start PCR Master Mix (2 x) of Fermentas GmbH (ready to use PCR buffer solution including thermostable DNA polymerase) was used for the *cytb* amplification and the BIOTAQ DNA polymerase of Bioline with 10 x reaction buffer and separate $MgCl_2$ solution for the *cox1* amplification. In addition to the recommended BIOTAQ DNA polymerase other mastermixes and polymerases were successfully used in the collaborative study.

2) Deoxynucleotide triphosphates can also be part of a commercial PCR master mix.

6 Apparatus

Apart from the usual laboratory equipment, the following equipment is required:

6.1 UV-spectrophotometer or fluorometer, to determine the concentration of DNA

6.2 Thermocycler

6.3 Gel electrophoresis device

6.4 Gel documentation system

6.5 DNA sequencer

7 Procedure

7.1 Sample preparation

It should be ensured that the test portion used for DNA extraction is representative for the laboratory sample. In composed samples (e.g. ready to use meals), single pure fish pieces have to be separated and analysed. With the analysis of samples composed of several pieces (e.g. bags with different fillets), test portions for every putative fish species are taken and analysed separately. To minimize the risk of amplifying adhering contaminants, test sample material shall not be taken from the surface of the laboratory sample. For further information regarding sample preparation, see ISO 20813.

7.2 DNA extraction

Concerning the extraction of DNA from the test sample, the general instructions and measures described in ISO 21571 should be followed, see ISO 20813. It is recommended to choose one of the DNA extraction methods described in ISO 21571:2013, Annex A. Alternatively, commercial kits can be used for the extraction and purification of DNA.

7.3 PCR

7.3.1 General

The primers used for the amplification of the section from the mitochondrial *cytb* gene are universal primers. The primer L14735 binds to a section in the neighbouring highly conserved tRNA-Glu gene. The primer H15149ad is a universal primer with fish-specific adaptations [1]. It is currently known that with some exceptions the primer pair L14735/H15149ad did not react with samples labelled as Barramundi (*Lates calcarifer*) or Nile perch (*Lates niloticus*) [7].

The primers used for the amplification of the section from the mitochondrial *cox1* gene were designed to amplify a segment from the 5' region of the *cox1* gene of fish [2]. The primer pair has been tested against a very broad taxonomic range of fish species, and has only failed in a small minority of cases (<5 % of species tested) [3].

7.3.2 PCR setup

The method was validated for a total volume of 20 µl (*cox1*) or 25 µl (*cytb*) per PCR. The reagents given in Table 4 and Table 5 should be used for the *cytb* and *cox1* PCR, respectively.

Reagents are completely thawed at room temperature and should be centrifuged briefly before usage. A PCR reagent mixture is prepared containing all PCR components in the given concentrations except for the DNA extract. The amount of PCR mixture depends on the total volume per PCR and the total number of the reactions including a sufficient pipetting reserve.