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Molecular biomarker analysis — DNA barcoding of fish and fish products using defined mitochondrial cytochrome b and cytochrome c oxidase I gene segments

Analyse de biomarqueurs moléculaires — Codes-barres d'ADN de poissons et de produits à base de poisson à l'aide de segments de gènes mitochondriaux de cytochrome b et cytochrome c oxydase I

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Contents

Page

Foreword

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This document was prepared by Technical Committee 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 460, *Food authenticity*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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 reliable, harmonized and standardized protocols for the authentication of fish products is necessary to ensure consumer protection and the detection of potential food fraud.

Molecular biomarker analysis — DNA barcoding of fish and fish products using defined mitochondrial cytochrome b and cytochrome c oxidase I gene segments

1 Scope

This document specifies a method for the identification of single fish and fish fillets to the level of genus or species. It allows the identification of a large number of commercially important fish species using DNA barcoding.

This method was validated on raw fish. Laboratory experience indicates additional applicability to processed fish products (e.g. cold smoked, hot smoked, salted, frozen, cooked, fried and deep-fried samples).

The described method 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 does not apply to complex fish products containing mixtures of two or more fish species.

The identification of fish species is carried out by PCR amplification of either a segment of the mitochondrial cytochrome b gene (*cytb*) or the cytochrome c oxidase I gene (*cox1, syn COI*), or both, followed by sequencing of the PCR products and subsequent sequence comparison with entries in databases.

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 16577, *Molecular biomarker analysis — Vocabulary for molecular biomarker analytical methods in agriculture and food production*

ISO 20813, *Molecular biomarker analysis — Methods of analysis for the detection and identification of animal species in foods and food products (nucleic acid-based methods) — General requirements 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 terminology databases for use in standardization at the following addresses:

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

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

3.1
alignment
sequence alignment
arrangement of nucleic acid sequences or protein sequences according to regions of similarity

Note 1 to entry: Alignment is a process or result of matching up the nucleotide residues of two or more biological sequences to achieve maximal levels of *identity* (3.3).

[SOURCE: ISO 16577:2022, 3.7.18, modified — “alignment” was added as the preferred term; Note 1 to entry was added.]

3.2
FASTA format
text-based format for representing either nucleotide sequences or amino acid (protein) sequences, in which nucleotides or amino acids are represented using single-letter codes

Note 1 to entry: A sequence in FASTA format begins with a single-line description, followed by lines of sequence data. The description line (defline) is distinguished from the sequence data by a greater-than (“>”) symbol at the beginning. It is recommended that all lines of text be shorter than 80 characters in length.

Note 2 to entry: An example sequence in FASTA format is:

```
>Sample_04_cytb
ATGGCCAGCCTCCGAAAAACTCATCCCCTTCTAAAGATTGCTAATGATGCATTAGTAGACCTTCCTGCCCCCTCT
AACCTCTCAACATTATGAAACTTCGGGTCTCTCCTAGGCCTCTGCTTAGCCGCCCAAATCTTAACAGGACTATTT
CTAGCGATACATTATACCGCAAACGTCGAGATAGCTTTCTCATCCGTCGTACACATCTGCCGCGACGTAAATTAC
GGATGACTAATCCGCAACATACACGCCAACGGCGCTTCTTTCTTCTTCATCTGCCTCTACCTACACATTGCACGA
GGCCTATATTACGGCTCCTACTTATTCATAGAGACCTGAAACATTGGAGTTGTACTATTCCTTTTAGTAATAATG
ACCGCCTTCGTAGGCTACGTCCTCCCT
```

Note 3 to entry: Blank lines are not allowed in the middle of FASTA input. Sequences are represented in the standard IUB/IUPAC amino acid and nucleic acid codes, with these exceptions:

— lower-case letters are accepted and are mapped into upper-case;

— a single hyphen or dash can be used to represent a gap of indeterminate length.

It is common to end the sequence with an “*” (asterisk) character and to leave a blank line between the description and the sequence.

[SOURCE: ISO 16577:2022, 3.1.2, modified — Example in Note 2 to entry was replaced; the third list item in Note 3 to entry was deleted.]

3.3
identity
extent to which two (nucleotide or amino acid) sequences have the same residues at the same positions in an *alignment* (3.1)

Note 1 to entry: Identity is often expressed as a percentage.

Note 2 to entry: In the sequence database of the Barcode of Life (BOLD), the term "similarity" is used instead of identity.

3.4
introgressed DNA
allele from one species incorporated in the gene pool of another, divergent species

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

3.5
query
sequence (or other type of search term) that is compared to entries in a database

3.6
query coverage
percentage of the *query* (3.5) covered by *alignment* (3.1) to the database sequence

4 Symbols and abbreviated terms

bp	base pairs
Glu	glutamic acid, glutamate
tRNA	transfer RNA
cox1, COI	cytochrome c oxidase I gene
cytb	cytochrome b gene
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
dNTP	deoxyribonucleotide triphosphate
A	adenine
C	cytosine
G	guanine
I	inosine
R	purine base (adenine or guanine)
T	thymine
Y	pyrimidine base (cytosine or thymine)
IUU	illegal, unreported and unregulated
PCR	polymerase chain reaction

5 Principle

DNA is extracted from fish and fish products applying a suitable method. Segments of approximately 460 base pairs of *cytb* and/or approximately 650 base pairs of *cox1* are amplified by PCR. For the amplification of the *cytb* segment a set of two primers is used. Amplification of *cox1* uses a set of four primers to ensure the amplification of DNA of as many fish species as possible. Some of the used primers include ambiguous bases to increase the number of species detected by the two methods. The nucleotide sequences of the PCR products are determined by a suitable DNA sequencing method (e. g. Sanger sequencing). The two PCR primers used to generate the *cytb* amplicon are also used for sequencing. The *cox1* primers have M13 tails that enable sequencing of the *cox1* amplicons using M13 sequencing primers. The determined 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 available sequences.

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

6 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized molecular biology grade and distilled, demineralized water or water of equivalent purity, in accordance with ISO 20813. Laboratory organization shall follow ISO 20813.

6.1 Thermostable DNA polymerase.[1)]

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

6.3 dNTP mix (dATP, dCTP, dGTP and dTTP).

NOTE dNTP can also be part of a commercial PCR master mix.

6.4 Oligonucleotides (see Tables 1 and 2).

NOTE 1 The abbreviations of the DNA bases in Tables 1 and 2 are based on the recommendations for unambiguous, uniform, and consistent nomenclature, published by the International Union of Pure and Applied Chemistry (IUPAC)[4].

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'

NOTE 2 Laboratory experience has shown that M13 tails can also be used with *cytb* primers.

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

Name	DNA sequence of oligonucleotide[a]
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'
[a] M13 tails of the primers are highlighted in bold and italic.	

6.5 Trehalose.

6.6 Agarose.

6.7 DNA size standard.

6.8 Sequencing primers (see Table 3).

Table 3 — Sequencing primers for *cox1* PCR products[3]

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 studies, the Maxima® Hot Start PCR Master Mix (2 ×) 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 × 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. Maxima® Hot Start PCR Master Mix (2 ×) of Fermentas GmbH (ready-to-use PCR buffer solution including thermostable DNA polymerase) and BIOTAQ™ DNA polymerase of Bioline are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.