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Soil quality — Identification of ecotoxicological test species by DNA barcoding

Qualité du sol — Identification des espèces par code-bare ADN dans les essais d'écotoxicologie

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Foreword

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*.

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

Currently, test species identification is usually based on morphological characters. However, this does not always give clear results because

a) few taxonomic experts are available,

b) closely related species can differ by a few, easily overlooked characters, and

c) even more importantly, several test species are in fact complexes of cryptic species.

A good example is the compost worm *Eisenia fetida/andrei* (used in ISO 11268-1, ISO 11268-2 and ISO 17512-1), in which morphological traits alone may not be sufficient to discriminate between both species[5][36]. Another well-known case is the predatory mite, *Hypoaspis* (*Geolaelaps*) *aculeifer*[50], which might get confused with *H. miles*, widely used in biological pest control[31].

Species misidentifications, the use of a morphospecies which is actually a complex of cryptic species, or even species mixing in lab cultures, can be a serious problem for the reliability of the ecotoxicological tests. Sibling species in a morphospecies complex can exhibit ecological, behavioural, and physiological differences, and can differ also in their response to toxicants (e.g. References [2], [17], [35], [40]). This also seems to be the case of the springtail *Folsomia candida* (used in ISO 11267 and ISO 17512-2), in which considerable levels of genetic differentiation have been found among natural populations of *F. candida* and among laboratory strains[9][19][41]. Although different laboratory strains have been found to exhibit only minor differences in the sensitivity towards some chemicals[12][9], other studies have detected significant variation in phenmedipham avoidance behaviour and divergent fitness responses to cadmium exposure among genetically differentiated strains[14][30]. Moreover, even if two species have similar responses to toxicants, the presence of two species within the same laboratory culture can result in the production of sterile hybrids, which will bias the outcome of reproduction tests[36].

Implementing species identification via DNA barcoding can help to overcome these obstacles, ensuring that the species or strain used for testing is well characterized. As a result, quality assurance can be improved, making the results obtained by different ecotoxicological laboratories far more reliable and comparable. For *Eisenia fetida/E. andrei* this work, including an international ringtest, has already been performed[36], see Annex A. The conclusions of this ringtest can be summarized as follows.

— DNA barcoding is a reliable and practical method for identifying *Eisenia* species.

— Only 17 out of 28 ecotoxicological laboratories were correct in their taxonomic assignment. Most laboratories with wrong or unknown assignments actually have *E. andrei* in stock.

— The existence of a cryptic species pair within *E. fetida* is a plausible hypothesis.

— It is important that earthworms used for ecotoxicological tests are regularly (re-)identified by DNA barcoding.

Very probably, similar experiences and recommendations can be drawn for other invertebrates species used in terrestrial ecotoxicology, as well as plants. Indeed, DNA barcoding has proven to be useful for specimen identification and species delimitation in many organism groups, including other earthworms[13][37], enchytraeids[16], mites[15], collembolans[32], molluscs[42], nematodes[28] and terrestrial plants[8].

Soil quality — Identification of ecotoxicological test species by DNA barcoding

1 Scope

This document specifies a protocol to identify ecotoxicological test specimens (mainly invertebrates and plants) to the species level, based on the DNA barcoding technique. This protocol can be used by laboratories performing DNA barcoding in order to standardize both the wet-lab and data analysis workflows as much as possible, and make them compliant with community standards and guidelines.

This document does not intend to specify one particular strain for each test method, but to accurately document the species/strain which was used.

NOTE 1 This does not imply that DNA barcoding is performed in parallel to each test run, but rather regularly (e.g. once a year, such as reference substance testing) and each time a new culture is started or new individuals are added to an ongoing culture.

This document does not aim at duplicating or replacing morphological-based species identifications. On the contrary, DNA barcoding is proposed as a complementary identification tool where morphology is inconclusive, or to diagnose cryptic species, in order to ensure that the results obtained from different ecotoxicological laboratories are referring to the same species or strain.

This document is applicable to identifications of immature forms which lack morphological diagnostic characters (eggs, larvae, juveniles), as well as the streamline identification of specimens collected in field monitoring studies, where large numbers of organisms from diverse taxa are classified.

NOTE 2 In principle, all species regularly used in ecotoxicological testing can be analysed by DNA barcoding. Besides the earthworms *Eisenia fetida* and *E. andrei*, further examples for terrestrial species are *Lumbricus terrestris*, *L. rubellus*, *Allolobophora chlorotica*, *Aporrectodea rosea,* and *A. caliginosa*, *Dendrodrilus rubidus, Enchytraeus albidus,* and *E. crypticus* (Haplotaxida); *Folsomia candida*, *F. fimetaria, Proisotoma minuta,* and *Sinella curviseta* (Collembola); *Hypoaspis aculeifer* and *Oppia nitens* (Acari); *Aleochara bilineata* and *Poecilus cupreus* (Coleoptera); *Scathophaga stercoraria, Musca autumnalis* (Diptera) or *Pardosa* sp. (Arachnida). Nematodes or snails and even plants can also be added to this list.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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

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

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

3.1
amplicon
specific DNA product generated by *PCR* (3.5) using one pair of *PCR primers* (3.6)

3.2
DNA barcode
unique pattern of DNA sequence that identifies each species

3.3
electropherogram
trace file
combination of a graphical representation of a Sanger DNA sequence composed of colour-coded peaks with each colour corresponding to one nucleotide

Note 1 to entry: They are automatically supplied by DNA sequencing programs.

3.4
Phred quality score
Q score
quality measure used to assess the accuracy of a sequencing reaction

Note 1 to entry: This quality measure indicates the probability that a given base is called incorrectly by the sequencer. Phred scores are on a logarithmic scale. Therefore, if Phred assigns a Q score of 30 (Q30) to a base, this is equivalent to the probability of an incorrect base call 1 in 1 000 times. A lower base call accuracy of 99 % (Q20) will have an incorrect base call probability of 1 in 100, meaning that every 100 base pairs sequencing read will likely contain an error.

3.5
polymerase chain reaction
PCR
molecular biology technique for rapidly synthesising multiple copies of a given DNA segment by using a DNA polymerase and an oligonucleotide primer pair

3.6
PCR primer
short oligonucleotides (usually 15 to 30 nucleotides in length) that allow PCR amplification of DNA between specific sites

Note 1 to entry: The two primers (a forward and a reverse) are base-paired to the top and bottom strand of the template DNA, and their 3'-OH ends are in convergent direction.

4 Principle

DNA barcoding is a molecular method that uses a short and standardized DNA region (the DNA barcode) as a genetic tag for species-level identification[22].

Since its inception in 2003 and the launch of the Barcode of Life project, DNA barcoding has systematically been applied not only to biological research, but also to several industrial fields where a correct identification of biological materials is essential, such as the food industry. For example, it is helping to detect fraud in herbal medicinal products[29], and it has been adopted by the Food and Drug Administration (FDA) for seafood and fish identification[21][44]. In fact, DNA barcoding is likely to become a routine test in many fields, in particular in food quality control and traceability[20].

Briefly, the goal of DNA barcoding is:

a) to obtain the nucleotide sequence of a standardised DNA region from an unidentified sample (a test specimen),

b) to compare that sequence with known sequences in a reference database by using bioinformatic methods, and

c) based on such comparison, to identify the sample to the species level.

Therefore, DNA barcoding cannot be a useful identification tool without a reliable and comprehensive reference database, which includes enough samples of each species from across its geographic range to account for intraspecific variability. Also, DNA barcoding relies on the premise that sequences in this barcode region are more similar between members of a species than to sequences of any other species (the so called barcode gap). Therefore, before applying DNA barcoding, a species delimitation study

of the target organismal group should have been carried out to assess its efficacy for discriminating species.

It is essential that the DNA barcoding method is carried out by trained staff. On the one hand, trained laboratory technicians are needed to optimize the wet-lab protocols for each organismal group. On the other hand, the wet-lab pipeline needs to be supervised by scientists trained in genomics and systematics. These scientists should also be in charge of the electropherogram and/or raw DNA sequence file analysis and species assignment.

5 Reagents and material

5.1 Biological material

Adequate specimen preservation is a critical factor to obtain good-quality DNA from samples. Whenever possible, specimen samples for DNA barcoding should be taken from freshly harvested or fresh-frozen tissue. Exposure to preservation agents such as ethyl acetate or formaldehyde should be avoided, as they destroy DNA.

Freezing at –80 °C or in liquid nitrogen (–196 °C) is the preferred method for long-term storage of tissue samples. DNA in dried specimens generally remains stable for at least one year, but degradation becomes increasingly problematic over time[23].

Ethanol-preserved material is easily analysed when fresh, but DNA will slowly become acidified and degraded unless ethanol is regularly refreshed or buffered. For proper tissue preservation, use an ethanol concentration of 95 % to 99 %, and ensure that the volume of ethanol is at least three times greater than the volume of tissue. In order to maintain the ethanol concentration to at least 95 %, it is necessary to replace the ethanol solution within the first days (at least three days) after sampling, and tightly seal the vial to avoid evaporation[23]. A combination of low temperatures (–20 °C) and ethanol will help preserve the samples for long-term storage and helps prevent degradation during thawing and re-freezing cycles.

As a general rule, DNA barcoding analysis should follow tissue collection as soon as possible, but specimens adequately preserved and stored for several months will perform well in DNA extraction[21][23].

5.2 Enzyme

Taq Polymerase from *Thermus aquaticus* is standard for PCR. Hot start Taq polymerases and/or high fidelity DNA polymerases have been shown to offer a high performance in DNA barcoding, allowing for greater amplification sensitivity and increased ease of reaction setup than standard polymerases (http://ccdb.ca/resources/).

Alternatively, pre-optimised commercial master mixes may be used. These consist of a premixed, ready-to-use solution containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates in routine PCR.

5.3 Oligonucleotide PCR primers

For Oligonucleotide PCR primers, see 8.3.2 and 8.3.3.

5.4 Reagents

5.4.1 **Nuclease-free water molecular grade water** (dd H_2O).

5.4.2 **TE buffer (Tris-EDTA buffer)**, 1-fold, pH 8,0.

Dissolve 1 ml of 1 mol/l Tris base (pH 8,0), 0,2 ml EDTA (0,5 mol/l) in 98,8 ml of molecular grade water. Adjust the pH to 8,0 with concentrated HCl.

5.4.3 Deoxynucleoside triphosphates (dNTPs).

5.4.4 PCR buffer, without Mg (500 mmol/l KCl, 100 mmol/l Tris-HCl, pH 8,3 at 25 °C).

Buffer is usually supplied with each enzyme as a 10-fold or fivefold concentrate. Use only the buffer supplied with each particular enzyme.

5.4.5 Magnesium chloride, $MgCl_2$.

5.4.6 PCR additives (optional): trehalose dihydrate, bovine serum albumin (BSA), formamide, dimethyl sulfoxide (DMSO).

5.4.7 Agarose (analytical grade, standard melting temperature).

5.4.8 TAE (gel-running buffer), 50-fold stock solution, pH 8,3.

Dissolve 242 g of Tris base [tris(hydroxymethyl)aminomethane], 57,1 ml of glacial acetic acid (17,4 mol/l), 100 ml of 500 mmol/l EDTA solution (pH 8,0) in 842,9 ml of molecular grade water.

5.4.9 Size standard 100 base pair (bp) DNA ladder, a commercially available molecular-weight marker suitable for sizing double-stranded DNA from 100 to 1 000 base pairs during gel electrophoresis.

5.4.10 6-fold Loading buffer, 3 ml of 100 % glycerol, 0,025 g of bromophenol blue, 0,025 g of xylene cyanol FF in 7 ml of molecular grade water.

5.4.11 Ethidium bromide solution (0,5 µg/ml) or any safer alternative nucleic acid stain.

5.4.12 PCR purification kit, either using enzymatic reactions, magnetic beads or silica-membrane-based cleanup.

5.4.13 5-fold Sequencing buffer (400 nm Tris-HCl, pH 9,0, 10 mmol/l $MgCl_2$).

5.4.14 BigDye® Terminator v3.1 cycle sequencing kit[1)].

5.4.15 Pop-7 Polymer for 3730 DNA analyzers[1)].

5.4.16 3730 DNA analyser capillary array, 50 cm[1)].

5.4.17 GeneScanTM 500 LIZTM DYE Size Standard[1)].

5.4.18 Highly deionized formamide.

6 Apparatus

The usual laboratory equipment, including micropipettes, centrifuge, and the following specific equipment.

6.1 Spectrophotometer, to measure the concentration and purity of double-stranded DNA at 260 nm.

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1) This protocol has been validated using the 3730 DNA Analyzer capillary electrophoresis system and the BigDye terminator chemistry. They are registered trademarks of Applied Biosystems. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.