Identification of *Anisakidae* Larvae at the species level by PCR/RFLP

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1. Aim and field of application

To identify the species or genotype of single Anisakidae larvae preserved in ethanol by a PCR/RFLP analysis. This method can be applied to larvae collected from human biopsies or from tissues of animal origin.

2. Principle of the method

The PCR is a molecular biology technique that allows for the amplification of specific nucleic acid fragments, of which the initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species (or genotype) has its own characteristic DNA portion, due to its composition and/or dimension, it is possible to choose an oligonucleotide pair allowing for its amplification. The PCR amplification is characterized by a high sensitivity and specificity.

It is possible to combine the “standard PCR” with the “Restriction Fragment Lenght Polymorphism” (RFLP), that means the analysis of DNA restriction fragments. The technique allow to distinguish PCR fragments of comparable length by enzymatic digestion with one or more endonucleases, enzymes able to cut DNA by recognition of short and specific oligonucleotide sequences. In our case it is possible to amplify the same portion of DNA from different species, identified on the basis of the size of DNA restriction fragments.

The larval stage of nematodes of the Anisakidae family parasitize fishes, cephalopods and shrimps, while the Anisakidae adult worms parasitize fishes, marine birds and mammals. The larvae belonging to the genus *Anisakis* and *Pseudoterranova* are responsible, if ingested by humans, of the disease known as anisakiasis. Larvae obtained from human patients or infected hosts can be morphologically identified, however larvae of the genus *Anisakis* and *Pseudoterranova* cannot be distinguished. Molecular methods based on the PCR/RFLP allowed the identification at the species level of single larvae of *Anisakis* spp. and *Pseudoterranova* spp., in particular 7 sibling species of *Anisakis*, *A. simplex* sensu-stricto (ss), *A. pegreffi*, *A. simplex* C, *A. ziphidarium*, *A. physeteris*, *A. typica*, *Anisakis* sp. A and *Pseudoterranova* spp (P. decipiens or P. krabbei or P. bulbosa). The *Anisakis* and *Pseudoterranova* species differ in the composition and/or size of the DNA sequence of the locus ITS, allowing the unambiguous identification of all anisakidae species epidemiologically relevant.

The size of the fragments produced by the amplification by nucleotide pairs are shown in Table A. In Table B, the fragments obtained by enzymatic digestion, with the specified enzymes, of ITS fragments, are reported.

*Table A - Size of the ITS fragments (base pairs) for each species*

<table>
<thead>
<tr>
<th>A. pegreffi</th>
<th>A. simplex s.s.</th>
<th>A. simplex C</th>
<th>A. ziphidarium</th>
<th>A. physeteris</th>
<th>A. typica</th>
<th>A. sp. A</th>
<th>Pseudoterranova spp. (P. decipiens s.s.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>951</td>
<td>951</td>
<td>953</td>
<td>931</td>
<td>898</td>
<td>956</td>
<td>926</td>
<td>906</td>
</tr>
</tbody>
</table>
Table B - Expected sizes of the ITS digestion fragments for each species (base pairs) obtained with Hinf and HhaI enzymes by agarose gel electrophoresis

<table>
<thead>
<tr>
<th>ITS fragments obtained by PCR</th>
<th>Hinf</th>
<th>HhaI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. pegreffii</strong></td>
<td>34, 67, 235, 284, 331</td>
<td>419, 532</td>
</tr>
<tr>
<td><strong>A. simplex ss</strong></td>
<td>34, 67, 235, 615</td>
<td>419, 532</td>
</tr>
<tr>
<td><strong>A. simplex C</strong></td>
<td>34, 67, 237, 615</td>
<td>142, 279, 532</td>
</tr>
<tr>
<td><strong>A. ziphidarium</strong></td>
<td>34, 273, 292, 332</td>
<td>413, 518</td>
</tr>
<tr>
<td><strong>A. physeteris</strong></td>
<td>34, 241, 263, 360</td>
<td>385, 513</td>
</tr>
<tr>
<td><strong>A. typica</strong></td>
<td>34, 328, 594</td>
<td>103, 153, 180, 212, 308</td>
</tr>
<tr>
<td><strong>A. sp A</strong></td>
<td>34, 269, 288, 335</td>
<td>137, 272, 517</td>
</tr>
<tr>
<td><strong>Pseudoterranova s.p.p.</strong></td>
<td>35, 179, 693</td>
<td>413, 493</td>
</tr>
</tbody>
</table>

Using the PCR/RFLP technique based on the size of the ITS digestion fragments with the Hinf enzyme, it is possible to distinguish larvae of the genus *Anisakis*, *A. pegreffii*, *A. ziphidarium*, *A. physeteris*, *A. typica* and *A. shupakovi* from *A. simplex C*, *A. simplex* sensu-stricto and *Pseudoterranova*. By ITS sequence digestion with *HhaI* enzyme, *A. simplex C* can be distinguished from *A. simplex* s.s.

3. References


ISO/FDI 20837:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for sample preparation for qualitative detection.
ISO/FDI 20838:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for amplification and detection for qualitative methods


4. Definitions

ITS (Internal Transcribed Spacer), interspaced sequence of the nuclear ribosomal gene, including the ITS-1, the 5.8S gene, the ITS-2 gene with the addition of 70 bp of the 28S gene

Oligonucleotide, short sequence (15/30 nucleotide bases) used to amplify a DNA specific fragment

SetA, mix of 2 oligonucleotide base pairs amplifying ITS from each species.

Reference larvae, larvae of Anisakis pegreffi in ethanol, supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma

Reference DNA, purified DNA from larvae of Anisakis pegreffi, supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma

DNA extraction positive control, a reference larva analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session

DNA/larva, DNA extracted from a single larva

Amplification positive control, a reference DNA; this control is used in the amplification session to verify the efficacy of the PCR

Amplification negative control, reagent grade water; this control is used in the amplification session to verify the efficacy of the PCR

PCR, Polymerase Chain Reaction

Restriction Enzymes. Restriction enzymes are enzymes of bacterial origin able to cut DNA at specific sites, that are sequences of 4-8 bases which length is different for each enzyme, allowing the DNA fragmentation in a reproducible and specific manner. Endonucleases cut inside the DNA chain. Enzyme concentration is measured as "enzymatic units" (U). In this case 1U corresponds to the amount of enzyme needed to completely digest 1 μg of DNA.

The definitions and terminology used in the UNI EN ISO 22174 standard are applied in the present protocol.

5. Devices/instruments

5.1 Stereo microscope, magnification 60×100x

5.2 Bench top refrigerated centrifuge for 1.5 mL tubes, minimum 10,000xg

5.3 Freezer ≤-15°C

5.4 Thermoblock with vibration, temperature range 25±100°C

5.5 Magnetic separation stand
5.6 PCR thermocycler
5.7 Refrigerator, temperature range +1 – +8°C
5.8 Horizontal electrophoretic apparatus
5.9 Analytical balance, readability 0.1g
5.10 UV transilluminator
5.11 Digital imaging system
5.12 Adjustable volume pipettes, volume range: 1-10µL, 2-20µL, 20-100µL, 50-200µL, 200-1000µL
5.13 Analytical grade water system production, resistivity \( \geq 18 \text{ Mohm/cm} \)
5.14 Vortex
5.15 Orbital shaker

6. Reagents and chemicals

6.1 **Incubation buffer.** Commercial solution: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Once prepared, label the solution with “IB+”. Store according to the manufacturer’s recommendations.

6.2 **Lysis buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with “LB+”. Store according to the manufacturer’s recommendations.

6.3 **Paramagnetic resin.** Commercial suspension: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Store according to the manufacturer’s recommendations.

6.4 **Washing buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with “WB+”. Store according to the manufacturer’s recommendations.

6.5 **Eluting buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Store according to the manufacturer’s recommendations.

6.6 **2x PCR master mix.** 2x commercial solution, Promega, codes: M7501, M7502, M7505 (composition: dATP 400 µM, dCTP 400 µM, dGTP 400 µM, dTTP 400 µM, MgCl2 3mM, Taq DNA polymerase 50 U/mL), other commercial PCR master mixes should be considered suitable for PCR amplification. Store according to the manufacturer’s recommendations.

6.7 **SetA.** The oligonucleotide mixture (6.8) used for the PCR; the mixture is obtained combining an equal volume of the 2 oligonucleotides NC5 and NC2 (6.8) in TE 0.1x (6.17); the final concentration corresponds to 20 pmol/µL; 100µL aliquots are prepared and stored frozen up to 24 months.

6.8 **Oligonucleotides.** Commercial preparation (Table C); the lyophilized products is reconstituted with TE 0.1x, according to the manufacturer’s recommendations, at a concentration of 100 pmol/µL; the lyophilized product can be stored frozen for up to 5 years; the reconstituted product can be stored frozen up to 18 months.

*Table C – Oligonucleotides present in the setA (6.7 e 6.8), their codes and amplified nucleotide sequences.*

<table>
<thead>
<tr>
<th>Sequenza oligonucleotidi</th>
<th>Codice</th>
<th>Sequenza amplificata</th>
</tr>
</thead>
<tbody>
<tr>
<td>S'-GTAGGTGAACCTGCGGAAGGATCATT-3'</td>
<td>NC5</td>
<td>ITS</td>
</tr>
<tr>
<td>S'-TTAGTTTCTTTTCCCGCT-3'</td>
<td>NC2</td>
<td></td>
</tr>
</tbody>
</table>

6.9 **Loading buffer 6x.** Commercial product allowing DNA molecule electrophoresis to be performed. Store according to the manufacturer’s recommendations.
6.10 Agarose and high resolution agarose. Commercial products suitable for performing DNA molecule electrophoresis. The high resolution agarose is suitable for the analysis of small DNA fragments (25-700 bp), improving their separation in gel electrophoresis. Store at room temperature for up to 24 months.

6.11 TAE solution 50x. Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store at room temperature for up to 24 months.

6.12 TAE solution 1x. 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. Store at room temperature for up to 1 month.

6.13 Ethidium bromide solution. Commercial product 10 mg/L. For the working condition, dilute 1:100,000; for 100 mL solution, add 1.0 µL. Store in the dark at room temperature for up to 24 months.

NOTE: Ethidium bromide is potentially mutagenous, carcinogenic and teratogenic; wear disposable gloves and handle very carefully the solution containing this substance.

6.14 L50. Commercial product containing markers for DNA molecular weight multiple of 50 bp. All commercial products containing molecules of multiples of 50 bp within the 50-500 bp range can be used. Store refrigerated according to manufacturer’s recommendations.

6.15 L1000. Commercial product containing markers for DNA molecular weight up to 1000 bp. All commercial products containing molecules of within the 50-1000 bp range can be used. Store refrigerated according to manufacturer’s recommendations.

6.16 TE 1x solution. Commercial product 10mM Tris-HCl (pH 8.0), 1mM EDTA-Na₂, pH 7.9–8.1 at 25°C. Store refrigerated for up to 12 months.

6.17 TE 0.1x solution. TE 1x diluted solution. For 100 mL preparation: take 10 mL from 1x solution and add 90 mL water. Filter with 0.22 µm filters and prepare aliquots of 10 mL. Store frozen for up to 24 months.

6.18 Milli-Q grade water. Resistivity $\geq$ 18 Mohm/cm

6.19 Reference Larvae. Larvae of A. pegreffi stored in ethanol (95-99%) supplied by the Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma. Store frozen for up to 10 years.

6.20 Reference DNAs. Genomic DNA purified from reference larvae. Reference DNAs are supplied by the Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma, and prepared according to the protocols described in Sambrook et al. (1989). Store frozen for up to 10 years.

6.21 Restriction enzymes Hinfl and Hhal. Commercial products suitable for DNA enzymatic digestion. Store refrigerated according to manufacturer’s recommendations. The oligonucleotide sequences recognized by each enzyme is reported in Table D.

Table D - Oligonucleotide sequences recognized by the reported enzymes

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Sequence recognized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hinfl/</td>
<td>5'...GANTC...3'</td>
</tr>
<tr>
<td></td>
<td>3'...CTNAG...5'</td>
</tr>
<tr>
<td>Hha/</td>
<td>5'...GCGC...3'</td>
</tr>
<tr>
<td></td>
<td>3'...CGCG...5'</td>
</tr>
</tbody>
</table>

6.22 Restriction enzyme buffers. Commercial products suitable for DNA enzymatic digestion with defined pH and saline concentration. The buffers are commonly sell together with the corresponding restriction enzyme. Store refrigerated according to manufacturer’s recommendations.
7. Procedure

7.1 Sample preparation

Test samples are inspected to verify the presence of larvae and the preservation conditions. If the conditions are not suitable, the test will not be performed.

The ethanol containing the larvae is transferred into a petri dish and observed under the stereo microscope. A maximum of 3 larvae are collected and placed in 1.5 mL conical tubes, one larva in each tube. Excess ethanol is removed and a minimum volume is left.

The tubes containing the larvae are spin at maximum speed for few seconds.

Store the tubes containing the frozen larvae. Under these conditions, larvae can be stored for the DNA extraction for up to 10 years.

7.2 Method

7.2.1 DNA extraction from one single larva

If not otherwise specified, the procedure is carried out at room temperature.

Each working session requires a reference larva, or portion, to be submitted to the DNA extraction procedure and identified as “positive control for the extraction”.

Before starting the procedure, prepare enough volume of the IB+ (6.1) and LB+ (6.2) solutions according to the manufacturer’s recommendations.

a) Centrifuge the tubes containing the larvae at maximum speed for a few seconds.

b) Add 100 µL of IB+ (6.1).

c) Incubate at 55°C for 30-60 min in the thermodblock. During incubation, shake at 1,400 vibrations/min.

d) Centrifuge, as in point “a”.

e) Add 200 µL of LB+ (6.2).

f) Add 10 µL of paramagnetic resin (6.3) after resuspending it by vortexing.

g) Incubate for 5-10 min at 25°C in the thermodblock. During incubation, shake at 1,400 vibrations/min.

h) Place the tubes in the magnetic separation stand and wait for 30-60 sec, so that the paramagnetic resin particles are blocked by the magnetic field on the tube wall.

i) Discard the liquid phase by aspirating, avoiding the dislodging of the resin particles.

j) Add 100 µL of LB+ (6.2) and resuspend the resin particles by vortexing.

k) Place the tubes in the magnetic separation stand, as in point “h”.

l) Discard the liquid phase by aspirating.

m) Add 100 µL of WB+ 1x (6.4) and resuspend the resin particles by vortexing.

n) Place the tubes in the magnetic separation stand, as in point “h”.

o) Discard the liquid phase by aspirating.

p) Repeat the washing step, from “m” to “o”, with WB+ (6.4) 3 times.

q) After the last washing, leave the tubes open to let the resin particles dry for 15-20 min.

r) Add 100 µL of the eluting buffer (6.5) and gently resuspend the resin particles, do not vortex.

s) Incubate at 65°C for 5 min. During incubation, shake at 1,400 vibrations/min.

t) Place the tubes in the magnetic separation stand, as in point “h”.

u) Collect the liquid phase (90-100 µL circa) and transfer it to a 1.5 mL tube.

v) The resulting extract is defined as “DNA/larva” and stored frozen. Under these conditions, it can be stored for up to 10 years.

7.2.2 PCR amplification
Unless otherwise clearly stated, keep tubes on ice; use tips with barrier and wear disposable gloves.

At each working session, use a positive and a negative amplification controls. Use reference DNA (6.19) as positive control and water (6.17) as negative control.

a) Thaw: DNA/larva, 2x PCR MasterMix, SetA, and positive amplification controls.

b) Mark with a progressive number an adequate number of 0.2 µL PCR tubes.

c) Prepare an adequate cumulative volume of the amplification mix. Evaluate the volume on the basis of a single sample amplification mix (Table C) and of the total number of samples plus two (1 for the positive amplification control and 1 for the negative one).

Table C – single sample amplification mix: components and volumes

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x PCR MasterMix (6.6)</td>
<td>25 µL</td>
</tr>
<tr>
<td>H2O</td>
<td>22 µL</td>
</tr>
<tr>
<td>SetA (6.7)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Total</td>
<td>48 µL</td>
</tr>
</tbody>
</table>

d) Mix the amplification mix by vortexing and centrifuge at maximum speed for a few sec.

e) Transfer 48 µL of the cumulative amplification mix to each PCR tube (point “b”).

f) Add 2 µL of the larval DNA to be tested to each tube.

g) Close the tubes, mix by vortexing and centrifuge at maximum speed for a few sec.

h) Start the amplifying cycle (Table D) on the thermocycler device; wait for the temperature to reach 95°C and insert the tubes in the thermoblock by pausing the instrument.

Table D – amplification cycles

<table>
<thead>
<tr>
<th>Step</th>
<th>Time/Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-denaturation</td>
<td>2 min/95°C</td>
</tr>
<tr>
<td>Amplification</td>
<td>30 s/95°C, 30 s/55°C, 75 s/72°C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>7 min/72°C</td>
</tr>
</tbody>
</table>

i) At the end of the amplification phase, centrifuge the tubes at maximum speed for a few sec.

j) To 10 ml of the amplification reaction add 2.0 µL of loading buffer 6x (6.9) if not contained in the PCR Master Mix (6.6).

m) Vortex and centrifuge the tubes at maximum speed for a few sec.

n) Keep tubes on ice or refrigerated until starting electrophoresis.

7.2.3 Result display

a) Assemble the electrophoresis apparatus according to the manufacturer’s recommendations. For the gel preparation, use a comb suited for the number of samples.

b) Add 1 gr agarose (6.10) in 100 mL TAE 1x (6.12) in a glass beaker.

c) Gently resuspend the powder by rotation.

d) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.

e) Restore with water the volume lost by boiling.

f) Allow the agarose solution to cool.

g) Before it solidifies (at about 47°C), add 1.0 µL of ethidium bromide solution (6.13).

h) Shake gently to dissolve uniformly the ethidium bromide and pour the agarose in the gel tray previously prepared (point “a”).
i) Wait for the gel to solidify, which requires at least 30 min.

j) Place the tray with the gel in the electrophoresis apparatus.

k) Cover the gel with TAE 1x buffer (6.12) and gently pull out the comb.

l) The first and, if possible, the last well are loaded with 15 µL of the L50 solution (6.14).

m) Load in each well 10 µL of the amplification product (point 7.2.2 “l”), respecting the progressive numbering of the tubes (point 7.2.2 “b”).

n) Connect the electrophoresis apparatus with the power supply and set 10 v/cm of gel.

o) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (6.9), reaches a distance of 1 cm from the gel border.

p) After 30 min, switch off the power supply, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if it is possible to easily distinguish all bands of molecular weight marker ranging from 50 and 500 bp. If the separation is incomplete, continue the run.

q) At the end of the run, transfer the gel to the imaging system and print the result.

7.2.4 Result Interpretation

The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight L1000 (6.14) and with the positive extraction and amplification controls. The visual evaluation is considered sufficient and adequate (see Table A).

The amplification test is considered valid if:

a) the amplification of the positive control shows an amplification product as in Table A;

b) the amplification of the negative control does not show any amplification product or, eventually, only bands related to unused oligonucleotides and/or primer dimers

c) the positive control of the extraction product shows an amplification product as in Table A;

The species identification is made after enzymatic digestion of the amplified fragments comparing the size of the band(s) produced by the sample(s) with those shown in Table B.

In case the sample shows a not expected band, the sample will not further processed and the identification will not be possible.

7.2.5 Enzymatic DNA digestion with endonucleases

Unless otherwise stated, keep tubes on ice; use tips with barrier and wear disposable gloves.

At each working session, independent digestions with HinfI and HhaI enzymes (6.21; 6.22) are performed. To verify the correct running of the digestion, the PCR amplification product of the reference DNA (positive control) is also digested.

In the procedure, the restriction enzymes are used at the initial concentration of 10-20 U/µl and concentrated restriction enzyme buffers at 10x. In case of different concentration, adjust the protocol according to the manufacturer's instruction.

a) Thaw PCR products, restriction enzymes and 10X restriction enzyme buffers.

b) Mark with a progressive number an adequate number of 1,5 mL tubes.

c) Prepare an adequate cumulative volume of the enzymatic digestion mix for each restriction enzyme. Evaluate the volume on the basis of a single sample enzymatic digestion mix (Table E) and of the total number of samples plus the positive control.

| Table E – Enzymatic digestion mix for a single sample: components and volumes |
10x buffer (6.23) 1,5 µL
Restriction enzyme (6.22) 5u (0,25-0,5 µL)
PCR product 10 µL
H₂O 3-3,25 µL
Total 15 µL

d) Mix each enzymatic digestion mix by vortexing and centrifuge at maximum speed for a few sec.
e) Transfer 5µL of the cumulative amplification mix to each tube (point “b”).
f) Add 10 µL of the PCR product to be tested to each tube.
g) Close the tubes, mix by vortexing and centrifuge at maximum speed for a few sec.
h) Incubate the tubes at 37°C for 2 h in the thermoblock (5.2) without shaking.
i) At the end of the amplification phase, centrifuge the tubes at maximum speed for a few sec.
j) Add 3 µL of loading buffer 6x (6.9).
k) Vortex and centrifuge the tubes at maximum speed for a few sec.
l) Keep tubes on ice or refrigerated until starting electrophoresis.

7.2.6 Result display
a) Assemble the electrophoresis apparatus according to the manufacturer’s recommendations. For the gel preparation, use a comb suited for the number of samples.
b) Add 3 gr of high resolution agarose (6.10) in 100 mL TAE 1x (6.12) in a glass beaker.
c) Gently resuspend the powder by rotation and leave at 4°C in the refrigerator for 30 min.
d) Boil the of high resolution agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.
e) Restore with water the volume lost by boiling.
f) Allow the agarose solution to cool with gentle shaking.
g) Pour the agarose in the gel tray previously prepared (point “a”).
h) Wait for the gel to solidify, which requires at least 30 min.
i) Place the tray with the gel in the electrophoresis apparatus.
j) Load in each well the enzymatic digestion product (point 7.2.1 “n”), respecting the progressive numbering of the tubes (point 7.2.1 “b”).
k) Load the first and, if possible, the last wells with 15 µL of the L50 solution (6.14).
l) Connect the electrophoresis apparatus with the power supply and set 10 v/cm of gel.
m) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (6.9), reaches a distance of 1 cm from the gel border.
q) Stain the DNA by transferring the gel in 400 ml of ddH₂O with 4 µL of ethidium bromide solution (6.13). Gently shake the gel on an orbital shaker (5.16).
r) After 30 min, remove the staining solution and replace it with 400 ml of ddH₂O. Gently shake the gel on an orbital shaker (5.16) for 15 min. Repeat once again this washing step.
s) After 30 min, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if all bands of molecular weight marker ranging from 25 and 1000 bp are easily distinguished. If the separation is incomplete, continue the run.
t) At the end of the run, transfer the gel to the imaging system and print the result.

7.2.7 Result Interpretation
The size of the restriction bands revealed by the electrophoresis is evaluated by their comparison with the
The enzymatic digestion test is considered valid if the digestion of the positive control shows a profile of bands product according to Table B.

The species identification is made after enzymatic digestion of the amplified fragments comparing the size of the band(s) produced by the sample(s) with those shown in Table B.

In case the sample shows a band different from those reported in Table B, the identification is not possible.

8. Results

The results are expressed as follows:

If the digestion profile with HinfI is 34, 67, 235, 615bp and with HhaI is 419, 532bp, the sample is identified as *A. simplex ss.*

If the digestion profile with HinfI is 34, 67, 235, 284, 331bp and with HhaI is 419, 532bp, the sample is identified as *A. pegreffii.*

If the digestion profile with HinfI is 34, 67, 237, 615bp and with HhaI is 142, 279, 532bp, the sample is identified as *A. simplex C.*

If the digestion profile with HinfI is 34, 273, 292, 332bp and with HhaI is 413, 518bp, the sample is identified as *A. ziphidarium.*

If the digestion profile with HinfI is 34, 241, 263, 360bp and with HhaI is 385, 513bp, the sample is identified as *A. physeteris.*

If the digestion profile with HinfI is 34, 328, 594bp and with HhaI is 103, 153, 180, 212, 308bp, the sample is identified as *A. typica.*

If the digestion profile with HinfI is 34, 269, 288, 335bp and with HhaI is 137, 272, 517bp, the sample is identified as *A. sp. A.*

If the digestion profile with HinfI is 35, 179, 693bp and with HhaI is 413, 493bp, the sample is identified as *Pseudoterranova spp.*

In case the digestion test was valid but the sample displays a band profile not comparable with those reported in Table B, identification at the species level will be considered “impossible”.

9. Characteristics of the method

This method has been characterised in terms of specificity, sensitivity and repeatability. The results of the validation process confirmed that the method is suitable for the specified aim, and were evaluated by the Italian accreditation body (ACCREDIA) to accredit it according to the ISO/IEC 17025.

10. Safety measures

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices while performing the test.