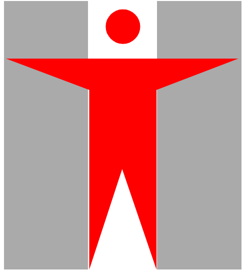

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GCMTI method publications

Differentiation of Two Genuine Deer Species of Cervi Cornu Pantotrichum by Specific-PCR

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Safety precautions: *This method involves the use of hazardous materials. It is the user's responsibility to apply appropriate precaution when handling such materials. Use eye and hand protection and where necessary carry out the work in a fume cupboard or biological safety cabinet.*

1. Introduction

Specific polymerase chain reaction (specific-PCR) analysis employs specific primers to recognize target species. Comparing to DNA sequencing approach like DNA barcoding, the procedure is relatively simple and fast as no DNA sequencing step is required, which makes specific-PCR commonly applied for rapid screening of species origin of pathogens, environmental microorganisms, food, and herbs. In Chinese Pharmacopoeia 2015, specific-PCR based methods for identification of species origins for animal-derived Chinese Materia Medica (CMM), such as *Deinagkistrodon acutus*, *Zaocys dhumnades*, *Bungarus multicinctus*, are recorded.

This method describes a screening test for the differentiation two genuine deer species, i.e. *Cervus nippon* and *Cervus elaphus*, of Cervi Cornu Pantotrichum from each other by specific-PCR analysis, which can be applied to Cervi Cornu Pantotrichum in whole or powdered form from the single species origin.

A typical protocol is included in the Supplementary Information as reference for ease of operation.

The analytical processes of this method can be broken down into the following steps: 1. DNA extraction; 2. amplification of target characteristic DNA region for differentiation by specific-PCR; 3. electrophoresis; and 4. result analysis.

2. Reagent and materials

All chemicals and reagents involved shall be of molecular biology / PCR grade and suitable for DNA analysis as a rule. The water used shall be double distilled or PCR grade water free from nuclease and nucleic acids. Chemicals, reagents and water shall be autoclaved prior to use, if feasible. Operator shall wear powder-free gloves for all operations. The use of aerosol-protected pipette tips as protection against cross contamination is recommended.

3. Apparatus

General laboratory equipment and apparatus for this method are listed in the Supplementary Information. Critical equipment in the entire analytical processes are stated as follows:

3.1 Laminar flow hood, to provide unidirectional flow of HEPA-filtered air over the work

area to prevent contamination.

3.2 **Temperature controlled incubator**, to provide experimental condition for nucleic acid extraction.

3.3 **Spectrophotometer**, to estimate the purity and yield of double-stranded DNA.

3.4 **PCR thermal cycler**, to use in PCR amplification of DNA.

3.5 **Electrophoresis system**, to check amplified PCR products.

4. General procedure

Having to guarantee the validity of test results, controls shall be run in parallel with test samples as described in Clause 5, where relevant, in order to detect DNA contamination of the reagents or sample-to-sample contamination.

The protocol, reagents, materials and apparatus described in the Supplementary Information is for reference purpose only. Any modification of the protocol, including but not limited to the use of other kits or changes of procedures, shall be subjected to adequate validation by the user who has the responsibility to assess the suitability of the testing items when adopting.

4.1 Preparation of test portion

Operator responsible for the preparation of test samples, such as reducing particle size, homogenization, and taking the test portion, shall have good laboratory practices and cross-contamination avoidance to preclude invalid test results.

4.2 DNA extraction

4.2.1 Acquisition of amplifiable DNA is a prerequisite for DNA method. The process in general can be carried out by using a commercial DNA isolation kit, which is much easier for quality control of extraction process and save time for troubleshooting as products' quality are controlled and guaranteed by manufacturers.

4.2.2 DNA extraction kits or protocols should be selected depending on the chemical/ cellular composition of herbal materials. It is advisable, where relevant, to remove the following compositions in herbal materials in order to obtain sufficient quality and yield of DNA extracts for subsequent PCR amplification process:

4.2.2.1 RNA and /or proteins, which may be removed by enzymatic

treatment using RNase and proteinase, respectively.

4.2.2.2 Tissue parts, such as antler, hair, scale, enriched in keratin, the use of dithiothreitol may facilitate the extraction efficiency.

4.2.3 Estimation of the quality of DNA extracts by means of

4.2.3.1 spectrophotometric analysis, and

4.2.3.2 amplification of endogenous gene of animal taxon.

4.3 Amplification of target characteristic DNA region by specific-PCR

4.3.1 This method provides 3 sets of primer pairs for the following purposes, deem it fit:

4.3.1.1 CNIP-f / CNIP-r is the specific primer pairs (SPP) for detection of *C. nippon* in this method. The primer pair amplifies a partial mitochondrial DNA region of *C. nippon* to produce a 223-bp DNA band, but not *C. elaphus*.

4.3.1.2 CELA-f / CELA-r is the SPP for detection of *C. elaphus* in this method. The primer pair amplifies a partial mitochondrial DNA region of *C. elaphus* to produce a 248-bp DNA band, but not *C. nippon*.

4.3.1.3 UnivP / UnivQ is an internal control primer pair (ICPP) that binds to animal mitochondrial genomes. This primer pair is to provide assurance that the DNA extract of sample is amplifiable. DNA extracts should be tested by this primer pair before proceed to specific-PCR analysis. This pair primer shall run with every test sample.

4.3.1.4 The information of all primer pairs used in this method are available in Annex A.

4.3.2 Prepare PCR reaction mix and perform PCR according to Annex A.

4.4 Electrophoresis

4.4.1 Resolve the PCR products by manual agarose gel electrophoresis system or automated electrophoresis system.

4.4.2 Capture the image and estimate the size of DNA band by reference to DNA size markers.

4.5 Result analysis

4.5.1 The differentiation between *C. nippon* and *C. elaphus* is based on the presence / absence of target DNA band (Clause 4.3.1) in electrophoresis profile.

5. Quality control parameters

The performance of each run is checked for conformance with the acceptance criteria of quality controls to determine if the results of analyses are acceptable and able to meet the objective of this method. In consideration of the compliance with quality control (QC) plan, user should determine the number of samples in a batch to give a reasonable handling capacity for operator to conduct DNA analysis.

5.1 Use of system controls

The system controls of this method are tabulated as below. The arrow (↓) indicates that this control should be applied in the particular analytical step.

Analytical steps	Extraction negative control[1]	Extraction positive control[2]	Sample duplicate control[3]	PCR negative control[4]
DNA extraction	↓	↓	↓	
Amplification of target characteristic DNA region by PCR	↓	↓	↓	↓
Electrophoresis	↓	↓	↓	↓

1. Extraction negative control is the negative control of DNA extraction process which does not contain any added sample. This control shall always be placed after the last DNA extraction sample. This control shall be analyzed in duplicate.

2. Extraction positive control serves as the positive control to reveal any defect or problem in the reagents used during the DNA extraction or in the operation of such process. This control uses a reference material of known taxonomic identity or come from the organisms of interest which has its taxonomic identity verified or certified by recognized museums, national authorities, universities or research institutions. This control shall be analyzed in duplicate.

3. Test sample shall be run in duplicate for entire analysis to demonstrate the consistence of test result obtained from the method.

4. PCR negative control is the negative control to reveal any defect and problems in the reagents used during the PCR or in the operation of such process. This control can demonstrate that the PCR reagent used be freed from any contaminating nucleic acids. This control shall be analyzed in duplicate.

5.2 Parameters

5.2.1 The system control is evaluated by electrophoresis. The following parameters shall be met:

	Parameter	**Acceptance criteria**
A	Extraction negative control	Absence of target DNA band
B	Extraction positive control	Presence of target DNA band in ICPP Presence of target DNA band in SPP for the corresponding species
C	Sample duplicate control	Consistent findings in duplicate samples
D	PCR negative control	Absence of target DNA band

6. References

Chinese Pharmacopoeia Commission. (n.d.) *Pharmacopoeia of the People’s Republic of China* (2015 ed.). Beijing: China Medical Science Press.

Annex A
(Normative)

Primer pairs and PCR conditions for this method

Oligo-nucleotide primers are detailed in below table. Primers may be ordered from a supplier who specializes in the synthesis of oligonucleotides. A standard desalted purification is sufficient for the use of specific-PCR analysis.

A1. Primer information.

Primer name	Primer direction	Oligonucleotide DNA sequence (5’-3’)	Amplicon size (bp)	Location of target site
UnivP	Forward	GGTTTACGACCTCGATGTTG	~104	Animal mitochondrial DNA
UnivQ	Reverse	CGGGTCTGAACTCAGATCAC		
CNIP-f	Forward	CTTACACATGCAAGCATCCA	223	Relative to *Cervus nippon* mitochondrion between bp 110 – 332 of DQ985076.1
CNIP-r	Reverse	TTAATCGTATGACCGCGGC		
CELA-f	Forward	GCAAGCATCCGCACYCCG	248	Relative to *Cervus elaphus* mitochondrion between bp 119 – 366 of KT290948.1
CELA-r	Reverse	AACACACTTTACGCCGTRKGC		

A2. PCR conditions for amplification of target characteristic DNA regions.

Reagent	UnivP / UnivQ	CNIP-f / CNIP-r	CELA-f / CELA-r
DNA template	~20 ng	~ 10 ng	~10 ng
10X PCR Buffer*	1 X	1 X	1 X
$MgCl_2$	2.0 mM	1.2 mM	1.2 mM
dNTPs	0.2 mM @ dNTP	0.2 mM @ dNTP	0.2 mM @ dNTP
Forward primer	0.4 µM	0.2 µM	0.2 µM
Reverse primer	0.4 µM	0.2 µM	0.2 µM
Taq DNA polymerase	0.25 µL	0.2 µL	0.2 µL
Sterilized Water	An appropriate amount to make up to 25 µL	An appropriate amount to make up to 25 µL	An appropriate amount to make up to 25 µL

*10X PCR Buffer shall contain no Mg^{2+} ion

A3. PCR cycling conditions for amplification of target characteristic DNA regions.

A. Animal DNA QC with UnivP/ UnivQ primer pair

Temperature	Time	No. of cycles
95°C	5 min	1
94°C 55°C 72°C	30 sec 30 sec 1 min	30
72°C	7 min	1
4°C	∞	--

B. Specific-PCR for *C. nippon* with CNIP-f / CNIP-r primer pair

Temperature	Time	No. of cycles
94°C	2 min	1
94°C 63°C 72°C	30 sec 30 sec 30 sec	30
72°C	5 mins	1
4°C	∞	--

C. Specific-PCR for *C. elaphus* with CELA-f / CELA-r primer pair

Temperature	Time	No. of cycles
94°C	2 min	1
94°C 63°C 72°C	30 sec 30 sec 30 sec	30
72°C	5 min	1
4°C	∞	--