SUPPLEMENTARY MATERIAL

Rapid Identification of Cervus antlers by Species-specific PCR Assay

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1. Experimental

1.1 Samples

Four antler species including CEL, CNT, RTL and RUK were used in this study, and relevant information of them was listed in Table S1. CE\textsubscript{i} was a certified raw material purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), and the others have been verified by COI barcoding. These samples were then handled according to the procedures required in the prevailing China Pharmacopoeia (Ch.P., 2015 edition, Vol. I).

Fourteen batches of commercial processed samples labelled Cervus antlers were mainly collected from wholesale market and Chinese medicine shops (Table S2), which were used for species identification by the newly proposed approach.
Table S1. Raw materials of antler species used in the study.

| Code | Species | Sources | Collection date |
|------|---------|---------|-----------------|
| CE₁  | CEL     | NICPBP  | March, 2017     |
| CE₂  | CEL     | Jinan, Shangdong | March, 2017    |
| CE₃  | CEL     | Changchun, Jilin | March, 2017    |
| CE₄  | CEL     | Changchun, Jilin | March, 2017    |
| CN₁  | CNT     | Jining, Shangdong | April, 2017   |
| CN₂  | CNT     | Tieling, Liaoning | March, 2018   |
| CN₃  | CNT     | Tonghua, Jilin | April, 2017    |
| CN₄  | CNT     | Tonghua, Jilin | April, 2017    |
| CN₅  | CNT     | Liaoyuan, Jilin | April, 2017    |
| RT₁  | RTL     | Baoding, Hebei | April, 2017    |
| RT₂  | RTL     | Wuxi, Jiangsu | April, 2017    |
| RT₃  | RTL     | Dalian, Liaoning | March, 2018   |
| RT₄  | RTL     | Baishan, Jilin | April, 2017    |
| RT₅  | RTL     | Tonghua, Jilin | April, 2017    |
| RT₆  | RTL     | Songyuan, Jilin | May, 2017     |
| RU₁  | RUK     | Southeast Asia | April, 2017    |
| RU₂  | RUK     | Southeast Asia | April, 2017    |
| RU₃  | RUK     | Tieling, Liaoning | March, 2018 |

Table S2. Commercial products tested in the study.

| No. | Form | Batch No. | Place of Origin | Manufacturer |
|-----|------|-----------|-----------------|--------------|
| 1   | Slice| 170101    | Liaoning        | Wangda TCM store, Tieling, Liaoning |
| 2   | Powder | 20180325 | Jilin           | Yuexiang TCM store, Tonghua, Jilin |
| 3   | Slice | 20180301 | Jilin           | Jiyong TCM store, Changchun, Jilin |
| 4   | Slice | 20180301 | Unknown         | Bozhou TCM store, Bozhou, Anhui |
| 5   | Powder | 20180325 | Unknown         | Qirui TCM store, Baoding, Hebei |
| 6   | Slice | 131105    | Liaoning        | Yonggang Co., Ltd., Bozhou, Anhui |
| 7   | Slice | 160501    | Jilin           | Baishixin Co., Ltd., Bozhou, Anhui |
| 8   | Slice | 170101    | Jilin           | Baishixin Co., Ltd., Bozhou, Anhui |
1.2 DNA extraction

18 batches of raw materials (CEL, CNT, RTL and RUK) and 14 batches of commercial products were smashed into their fine powder in liquid N\(_2\), and then subject to genomic DNA extraction by improved SDS-based protocols. In details, 50 mg of the powder was mixed with 10 \(\mu\)L of 20 mg·mL\(^{-1}\) Proteinase K and 990 \(\mu\)L of extraction buffer composed of 100 \(mM\) NaCl, 10 \(mM\) Tris-HCl (pH 8.0), 25 \(mM\) EDTA and 0.5% SDS (w/v), and the mixture was constantly incubated at 56°C under horizontal shaking at 100 rpm for 6 hrs. After centrifugation at 12,000 rpm for 15 mins, 800 \(\mu\)L of the supernatant was transferred to a 2.0 mL centrifuge tube. An equal volume of Tris-phenol solution, PCI solution and CI solution were sequentially mixed with the supernatant for further purification. Then, 500 \(\mu\)L of the supernatant was precipitated by 1000 \(\mu\)L of 96% EtOH and 50 \(\mu\)L of 5.0 \(M\) KAc solution. The supernatant was discarded after centrifugation at 12,000 rpm for 15 mins, and the resulting DNA pellet was washed with 70% EtOH and finally reconstituted in 25 \(\mu\)L of TE buffer for subsequent PCR amplification.

Nucleic acid & protein spectrophotometer (BioSpec-mini, Shimadzu) were used to quantify the purity and concentration of the extracted DNA. These purified DNA samples from raw materials or commercial products were then diluted to 100 ng·\(\mu\)L\(^{-1}\) as template in further PCR assays.

1.3 Primers design and screening

Mitochondrial complete gene sequences from four species in the antler were incorporated to develop an accurate and rapid method for their identifications.
Therefore, sixteen mitochondrial genome sequences of four species, including CNT (Accession No.: NC_006993.1, NC_006973.1, NC_013834.1, NC_016178.1, NC_007179.1, NC_018595.1, NC_008462.1), CEL (Accession No.: NC_013836.1, NC_013840.1, NC_014703.1, NC_007704.2), RTL (Accession No.: NC_007703.1, KM506758.1), and RUK (Accession No.: NC_031835.1, NC_008414.3, EF035448.1) were used to as targets. Suitable areas for designing species-specific primers were identified by DNAMAN software (v. 8.0.8.789), and species-specific primers for species identification were then designed using Oligo software (v. 7.60). Primer sets were evaluated by Oligo and online NCBI Primer-BLAST. The preliminary theoretical screening of designed primer pairs was carried according to the conditions such as the primer score and the length of the amplified product, and characteristic information of partial primers with the highest score were summarized in Table S3. All the primers were then synthesized by Sangon Biotech (Shanghai) Co., Ltd. Subsequent screening process of each primer set was performed against five selected samples (Table S1).

**Table S3.** Partial primer sets designed in this study.

| Species | No. | Sequence (5’-3’) | Target gene | Length (bp) |
|---------|-----|------------------|-------------|-------------|
| CEL     | 1   | F TATATATACACTCAGACCCCA | ND6         | 187 bp      |
| CNT     |     | R GCCTATTTTGGCTCTGCTC |             |             |
| CEL     | 2   | F TATAGAGGAAACCCGAAACCA | COX3        | 146 bp      |
| CNT     |     | R CTACAAAGAAAGTTGAGCCAT |             |             |
| RTL     | 3   | F CGCTAACACGTACCATACCAA | ND5         | 200 bp      |
| RTL     |     | R CTGCGAATGACCTGCCAT   |             |             |
| RTL     | 4   | F TGCACCTCTAGGACAACCT | ND5         | 109 bp      |
| RUK     | 5   | F CATTTACATCGCGGCACCT | CYTB        | 101 bp      |
| RUK     |     | R AGGAATTATTCCTGCGCTGTA |             |             |
| RUK     | 6   | F ATCATTTTTAATTAGCTCCC | ND2         | 116 bp      |
| RUK     |     | R GCTGCGCTCTGAGCTC    |             |             |
| RUK     | 7   | F GTTTGCGTGCAACGAGA   | COX1        | 70 bp       |
1.4 PCR Amplification

PCR amplification was carried out in a final reaction volume of 25 μL composed of 2.5 μL of 10 × PCR buffer, 2.0 mM of MgCl₂, 0.2 mM of each dNTP, 0.2~0.4 μM of each primer, 0.625~1 unit of Taq polymerase, 19.875 μL of ultrapure water and 1 μL of DNA template. The PCR cycler conditions used were an initial denaturation at 95°C for 3 mins, followed by 30~35 cycles of 95°C for 30 s, 60°C~65°C for 30 s and 72°C for 1 min with a final extension at 72°C for 7 mins. After resolution by 2%~3% agarose gel electrophoresis and staining in ethidium bromide, the resulting amplicons were visualized under UV light.

1.5 Specificity and sensitivity

The specificity test of three primer sets were further validated under the optimum conditions using different batches of antler species, including separate four batches of CNT and CEL for the verification of common primers, while six batches for primer set of RTL and another three batches for primer pair of RUK. Furtherly, in order to verify the sequences of short-length fragments amplified by the species-specific primers, PCR products of raw material of CE₁, CN₂, RT₁ and RU₁ were subject to sequencing in both directions by Sangon Biotech (Shanghai) Co., Ltd. Sensitivity of a selected specific primer set for each of the four species was determined in a concurrent PCR run with DNA template of a series of concentrations (100 ng·μL⁻¹, 10 ng·μL⁻¹, 1 ng·μL⁻¹, 0.1 ng·μL⁻¹, and 0.01 ng·μL⁻¹) while the primer remained unchanged.

1.6 Analysis of reference sample mixtures

Prior to mixing to create reference mixtures, sample of different species was collected to undergo DNA extraction and serve as a positive control. Four reference
antler mixtures (CNT:RTL, CNT:RUK, CEL:RTL and CEL:RUK) were prepared in five levels (9:1, 7:3, 1:1, 3:7 and 1:9) of one species mixed with the second species, with a total weight of 50 mg per sample. Individual sample was homogenized with 1 mL of SDS extraction buffer for DNA extraction by SDS-based protocols as aforementioned. Then, the selected primers and the optimized PCR conditions were applied to these reference samples.

1.7 Application of PCR assay to commercial products

Finally, the developed method was used to assess the authenticity of commercially available products, for the identification of animal origins and the verification of labelling compliance. 50 mg of these samples were individually subject to DNA extraction. And the optimized PCR conditions for each species were then applied to the DNA sample.

| Table S4. Primer sets used for PCR assay in this study. |
|-------------------------------------------------------|
| Species | Code | Sequence (5'-3') | Target gene | Length (bp) |
|---------|------|------------------|-------------|-------------|
| CEL | PC | TGATATATAACACTCAGACCCCA | ND6 | 187 |
| CNT | R | GCTGTATTTGCGTCTGTC | ND5 | 200 |
| RTL | PRT | CGCTAACAGTCATATACAAA | CYTB | 101 |
| RUK | PRU | CATTTATCATCAGGACT | CYTB | 101 |

| Table S5. Optimized PCR conditions for three primer sets analysed in this study. |
|-------------------------------------------------------------|
| Primers | Program Step | PC | PRT | PRU |
|---------|-------------|----|-----|-----|
| Amplification | -Initial denaturation | 95°C (3 min)\(a\) |
| | -Denaturation | 95°C (30 s)\(a\) |
Annealing
- 64°C (30 s) 63°C (30 s) 61°C (30 s)

Extension
- 72°C (1 min)*

Cycle number
- 33*

Final extension
- 72°C (7 min) *

* These conditions were the same for all primers.

Table S6. Results of species identification in commercial products. The results of PCR are reported as positive (+) or negative (-).

| No. | PC | PRT | PRU |
|-----|----|-----|-----|
| 1   | -  | +   | -   |
| 2   | -  | +   | -   |
| 3   | -  | +   | -   |
| 4   | -  | +   | -   |
| 5   | -  | +   | -   |
| 6   | +  | -   | -   |
| 7   | +  | -   | -   |
| 8   | +  | -   | -   |
| 9   | +  | -   | -   |
| 10  | +  | -   | -   |
| 11  | +  | -   | -   |
| 12  | +  | -   | -   |
| 13  | +  | -   | -   |
| 14  | +  | -   | -   |
Figure S1. Flow chart for the establishment of the novel strategy.

Figure S2. Screening of designed primer sets for species-specificity (M: Low ladder, SN127; N: Negative control).
Figure S3. Optimized PCR conditions for three primer sets.

Figure S4. Specificity test for three specific primer sets.
Figure S5. Amplicons of specific primer sets sequenced and aligned.

Figure S6. Sensitivity test for three specific primer sets. The concentration of template DNA from lane A to lane E was 100, 10, 1, 0.1, 0.01 ng·μL⁻¹.

Figure S7. Analysis of reference sample mixtures.
Figure S8. Analysis of commercial products by PCR.