



Research Article

Development of a species-specific PCR assay for authentication of *Agkistrodon acutus* based on mitochondrial *cytochrome b* geneYingnuo Li^{a,e}, Wang Yanshuang^b, Li Mingcheng^c, Zhang Lihua^d, Yuan Guang Xin^{a,*}^a School of Pharmacy, Beihua University, Jilin 132013, China^b School of Medicine, Beihua University, Jilin 132013, China^c School of Medical Technology, Beihua University, Jilin 132013, China^d Jilin City Leibo Science and Technology Co., Ltd., Jilin 132013, China^e Department of Pharmacy, Changchun Medical College, Changchun 130031, China

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ABSTRACT

Background: *Agkistrodon acutus*, a traditional Chinese medicine, clinically used in the treatment of rheumatism, tumor, and cardiovascular and cerebrovascular diseases. Due to the unique medicinal value and the difficulty of artificial breeding of *Agkistrodon acutus*, the supply of *Agkistrodon acutus* on the market exceeds the demand, and a large number of its adulterants are found on the market. In this study, the *cytb* gene sequences of *Agkistrodon acutus* and 9 snakes were compared and analyzed, specific primers were designed, and specific PCR methods were established to detect *Agkistrodon acutus* medicinal samples on the market.

Results: This method was successfully applied to distinguish the snake from other adulterated species, and tested 18 *Agkistrodon acutus* samples randomly purchased from six cities. Twelve samples were counterfeit and six were genuine. The standard reference material of *Agkistrodon acutus* was cloned by molecular cloning and sequencing, and the gene sequence difference with other species was significant. It shows that the region could be used as the fingerprint region of the target species.

Conclusions: The proposed method can be used as a species-specific marker and can be highly distinguished from other adulterated snake species, which is helpful to effectively avoid the problem of false sale of *Agkistrodon acutus*.

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1. Introduction

Agkistrodon acutus, nicknamed deinagkistrodon acutus, white flower snake or chessboard snake is a dried body of *Agkistrodon acutus* (Guenther). *Agkistrodon acutus* has a long medical history, clinically used in the treatment of rheumatism, tumor, and cardiovascular and cerebrovascular diseases. Current studies on *Agkistrodon acutus* mainly focus on the immunomodulation, antitumor and cardiovascular protection of *Agkistrodon acutus* venom [1,2,3].

Nowadays, *Agkistrodon acutus* is one of the most endangered snakes listed in Convention on International Trade in Endangered

species of Wild Fauna and Flora (CITES) [4]. In order to protect the endangered snake species, the commercial pythons are mostly farmed. Because of the unique medicinal value and the difficulty of artificial breeding of *Agkistrodon acutus*, the supply of *Agkistrodon acutus* on the market exceeds the demand, and a large number of its adulterants (mostly *Bungarus multicinctus*, *Naja*, *Agkistrodon halys*, *Dinodon rufozonatum*, etc.) are found on the market [5].

The traditional identification method of *Agkistrodon acutus* is based on its morphological or histological features, which is subjective and dependent on the experience of the authenticators. However, most of the *Agkistrodon acutus* medicines sold on the market have been processed, thus losing their integrity and changing in its external morphology, so that it is difficult to distinguish their characters [6]. Because the structure and chemical composition of different snake samples are similar, and their histocyte characteristics are not obvious [7], it is difficult to distinguish

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Table 1
Sources of standard and counterfeit *Agkistrodon acutus*.

Species	Code	Source
<i>Agkistrodon acutus</i> (Guenther)	ZPQS-1	National Institute for the Control of Pharmaceutical and Biological Products
	QS1, QS2	Yixing City, Guizhou Province
	QS3, QS4	Nanning City, Guangxi Province
	QS5, QS6	Dehui City, Jilin Province
<i>Bungarus multicinctus</i>	201204-71	National Institutes for Food and Drug Control
	YHS1	Yixing City, Guizhou Province
	YHS2	Nanning City, Guangxi Province
<i>Naja</i>	YHS3	Dehui City, Jilin Province
	210206-101	National Institutes for Food and Drug Control
	YJS1	Yixing City, Guizhou Province
<i>Agkistrodon halys</i>	YJS2	Nanning City, Guangxi Province
	YJS3	Dehui City, Jilin Province
	201204-078	National Institutes for Food and Drug Control
<i>Dinodon rufozonatum</i>	FS1	Yixing City, Guizhou Province
	FS2	Nanning City, Guangxi Province
	FS3	Dehui City, Jilin Province
	201204-087	National Institutes for Food and Drug Control
<i>Vipera berus</i>	CLS1	Yixing City, Guizhou Province
	CLS2	Nanning City, Guangxi Province
	CLS3	Dehui City, Jilin Province
<i>Ovophis monticola</i>	KS1	Yixing City, Guizhou Province
	KS2	Nanning City, Guangxi Province
	KS3	Dehui City, Jilin Province
<i>Elaphe moellendorffi</i>	SLS1	Yixing City, Guizhou Province
	SLS2	Nanning City, Guangxi Province
	SLS3	Dehui City, Jilin Province
<i>Zaocys dhumnade</i>	BHS1	Yixing City, Guizhou Province
	BHS2	Nanning City, Guangxi Province
	BHS3	Dehui City, Jilin Province
<i>Ptyas mucosus</i>	WSS1	Yixing City, Guizhou Province
	WSS2	Nanning City, Guangxi Province
	WSS3	Dehui City, Jilin Province
	HSS1	Yixing City, Guizhou Province
	HSS2	Nanning City, Guangxi Province
	HSS3	Dehui City, Jilin Province

Agkistrodon acutus from its adulterants by the microscopic identification and physicochemical identification.

The molecular identification of Traditional Chinese Medicines (TCMs) is characterized by high accuracy, specificity and sensitivity, not limited by the traditional morphological identification, and can be used as raw medicinal materials, medicinal slices, powders and preparations (pills, granules, etc.) [8]. The reported methods for the molecular identification of TCMs, such as RAPD [9], SSR [10], PCR-RFLP [11], AS-PCR [12], AFLP [13], Multiplex PCR [14] and DNA barcode analysis [15], are all dependent on PCR [16]. The *cytochrome b* (*cytb*) gene in mitochondrial DNA (mtDNA), which evolves at a moderate rate, has become a hot spot in recent studies as an effective molecular genetic marker [17].

In this study, the mtDNA *cytb* gene of *Agkistrodon acutus* was used as the target gene, and its specific fingerprint region was selected to design the specific primer to obtain the specific amplified fragment of *Agkistrodon acutus*. This method could effectively distinguish *Agkistrodon acutus* from its common adulterants, with a stable result of the amplification and a high resolution of electrophoresis. On this basis, we developed a DNA detection method for *Agkistrodon acutus*, which may make the identification process simpler, faster, more accurate and more suitable for its popularization [18].

2. Materials and methods

2.1. Collection of samples

Agkistrodon acutus standard reference substance (ZPQS-1) was purchased from National Institute for the Control of Pharmaceutical and Biological Products. *Bungarus multicinctus* (201204-71),

Naja (210206-101), *Agkistrodon halys* (201204-078) and *Dinodon rufozonatum* (201204-087) standards were purchased from National Institutes for Food and Drug Control. *Agkistrodon acutus* (QS1-QS6), *Bungarus multicinctus* (YHS1-YHS3), *Naja* (YJS1-YJS3), *Agkistrodon halys* (FS1-FS3), *Vipera berus* (KS1-KS3), *Ovophis monticola* (SLS1-SLS3), *Dinodon rufozonatum* (CLS1-CLS3), *Elaphe moellendorffi* (BHS1-BHS3), *Zaocys dhumnade* (WSS1-WSS3) and *Ptyas mucosus* (HSS1-HSS3), which all were genuine, were provided by three different serpentine breeding bases (Table 1).

Agkistrodon acutus medicinal materials, totally 18 batches, were randomly purchased from 6 Chinese cities from May 2017 to October 2017. The purchased medicinal materials were washed with water 3 times and dried at 40–50°C, and then stored in a ventilated, shady, cool and dry place for use.

2.2. Preparation for genomic DNA

All samples collected in the study were fully washed with double distilled water to remove the sediment and impurities and dried at 40–50°C, and then ground into powders with a sterilized pulverator and the sample powders were stored in containers. 0.5 g of *Agkistrodon acutus* sample was placed in a mortar and ground into powders fully; 0.1 g of the sample powders was placed in a 1.5 mL centrifuge tube and 275 µL P1 digestible solution [200 µL nucleus lysis buffer, 50 µL of 0.5 mol/L ethylenediamine tetraacetic acid disodium solution, 20 µL protease K solution (20 mg/mL) and 5 µL RNAase solution] was added into the centrifuge tube, which was incubated at 55°C for 1 h, and then 250 µL P2 lysis buffer was added into the centrifuge tube, which was mixed fully and placed into the purified DNA column to be centrifuged (10,000 rpm) for 3 min; the filtrate was discarded and 800 µL P3 eluent [26 µL of 5 mol/L potassium acetate solution,

18 μL of 1 mol/L Tris-hydrochloric acid solution (pH 7.5), 3 μL of 0.5 mol/L ethylenediamine tetraacetic acid disodium solution (pH 8.0), 480 μL anhydrous ethanol and 273 μL aseptic double distilled water] was added into it, which was centrifuged (10,000 rpm) for 1 min; the filtrate was discarded, and the residue was repeatedly eluted with the above eluent 3 times, in which the solution was centrifuged at 10,000 rpm for 1 min each time; the filtrate was discarded, the residue was centrifuged for 2 min again, and then the DNA purified column was transferred into another centrifuge tube; 100 μL P4 sterilized double distilled water was added into the tube, which was left standing at room temperature for 2 min, then centrifuged at 10,000 rpm for 2 min to obtain the supernatant as the test solution, and the supernatant was kept at 20°C for use. 0.1 g *Agkistrodon acutus* control medicinal material was prepared into the template DNA solution of control medicinal material according to the same method as described above.

2.3. PCR reaction system and reaction conditions

The primers were synthesized by Shanghai Bioengineering Co., Ltd.

Upstream: 5'-GGCAATTCACACAGCCAACATCAACT-3';

Downstream: 5'-CCATAGTCAGGTGGTTAGTGATAC-3'.

The PCR reaction system was carried out in a 200 μL centrifuge tube, the total volume of the reaction was 25 μL , and the reaction system consisted of 2.5 μL 10 \times PCR buffer, 2 μL dNTP (2.5 mmol/L), 0.5 μL of each identification primer (10 $\mu\text{mol/L}$), 0.2 μL high fidelity Taq DNA polymerase (5 U/mL), 2 μL template and 17.3 μL sterilized double distilled water. The centrifuge tube was placed in a PCR instrument, and the PCR reaction parameters included pre-denaturation at 95°C for 5 min, circular reaction 30 times (at 95°C for 30 s, at 63°C for 45 s) and extension (at 72°C) for 5 min.

2.4. Specificity test

The specificity of the primer pairs was tested by cross-amplification with DNA from 9 non-target species (*Bungarus multicinctus*, *Naja*, *Agkistrodon halys*, *Dinodon rufozonatum*, *Vipera berus*, *Ovophis monticola*, *Zaocys dhumnade*, *Ptyas mucosus* and *Elaphe moellendorffi*).

2.5. Cloning and sequencing of *Agkistrodon acutus* DNA fragment

The PCR reaction of *Agkistrodon acutus* positive DNA was conducted. 5 μL PCR product was used for the agarose gel electrophoresis. The DNA band was cut by a UV transmission

instrument and the target gene was recovered by gel recovery kit (AxyPreo DNA Gel Extraction Kit 50-prep). The target gene was ligated with the pGM-T vector for cloning, and the plasmid DNA was extracted to confirm the sequence accuracy by sequencing.

3. Results

3.1. Analysis of genomic DNA

The UV spectrophotometry showed that the value of A260/A280 was 1.66 ± 0.10 and the concentration of extracted genomic DNA was 178 ± 10 ng/ μL , indicating that the extracted DNA samples were not contaminated by proteins, with a high yield, good purity and good integrity.

3.2. Specificity test

The results of agarose gel electrophoresis verified that the specificity of the species primers is good, and there is no cross reaction with different adulterants such as *Bungarus multicinctus* and *Naja*, which can be well distinguished (Fig. 1).

3.3. Cloning and sequencing of *Agkistrodon acutus* samples

The results of plasmid sequencing were spliced by BioEdit software and proofread manually. The sequence length of the intercepted samples was 343 bp, which was imported into NCBI (<http://www.NCBI.nlm.nih.gov/>) for BLAST. There was no base insertion, deletion and mutation in the monoclonal DNA sequence of the target gene. The *cytb* gene sequences of different snakes were aligned by MEGA 7.0 software for multiple sequence comparative analysis, and statistical base composition and analysis similarity score (number of identical residues/compared total number of residues \times 100%) (Table 2).

3.4. Sample identification

Market sample detection: 18 batches of *Agkistrodon acutus* samples were successfully detected and identified (Fig. 2). (Source and the authenticities of the samples on the market are shown in Table 3.)

4. Discussion

Agkistrodon acutus is a valuable Chinese herbal medicine, but in recent years, its wild resources are scarce, and the counterfeits and adulterants of *Agkistrodon acutus* on the market are increasing, so the investigation into the quality of *Agkistrodon acutus* on the market is significant [6]. This study analyzed the quality of 18 samples from medicinal materials markets and pharmacies in six cities, in which the failure rate of samples detected at DNA molecular level was 66.6% (Table 3), indicating that there was a serious confusion in the market of *Agkistrodon acutus*, and the authentic *Agkistrodon acutus* should be further standardized.

The identification of the origin of a species is also important to consumers because of the economic losses caused by fraudulent substitution. The use of rapid, efficient and reliable analytical methods is a valuable and irreplaceable tool for ascertaining the origins or authenticity of crude drugs as a guarantee of the quality of proprietary Chinese medicines. In the actual market survey, although there are many current quality evaluation methods, they all have different limitations [19].

DNA is the carrier of genetic materials in organisms, with a high conservation and specificity [20], *Cytochrome b* gene is the gene encoding *cytb* protein in organisms, with a moderate evolutionary

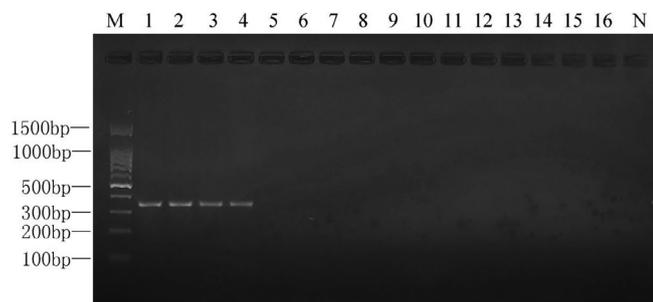


Fig. 1. PCR amplification for detecting primer specificity of *Agkistrodon acutus*. M. 100 bp DNA Ladder; 1. *Agkistrodon acutus* standard reference ZPQS-1; 2. *Agkistrodon acutus* QS1; 3. *Agkistrodon acutus* QS2; 4. *Agkistrodon acutus* QS3; 5. *Bungarus multicinctus* 201204-71; 6. *Bungarus multicinctus* YHS1; 7. *Naja* 210206-101; 8. *Naja* YJS1; 9. *Agkistrodon halys* 201204-078; 10. *Agkistrodon halys* FS1; 11. *Dinodon rufozonatum* CLS1; 12. *Vipera berus* KS1; 13. *Ovophis monticola* SLS1; 14. *Elaphe moellendorffi* BHS1; 15. *Zaocys dhumnade* WSS1; 16. *Ptyas mucosus* HSS1; N. negative control.

Table 2
Alignment analysis of the sequencing results of the *Agkistrodon acutus* sample and the *cytb* sequences of different species.

Species	T (U) %	C %	A %	G %	Similarity score %
<i>Agkistrodon acutus</i>	27.1	29.4	29.5	14.0	99.1
<i>Bungarus multicinctus</i>	30.3	23.9	31.8	14.0	79.0
<i>Naja</i>	25.7	29.4	30.9	14.0	79.6
<i>Agkistrodon halys</i>	30.3	29.2	27.1	13.4	82.8
<i>Dinodon rufozonatum</i>	30.3	26.5	29.7	13.4	78.1
<i>Vipera berus</i>	26.5	30.0	28.9	14.6	79.9
<i>Ovophis monticola</i>	25.1	32.4	27.1	15.5	83.7
<i>Elaphe moellendorffi</i>	27.4	26.5	32.4	13.7	78.1
<i>Zaocys dhumnade</i>	28.0	28.6	30.0	13.4	79.9
<i>Ptyas mucosus</i>	29.2	24.8	32.9	13.1	78.4

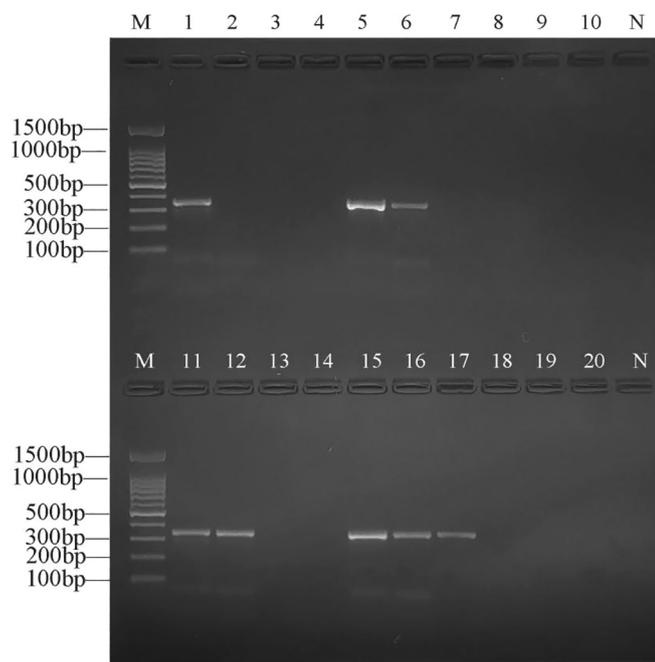


Fig 2. Amplification of diagnostic PCR product of *Agkistrodon acutus*. M. 100 bp DNA Ladder; 1. Positive control; 2–10. Sample 1–9; 11. Positive control; 12–20. Sample 10–18 (Sample information were shown in Table 3); N. Negative control.

rate and a larger proportion of conversion or transversion, and has been widely used in the research on phylogeny, genetic diversity and species identification, and it is one of the ideal molecular

Table 3
Source and test results of *Agkistrodon acutus* samples on the market.

Number	Code	Source	Size (g)	Genuine of counterfact
Sample 1	JLJLQS-1	Jilin	5	Counterfact
Sample 2	JLJLQS-2	Jilin	5	Counterfact
Sample 3	GXGLQS-1	Guilin	5	Counterfact
Sample 4	GXGLQS-2	Guilin	5	Genuine
Sample 5	GXGLQS-3	Guilin	5	Genuine
Sample 6	GXGLQS-4	Guilin	5	Counterfact
Sample 7	JLCCQS-1	Changchun	5	Counterfact
Sample 8	JLCCQS-2	Changchun	5	Counterfact
Sample 9	JLCCQS-3	Changchun	5	Counterfact
Sample 10	HJMJQS-1	Mudanjiang	5	Genuine
Sample 11	HJMJQS-2	Mudanjiang	5	Counterfact
Sample 12	HJMJQS-3	Mudanjiang	5	Counterfact
Sample 13	SCCDQS-1	Chengdu	5	Genuine
Sample 14	SCCDQS-2	Chengdu	5	Genuine
Sample 15	SCCDQS-3	Chengdu	5	Genuine
Sample 16	SCCDQS-4	Chengdu	5	Counterfact
Sample 17	SCCDQS-5	Chengdu	5	Counterfact
Sample 18	JLSPQS-1	Siping	5	Counterfact

genetic markers and widely used for the identification of animal medicinal materials [21,22,23,24]. DNA sequence analysis can be used to look for the conservative and specific fingerprint region of *Agkistrodon acutus* adulterates by analyzing their genomic sequences, and the genetic characteristics of the identified objects were detected and identified at the molecular level by PCR method, with a high species-specificity [25,26,27,28].

At present, some scholars have studied the DNA fingerprinting method based on mtDNA *cytb* gene, and successfully established the PCR detection methods for Chinese medicinal materials, such as *Panax ginseng* [29], *Cornu Cervi Pantotrichum* [30], *Fetus cervi* [31], and *Colla Corii Asini* [32]. Li et al. [33] successfully developed the DNA detection kit for *Zaocys dhumnades*, and Hou et al. [34] developed a kit for *Ophiocordyceps sinensis* and evaluated the effect of the kit.

We compared and analyzed the *cytb* gene sequences of *Agkistrodon acutus* and 9 snake species, and identified specific SNP regions for designing detection primers. The amplification sequence obtained from the positive sample was more than 99% identical with the sequence of *Agkistrodon acutus* by comparison, and less than 85% similar to other species, the base difference is large, and there was no cross species amplification with the fake product. It shows that the region could be used as the fingerprint region of the target species, and other adulterated snake species could be highly distinguished.

In this study, under the premise of ensuring the sensitivity and specificity of PCR reaction, the PCR time was shortened as much as possible to improve the detection efficiency and to achieve the accurate results. The purpose of simple operation and shortened experiment time to ensure the smooth progress of molecular biology identification and reduce the requirements on the external environment [35].

5. Conclusion

Based on the mtDNA *cytb* gene, a species-specific PCR method for the determination of the processed *Agkistrodon acutus* medicinal materials was successfully developed, which can be distinguished from other adulterated species. The difference of gene sequence between primer amplification region and other species was significant. The operation process is simple, and the detection accuracy, stability and reproducibility are high. The popularization and application of this method can effectively avoid the problem of false *Agkistrodon acutus* sales, and also contribute to the application and development of DNA detection methods in the determination of TCMs.

Conflict of interest

The author(s) declare no competing interests.

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References

- Lin X, Qi J, Chen M, et al. A novel recombinant fibrinogenase of *Agkistrodon acutus* venom protects against hyperacute rejection via degradation of complements. *Biochem Pharmacol* 2013;85:772–9. <https://doi.org/10.1016/j.bcp.2012.11.012>. PMID: 23178656.
- Zhang Y, Xu X, Shen D, et al. Anticoagulation factor I, a snake C-type lectin from *Agkistrodon acutus* venom binds to FIX as well as FX: Ca²⁺ induced binding data. *Toxicon* 2012;59:718–23. <https://doi.org/10.1016/j.toxicon.2012.03.006>. PMID: 22445822.
- Kong Y, Huo J, Xu W, et al. A novel anti-platelet aggregation tripeptide from *Agkistrodon acutus* venom: isolation and characterization. *Toxicon* 2009;54:103–9. <https://doi.org/10.1016/j.toxicon.2009.03.027>. PMID: 19345702.
- Yau FCF, Wong KL, Shaw PC, et al. Authentication of snakes used in Chinese medicine by sequence characterized amplified region (SCAR). *Biodivers Conserv* 2002;9:1653–62. <https://doi.org/10.1023/A:1016836017903>.
- Jiang C, Yuan Y, Liu L, et al. Homogeneous fluorescent specific PCR for the authentication of medicinal snakes using cationic conjugated polymers. *Sci Rep* 2015;5:16260. <https://doi.org/10.1038/srep16260>. PMID: 26537289.
- Xu Y, Lu Y, Wu HP, et al. Progress in molecular identification of snake drugs. *Chin J Chin Mater Med* 2017;42:2930–3.
- Wang YS, Yuan GX, Zhang LH, et al. Establishment of mink heart identification method based on mitochondrial cytochrome b gene and development of its detection kit. *Mitochondrial DNA Part A* 2019;30:325–31. <https://doi.org/10.1080/24701394.2018.1504932>. PMID: 30318961.
- Lam KY, Chan GK, Xin GZ, et al. Authentication of *Cordyceps sinensis* by DNA analyses: comparison of ITS sequence analysis and RAPD-derived molecular markers. *Molecules* 2015;20:22454–62. <https://doi.org/10.3390/molecules201219861>. PMID: 26694332.
- Liu Y, Zhang P, Song M, et al. Transcriptome analysis and development of SSR molecular markers in *Glycyrrhiza uralensis* Fisch. *PLoS ONE* 2015;10:1–12. <https://doi.org/10.1371/journal.pone.0143017>. PMID: 26571372.
- Zhou XW, Li QZ, Yin YZ, et al. Identification of medicinal *Ganoderma* species based on PCR with specific primers and PCR-RFLP. *Planta Med* 2008;74:197–200. <https://doi.org/10.1055/s-2008-1034289>. PMID: 18247261.
- Heubl G. New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. *Planta Med* 2010;76:1963–74. <https://doi.org/10.1055/s-0030-1250519>. PMID: 21058240.
- Pang YX, Wang WQ, Zhang YB, et al. Genetic diversity of the Chinese traditional herb *Blumea balsamifera* (Asteraceae) based on AFLP markers. *Genet Mol Res* 2014;13:2718–26. <https://doi.org/10.4238/2014.April.14.1>. PMID: 24782086.
- Gao L, Yin Y, Li J, et al. Identification of traditional Chinese medicinal pipefish and exclusion of common adulterants by multiplex PCR based on 12S sequences of specific alleles. *Mitochondrial DNA Part A* 2018;29:340–6. <https://doi.org/10.1080/24701394.2016.1278538>. PMID: 28129724.
- Hou F, Wen L, Peng C, et al. Identification of marine traditional Chinese medicine dried seahorses in the traditional Chinese medicine market using DNA barcoding. *Mitochondrial DNA Part A* 2018;29:107–12. <https://doi.org/10.1080/24701394.2016.1248430>. PMID: 27871217.
- Sungmin K, Hae-Seok E, Hyeoung K, et al. DNA barcode-based molecular identification system for fish species. *Mol Cells* 2010;30:507–12. <https://doi.org/10.1007/s10059-010-0148-2>. PMID: 21110132.
- Liu Y, Staerk D, Nielsen MN, et al. High-resolution hyaluronidase inhibition profiling combined with HPLC-HRMS-SPE-NMR for identification of anti-necrosis constituents in Chinese plants used to treat snakebite. *Phytochemistry* 2015;119:62–9. <https://doi.org/10.1016/j.phytochem.2015.09.005>. PMID: 26386983.
- Ashwin N, Robert RF, Adrian MV, et al. Novel primers for complete mitochondrial cytochrome b gene sequencing in mammals. *Mol Ecol Resour* 2012;12:191–6. <https://doi.org/10.1111/j.1755-0998.2011.03078.x>. PMID: 21974833.
- Han K, Wang M, Zhang L, et al. Application of molecular methods in the identification of ingredients in Chinese herbal medicines. *Molecules* 2018;23. <https://doi.org/10.3390/molecules23102728>. PMID: 30360419.
- Zhang X, Zhou T, Yu W, et al. Development and evaluation of a PCR-based assay kit for authentication of *Zoocys dhumnades* in traditional Chinese medicine. *Mitochondrial DNA Part A* 2018;29:102–6. <https://doi.org/10.1080/24701394.2016.1248429>. PMID: 28035866.
- Zhang NP, Zhang WJ, Wei F, et al. The standard method of DNA molecular identification for traditional Chinese medicine. *Chin J Pharm Anal* 2014;34:2066–70.
- Emerling CA. Genomic regression of claw keratin, taste receptor and light-associated genes provides insights into biology and evolutionary origins of snakes. *Mol Phylogenet Evol* 2017;115:40–9. <https://doi.org/10.1016/j.ympev.2017.07.014>. PMID: 28739369.
- Lopez-Oceja A, Gamarrá D, Borrigan S, et al. New cyt b gene universal primer set for forensic analysis. *Forensic Sci Int Genet* 2016;23:159–65. <https://doi.org/10.1016/j.fsigen.2016.05.001>. PMID: 27206224.
- Yacoub HA, Fathi MM, Sadek MA. Using cytochrome b gene of mtDNA as a DNA barcoding marker in chicken strains. *Mitochondrial DNA* 2015;26:217–23. <https://doi.org/10.1019/19401736.2013.825771>. PMID: 24020964.
- Kappel K, Haase I, Käppel C, et al. Species identification in mixed tuna samples with next-generation sequencing targeting two short cytochrome b gene fragments. *Food Chem* 2017;234:212–9. <https://doi.org/10.1016/j.foodchem.2017.04.178>. PMID: 28551228.
- Lopez-Oceja A, Nuñez C, Baeta M, et al. Species identification in meat products: a new screening method based on high resolution melting analysis of cyt b gene. *Food Chem* 2017;237:701–6. <https://doi.org/10.1016/j.foodchem.2017.06.004>. PMID: 28764056.
- Lyons LA, Grahn RA, Kun TJ, et al. Acceptance of domestic cat mitochondrial DNA in a criminal proceeding. *Forensic Sci Int Genet* 2014;13:61–7. <https://doi.org/10.1016/j.fsigen.2014.07.007>. PMID: 25086413.
- Di LP, Lancioni H, Ceccobelli S, et al. Mitochondrial DNA variants of Podolian cattle breeds testify for a dual maternal origin. *PLoS ONE* 2016;13. <https://doi.org/10.1371/journal.pone.0240156>. PMID: 29462170e0192567.
- Yang L, Kong XY, Yang S, et al. Haplotype diversity in mitochondrial DNA reveals the multiple origins of Tibetan horse. *PLoS ONE* 2018;13. <https://doi.org/10.1371/journal.pone.0201564>. PMID: 30052677e0201564.
- Shim YH, Park CD, Kim DH, et al. Identification of *Panax* species in the herbal medicine preparations using gradient PCR method. *Biol Pharm Bull* 2005;28:671–6. <https://doi.org/10.1248/bpb.28.671>. PMID: 15802808.
- Gao L, Xia W, Ai J, et al. Development of multiplex PCR assay for authentication of *Cornu Cervi Pantotrichum* in traditional Chinese medicine based on cytochrome b and C oxidase subunit 1 genes. *Mitochondrial DNA Part A* 2016;27:2989–92. <https://doi.org/10.1019/19401736.2015.1060475>. PMID: 26287950.
- Ai JX, Wang XS, Gao LJ, et al. PCR-fingerprint profiles of mitochondrial and genomic DNA extracted from Fetus cervi using different extraction methods. *Mitochondrial DNA Part A* 2017;28:781–6. <https://doi.org/10.1080/24701394.2016.1186666>. PMID: 27247076.
- Luo HM, Xiao BY, Nie P, et al. Application of DNA molecular identification method to distinguish donkey-derived components in Ejiao. *Chin J Pharm Anal* 2017;37:202–11.
- Li ZT, Sun JY, Zhou TT, et al. Development of *Zoocys dhumnades* (Cantor) DNA test kit and its application in quality inspection of commercial products. *Chin Pharm J* 2017;28:6295–9.
- Hou FX, Cao J, Wang SS, et al. Development and evaluation of a rapid PCR detection kit for *Ophiocordyceps sinensis*. *China J Chin Mater Med* 2017;42:1125–9.
- Li MC, Gao L, Qu L, et al. Characteristics of PCR-SSCP and RAPD-HPCE methods for identifying authentication of Penis et testis cervi in Traditional Chinese Medicine based on cytochrome b gene. *Mitochondrial DNA Part A* 2016;27:2757–62. <https://doi.org/10.1019/19401736.2015.1053053>. PMID: 26309015.