Application of DNA Barcoding for the Identification of a Traditional Chinese Medicine Shedan

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Research

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Abstract

Background

Shedan has a long history of application in Traditional Chinese medicine (TCM), however, Shedan from different original source has been indiscriminately used. So far, there is still a lack of an effective tool to differentiate the original source of Shedan medicinal materials, which brings great risk to the safety and effectiveness of clinical applications. Hence, it is imperative to develop a practicable approach to identify Shedan medicinal materials.

Methods

The specificity of two pairs of primers, including Folmer's universal primers and a pair of originally designed primers COISNFF/COISNFR, was tested to screen the more specific primers for further origin identification of Shedan. A total of 253 fresh snake gallbladder samples from 31 morphologically identified snake species were collected and authenticated. Moreover, 51 fresh snake bile samples and 17 fresh bile samples from five other common domestic poultry and livestock (cattle, chicken, duck, pig and sheep) were collected and distinguished using the more specific primers. Additionally, a total of 195 market Shedan samples randomly selected from 18 batches of Shedan medicinal materials were investigated. Sequence definition was executed by querying sequence similarities in GenBank and the Barcode of Life Data System (BOLD), respectively.

Results

It turned out that the standard COI barcode obtained by COISNFF/COISNFR primers, rather than Folmer's universal primers, can distinguish all the testing samples from each other in fresh Shedan samples, and COISNFF/COISNFR primers were also specific to snake species and the other four animal species except duck. In terms of market Shedan, 84.6% (165/195) samples can be attributed to 13 snake species from four families and 4.6% (9/195) can be attributed to adulterated chicken species.

Conclusion

The COI-based DNA barcoding was practicable for species identification of Shedan used in traditional Chinese medicine. The original source of current market Shedan, including adulterated species, has been preliminarily clarified, which provides a foundation for quality control of Shedan medicinal materials.

Highlights

1. The originally designed specific primers COISNFF/COISNFR are proposed as an universal primers for origin identification of Shedan.
2. The snake gallbladder and snake bile of Shedan crude drugs have been identified respectively.
3. 304 COI barcodes (658 bp) belonging to 31 snake species have been obtained and expanded the reference barcode sequences of snakes in GenBank database.
4. The original source of Shedan crude drugs from the market has been preliminarily clarified.

Background

Shedan, a precious traditional Chinese medicine (TCM), was initially documented in the herbal Mingyi Bielu and it has been used for more than two thousand years. Previous studies have revealed that Shedan possesses satisfying therapeutic effects of clearing away heat and detoxification, reducing phlegm, and relieving cough, and it has been commonly utilized in the management of mycoplasma pneumonia in children [1–3]. Shedan is one of the major ingredients of more than 30 Chinese patent medicines, including the widely acclaimed Niu Huang Shedan Chuanbei solution [4], Pianzai Huang [5], and Shedan

Chuanbei powder [6]. Although it pointed out that snake bile should be derived from snakes in the three families of Colubridae, Elapidae, or Viperidae in the Chinese Pharmacopoeia (2020), the quality standard of Shedan medicinal materials has not yet been established [7]. For decades, the demand for snakes and their relevant products is rising [8]. However, the excessive and indiscriminate hunting, habitat loss and the implementation of Law of the People's Republic of China on the Protection of Wildlife have greatly affected the snake medical supplies [9], and further lead to the market adulteration of snake drugs and snake-relevant medicinal materials [10–11]. Bile acids are the predominant biologically active ingredients in snake bile, but the bile acid profile varied greatly among different snake species [12–13]. Clarifying the original source of Shedan medicinal materials is an essential prerequisite for ensuring safe and effective medication use, because snake bile raw material has always been derived from mixture gallbladders. So far, several conventional identification methods, such as character identification, microscopic identification and chemical analysis [10, 12], have been developed to distinguish snake gallbladder or snake bile from different original sources, still, a more effective and dependable method is required to identify the original source of Shedan raw materials without definite morphological features or characteristic chemical constituents.

DNA Barcoding technique [14], differentiating species by comparing the congruence between the query sequences derived from samples and reference barcodes of the known identity in public libraries, is a powerful tool for biological identification. Thereinto, a fragment (~650 bp) of the barcode region in mitochondrial cytochrome c oxidase submit I (COI) gene, has been used as the most effective DNA barcode marker for identifying and classifying animal-derived medicinal materials even with highly similar or incomplete morphological traits [15–18]. Previous studies have displayed that DNA barcoding can accurately identify different snake species and distinguish snake-related medicinal materials from adulterants and substitutes through amplifying the COI sequence of the muscle tissue or periosteum serpentis [19–24], and none of these studies focused on snake gallbladder or snake bile. It was worth noting that the amplification primers of these studies were incompletely identical, but COI gene universal primers (LC01490/HCO2198) [25] were mostly adopted.

In the present study, we explored the feasibility of applying COI-based DNA barcoding to identify Shedan by detecting the fresh snake gallbladder or snake bile samples, and the barcoding method developed here was further applied to authenticate the market Shedan medicinal materials.

Materials And Methods

Sample collection and processing

This study was approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. All operations were executed according to the guidelines for the care and treatment of experimental animals of the Center for Laboratory Animal Care, Tongji Medical College, Huazhong University of Science and Technology. All the original animals were morphologically identified by Professor Jiachun Chen (Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Huazhong University of Science and Technology), and all voucher samples were deposited in the herbarium of School of Pharmacy, Huazhong University of Science and Technology.

For the fresh snake gallbladder samples, a total of 253 fresh gallbladders (Table 1) belonging to 31 snake species in three families were collected from four snake farms in Hubei, Hunan, Jiangxi, and Zhejiang province, respectively. These four provinces were located in the middle and lower reaches of the Yangtze River area where was the main Shedan producing area in China. After dissecting out from the morphologically identified original animals, the fresh snake gallbladders were washed with sterile water, preserved in 95% ethanol, and stored at -20°C until used for DNA extraction.

For the fresh gallbladder bile samples, a total of 68 fresh gallbladder bile samples (Table 2), were collected. Specifically, 51 fresh snake gallbladders belonging to 17 snake species were collected from one snake farm in Hunan province, China. Moreover, 17 fresh gallbladders from five other common adulterated domestic poultry and livestock (cattle, chicken, duck, pig and sheep), were collected from one Animal Husbandry Co., Ltd. in Hubei province, China. The gallbladder

bile derived from these 68 fresh gallbladders was freeze-dried into powder using lyophilizer and stored at -20°C until used for DNA extraction.

For the market Shedan samples, a total of 18 batches of market Shedan medicinal materials (Table 3), were collected from Chinese medicinal material markets or commercial companies related to the production of Shedan and its preparations, such as Bozhou herb market, and Deqing Moganshan Snakes Industrial Co., Ltd. In the market, each batch of commercial Shedan contained different numbers of gallbladders, and they were mixed and stored in liquor with an alcohol content of more than 50%. The market gallbladders were divided into three categories according to their size per batch, and then approximately 1/3 of which were randomly selected. Among these chosen gallbladders from the 18 batches of market Shedan, 13 batches of gallbladder bile were used for another purpose for chemical analysis, and the rest gallbladders (gallbladder tissues) were stored at -20 °C until used for DNA extraction, and the other five batches of gallbladder bile were also freeze-dried into powder and stored at -20°C until used for DNA extraction. Altogether, 134 market Shedan gallbladder samples of 13 batches of Shedan medicinal materials and 71 gallbladder bile samples of the other five batches of Shedan medicinal materials were detected through DNA barcoding.

DNA extraction

Gallbladder materials were pretreated following the principles of molecular identification using DNA barcoding [26], and the total genomic DNA was isolated from the gallbladder tissue or gallbladder bile through the phenol-chloroform method [27] with slight modifications. For the fresh snake gallbladder samples, a small piece of gallbladder tissue was ground into powder in liquid nitrogen, placed in 700 µL of extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 2% CTAB (pH 8.0), 2% PVP, 2% β-mercaptoethanol, and 10 µL of 20 mg/mL proteinase K), and then incubated at 65°C for about 30 min. DNA was subsequently purified by phenol-chloroform-isoamylalcohol (25:24:1, v/v/v) and ethanol precipitation. Finally, the pellet was dried, dissolved in 50 µL of sterile TE buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA), and stored at -20°C before use. For the fresh gallbladder bile samples, the total genomic DNA was extracted from bile (approximately 5 mg) following the DNA extraction method of the foregoing fresh gallbladder samples.

Barcode sequences amplifying and sequencing

Initially, a pair of specific primers (COISNFF: 5'-TCAACAAACCACAAAGAYATYGG-3', COISNFR: 5'-ACTTCYGGRTGKCCRAARAATCA-3') had been originally designed based on COI gene universal primers and their corresponding base sites in snake COI sequences using MEGA 5.0 [28] and Primer 5.0 software (Premier Biosoft International, Palo Alto, CA), and the specificity of these two pairs of primers was tested by partial snake species (see Additional file 1: Table S1). PCR amplification was carried out in a Bio-rad T100 Thermal Cycler (Bio-rad, USA) with a 25 µL reaction mixture, which contained 2.5 µL 10× PCR Buffer, 2.5 µL dNTPs (2 mM), 1.5 µL MgSO₄ (1.5 mM), 0.5 U Taq polymerase (1 U/µL) (TOYOBO, Osaka, Japan), 0.75 µL of each forward and reverse primer (10 pmol/µL each), 15.5 µL of sterilized distilled water, and 1 µL of template DNA. The PCR amplification of LC01490/HCO2198 primers was under the following conditions: 94°C for 2 min, followed by 35 cycles of 98°C for 10 s, 53°C for 1 min, and 68°C for 1 min, and a final extension at 68°C for 5 min. And the PCR amplification of COISNFF/COISNFR primers was under the following conditions: 94°C for 2 min, followed by 35 cycles of 98°C for 10 s, 51°C for 50 s, and 68°C for 50 s, and a final extension at 68°C for 5 min. The PCR products were confirmed on a 1.0% agarose gel, purified with the TIANGel Midi Purification Kit (Tiangen Biotech Co., Beijing, China), and bidirectionally sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, USA).

Species identification and data analysis

Consensus sequences and contig generation were accomplished using CodonCode Aligner V 4.0 (CodonCode Co., USA). After trimming the amplification primers, sequences obtained were queried to GenBank and the Barcode of Life Data System (BOLD) for species identification, respectively, and their species would be confirmed based on the best match ≥ 98%, otherwise, the species of the query sequence could not be defined. The average intra- and interspecific genetic distance of the barcodes of the fresh snake gallbladder samples were calculated based on Kimura-2-parameter (K2P) distance model using MEGA 5.0 and

they were used to evaluate the DNA barcoding gap. Sequences generated by COISNFF/COISNFR primers were deposited in the GenBank database.

Phylogenetic tree reconstruction

To generate the phylogenetic relationships and ascertain the accuracy of the potential barcode for species identification, a neighbor-joining (NJ) tree was constructed in MEGA 5.0 and the bootstrap values were evaluated based on 1000 replicates. *Acrochordus javanicus* from the family Acrochordidae (GenBank accession number: KX752053) [29] was selected as the outgroup in the NJ tree. To provide additional insights about the taxonomic identity of our material: we randomly downloaded one conspecific COI barcode sequence of the 31 snake species previously identified by morphology from GenBank, and then analyzed them together with the barcode sequences obtained from the fresh snake gallbladder samples in the NJ tree analysis.

Investigating the market Shedan medicinal materials

The DNA extraction, PCR amplification with COISNFF/COISNFR primers and sequencing of the market Shedan samples were the same as described above. The sequences obtained were queried to GenBank and BOLD Systems for species determination, respectively, and they were also submitted to the GenBank database. In the process of sequence definition, we also paid attention to the similarities between the query sequences obtained from market Shedan samples and the reference barcode sequences submitted to the GenBank database by this study.

Results

Identifying the fresh snake gallbladder samples by DNA barcoding

Genomic DNA was isolated from the fresh snake gallbladder tissue per sample. For the testing snake species, although the testing samples could be amplified with both LCO1490/HCO2198 primers and COISNFF/COISNFR primers (see Additional file 2: Figure. S1 and see Additional file 3: Figure. S2) and a standard barcode sequence could be obtained from each testing specimen, the identification results showed that LCO1490/HCO2198 primers were not as specific as COISNFF/COISNFR primers to each testing snake species (see Additional file 1: Table S1). Therefore, COISNFF/COISNFR primers were selected as the optimal amplification primers for species identification of Shedan.

A total of 253 COI sequences (658 bp) were eventually generated from the fresh snake gallbladder samples and analyzed. No insertions, deletions or stop codons were present in any sequence, indicating that nuclear pseudogenes did not appear in the analysis. The overall average nucleotide frequencies were A (26.7%), T (27.8%), C (29.1%) and G (16.4%), with an average GC-lowest of 45.5%. The 253 sequences were attributed to 31 snake species with 98–100% best matches in at least one database of GenBank and BOLD Systems, and the molecular identification results were consistent with the morphological classification results of their original animals. These 253 barcode sequences were deposited in the GenBank database and their GenBank accession numbers were shown in Table 1.

The intra- and interspecific genetic distance of the snake species of the fresh snake gallbladder samples based on COI barcode sequences were summarized in Table 4. The average genetic distance within species (0.9%) was much smaller than the mean genetic distance between species (20.2%), and the highest genetic distance within species (8.5%) was less than the smallest interspecies genetic diversity (9.1%), evincing a distinct barcode gap due to the no overlap between intra-and interspecies genetic distance.

One COI barcode sequence of the identical species in the same collection region was randomly selected for phylogenetic analysis, and 52 COI sequences were finally picked from the enormous 253 barcode sequences. Then, these 52 COI sequences combined with the additional 32 COI barcode sequences obtained from GenBank were used to construct a NJ tree (Fig. 1). In the tree, sequences within species were preferentially clustered together as 32 monophyletic clades with strong support (94–100) (Fig. 1), and the monophyletic clades were further consisted of four paraphyletic groups (Colubridae, Elapidae, Viperidae

and Acrochordidae), demonstrating that these fresh snake gallbladder samples had been well authenticated to the species level. In other words, snake species could be authenticated by COI-based barcoding molecular method.

Distinguishing the fresh snake gallbladder bile samples using DNA barcoding

Genomic DNA was extracted from the fresh snake gallbladder bile per sample. For the 51 bile samples from snakes, desired PCR products were amplified using COISNFF/COISNFR primers (Fig. 2). In the end, 51 COI sequences (658 bp) were attained and verified as 17 snake species in GenBank and BOLD Systems with both high sequence similarities (99–100%), which was consistent with their original animal species by morphological classification.

Authenticating the fresh gallbladder bile samples from common adulterated animals using DNA barcoding

Among the 17 fresh gallbladder bile samples from the other five common adulterated animals, genomic DNA was extracted from each fresh gallbladder bile sample. Except three duck bile samples, the rest 14 samples showed positive PCR performance with COISNFF/COISNFR primers (Fig. 3). As a result, 14 COI sequences (658 bp) were achieved and classified as four species (*Gallus gallus*, *Sus scrofa*, *Bos taurus* and *Ovis aries*, Table 2) in GenBank and BOLD Systems with best match $\geq$ 99%, which was consistent with their previous morphological taxon data.

Sensitivity of COISNFF/COISNFR primers

To detect the sensitivity of COISNFF/COISNFR primers, template DNA was diluted to a series of concentrations ranging from 100 ng/μL to 1pg/μL. The amplification results exhibited that the minimum effective concentration for positive amplification was 10 pg/μL, and no amplifications were detected below this concentration for COISNFF/COISNFR primers (Fig. 4).

Authenticating market Shedan medicinal materials samples through DNA barcoding

For the 134 market Shedan gallbladder samples, genomic DNA had been extracted from each gallbladder tissue sample and they could be amplified by COISNFF/COISNFR primers to generate the desire PCR products. In the end, 134 COI sequences (658 bp) were gained and assigned to 13 snake species (Table 3) based on the best match (98–100%) in GenBank and BOLD Systems, respectively, including eight snake species (*A. stolatum* (n=1), *C. radiatus* (n=3), *E. carinata* (n=39), *E. taeniura* (n=13), *L. rufozonatus* (n=22), *P. dhumnades* (n=33), *P. korros* (n=5) and *P. mucosus* (n=4)) from the family Colubridae, one snake species (*N. atra* (n=3)) from the family Elapidae, three snake species (*D. acutus* (n=5), *G. brevicaudus* (n=2) and *P. mucrosquamatus* (n=1)) from the family Viperidae, and one snake species (*X. unicolor* (n=3)) from the family Xenopeltidae. In total, 97.8% (131/134) of the market Shedan gallbladder samples were derived from the first three families stated in the Chinese Pharmacopoeia.

For the 61 gallbladder bile samples of market Shedan, except two bile samples with the batch number GD20150801 and one bile sample with the batch number AH20181024, genomic DNA had been extracted from the remaining bile samples, and 31 samples of which could be amplified with COISNFF/COISNFR primers to generate the desire PCR products. As a result, 31 COI sequences were obtained and assigned as six animal species (Table 3) based on the best match (98–100%) in GenBank and BOLD Systems, respectively, including three snake species (*C. radiatus* (n=2), *E. carinata* (n=3) and *P. mucosus* (n=5)) from the family Colubridae, one snake species (*N. atra* (n=2)) from the family Elapidae, and one snake species (*X. unicolor* (n=10)) from the family Xenopeltidae, and one adulterated species (*G. gallus* (n=9)). In total, 19.7% (12/61) of the market Shedan bile samples were derived from the first two families recorded in the Chinese Pharmacopoeia. It was worth noting that 16.4% (10/61) of the market Shedan bile samples were derived from the family Xenopeltidae, and 14.8% (9/61) were adulterants. Moreover, the remaining 30 market bile samples could not be discerned due to the inability to extract DNA or the failure of PCR amplification.

To sum up, 100% (134/134) of market Shedan gallbladder samples and 50.8% (31/61) of market Shedan bile samples were classified to the species level. In total, 84.6% (165/195) of market Shedan samples had been successfully distinguished and attributed to 13 snake species from four families (Colubridae, Elapidae, Viperidae and Xenopeltidae), along with one

adulterated species (*G. gallus* (chicken)) (Table 3). Thereinto, the market Shedan derived from the three families (Colubridae, Elapidae and Viperidae), the family Xenopeltidae and adulterated species accounted for 73.3% (143/195), 6.7% (13/195) and 4.6% (9/195), respectively. Moreover, it was worth noting that two protected snake species (*C. radiatus* and *X. unicolor*) listed as Class II of List of key protected wild animals in China were found and they accounted for 9.2% (18/195) of market Shedan samples. The 165 COI sequences were also deposited in the GenBank database and their accession numbers were shown in Table 3.

Discussions

A reliable library of reference DNA barcode sequences from morphologically pre-identified samples is an essential prerequisite for the application of DNA barcoding technique [30–31]. In the present study, considerable fresh Shedan of original animals identified by morphology were collected. The previous studies had reported that COI gene universal primers (LC01490/HCO2198) were appropriate for distinguishing snake species from the families of Colubridae and Elapidae [19, 20, 22]. Therefore, COISNFF/COISNFR primers were optimized based on LC01490/HCO2198 primers and their corresponding region in COI gene of snakes by this study. COISNFF/COISNFR primers (degenerated primers) could provide more possible and precise binding sites with divergent sequences of different snake species and even other potential animal species, and they exhibited better discrimination ability than LC01490/HCO2198 primers in identifying snake species. Subsequently, the identification results of the fresh Shedan through DNA barcoding with COISNFF/COISNFR primers were coherent with their original data of morphological taxon, indicating that DNA Barcoding could act as a practical and reliable molecular method for origin identification of Shedan. Remarkably, this study has complemented the first COI barcode record of four snake species (*C. septentrionalis*, *O. cheni*, *O. rufodorsatus* and *S. kelloggii*) in the GenBank database.

In addition to being useful for identifying the fresh Shedan sample from the 31 snake species, the originally designed specific primers COISNFF/COISNFR were also suitable for discriminating the fresh bile samples from four other common domestic poultry and livestock (cattle, chicken, pig and sheep). Moreover, 13 snake species and adulterated chicken species were identified in market Shedan samples, indicating that this method can be workable for the origin identification of Shedan medicinal materials.

In this study, the snake gallbladder and snake bile of Shedan crude drugs were investigated. It turned out that most of the market Shedan samples were identified to the species level, and the original species of current market Shedan medicinal materials were not only from the three families (Colubridae, Elapidae and Viperidae) stated in the Chinese Pharmacopoeia, but also from the other family Xenopeltidae. Unexpectedly, a small amount of low-value chicken gallbladders was found to be adulterated in high-value commercial Shedan medicinal materials by this study. These identification results revealed that the original source of Shedan crude drugs in the market is relatively complicated, and from which the key protected snake species and adulterated chicken species were detected, indicating that more attention should be paid to strengthen the protection of wild snake resources during the development and utilization of snake resources and simultaneously reinforce the supervision of the source of market Shedan medicinal materials.

Except for a few for fresh use, most medicinal materials are subject to different traditional processing procedures in time after collecting, such as fumigating, sun-drying, slicing and powdering, to ensure the quality and facilitate storage and transportation [32]. However, some conventional processes usually make the morphological characteristics blurred or even lost, which further hinders the morphological identification of medicinal materials. Simultaneously, DNA degradation or fragmentation might appear during processing, which significantly impedes the amplification of full-length barcodes of highly processed materials [33–34]. Moreover, although genomic DNA was extracted from the duck bile specimen and the amplification conditions were adjusted repeatedly, no PCR products could be amplified with COISNFF/COISNFR primers, which might be caused by the inability of duck template to bind with COISNFF/COISNFR primers. Therefore, some market Shedan bile samples could not be identified due to the failure of PCR amplification might be mainly caused by the serious DNA degradation, and even complete DNA degradation as three market Shedan bile samples from which no genomic DNA could be extracted, or they were from the animal species that could not be identified through DNA barcoding with COISNFF/COISNFR

primers. In future studies, it is supposed to develop other constructive methods such as mini-barcoding method [35–37] to clarify the original source of Shedan medicinal materials more comprehensively.

Conclusion

This research has established a molecular identification approach of the COI-based DNA barcoding on differentiating Shedan used in TCM. Meanwhile, it also suggested that COISNFF/COISNFR primers could be used as a pair of candidate universal primers for origin identification of Shedan. The original source of market Shedan has been firstly reported, which provides a preliminary basis for further studies on quality control of Shedan crude drugs.

Abbreviations

Not applicable

Declarations

Supplementary Information

Supplementary data associated with this article can be found in the attachment files.

Acknowledgements

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Authors' contributions

JC was the study supervisor and was responsible for designing the study. XW contributed to the conception, design, and collection of samples of the study. CZ, SG, JZ, YF and JN also contributed to the collection of samples. CZ and SG performed the experiment, and the former carried out the statistical analysis and wrote the manuscript. BL and LW were required for manuscript revision. All authors read the manuscript and approved the final version.

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Availability of data and materials

The datasets are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental operations were executed according to the guidelines for the care and treatment of experimental animals of the Center for Laboratory Animal Care, Tongji Medical College, Huazhong University of Science and Technology. All experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 The fresh snake gallbladder samples used and their GenBank accession numbers in this study

Family	Species	Voucher number	Locality	Date of collection	Quantity	GenBank accession number
Colubridae	<i>Amphiesma stolatum</i>	SR85, H-75	Hunan	May. 2014, Nov. 2016	2	KR045946, MH153695
	<i>Boiga kraepelini</i>	JHLS01	Hunan	Aug. 2017	1	MG788988
	<i>Calamaria septentrionalis</i>	DWLTS01	Hunan	Aug. 2017	1	*MG788985
	<i>Cyclophiops major</i>	SR56–SR60	Hunan	May. 2014	5	KR045919–KR045923
	<i>Elaphe carinata</i>	SR19–SR20, SR89–SR94, SR122–SR123, SR207–SR212, SR266–SR268, H-68, WJS1–WJS4	Hubei, Hunan, Jiangxi, Zhejiang	Sep. 2013, May. 2014, Jun. 2014, Oct. 2015, Nov. 2016	24	KR045883–KR045884, KR045950–KR045955, KR045982–KR045983, KR046040–KR046045, KR046086–KR046088, MH153693, MH153665–MH153668
	<i>Elaphe taeniura</i>	SR44–SR49, SR134–SR137, SR165–170, SR260–262, HMJS1–HMJS 5	Hubei, Hunan, Jiangxi, Zhejiang	May. 2014, Jun. 2014, Mar. 2015, Oct. 2015	24	KR045908–KR045913, KR045991–KR045993, KR046016–KR046019, KR046080–KR046082, MH153646, MH153649–MH153650, MH153660, MH153656–MH153659
	<i>Euprepophis mandarinus</i>	SR41–SR43, SR235–SR237, SR257–SR259	Hubei, Hunan, Jiangxi	May. 2014, Jun. 2014	9	KR045905–KR045907, KR046058–KR046059, KR046077–KR046079, MH159196
	<i>Lycodon flavozonatus</i>	SR95, H-57–H-59	Hunan	May. 2014, Nov. 2016	4	KR045956, MH153685, MH185201–MH185202
	<i>Lycodon futsingensis</i>	FQBHS01	Hunan	Aug. 2017	1	MG788981
	<i>Lycodon rufozonatus</i>	SR1, SR8, SR35–SR40, SR143–SR147, SR158, SR160–SR164, CLS1–CLS4	Hunan, Jiangxi, Zhejiang	Oct. 2013, May. 2014, Aug. 2015, Oct. 2015	22	KR045865, KR045872, KR045899–KR045904, KR046010–KR046015, KR045999–KR046003, MH153669–MH153672
	<i>Myrophis chinensis</i>	SR117–SR121, SR232	Hunan, Jiangxi	May. 2014	6	KR045977–KR045981, KR046055
	<i>Oligodon chinensis</i>	ZGXTS01	Hunan	Aug. 2017	1	*MG788986
	<i>Opisthotropis cheni</i>	MSHLS01	Hunan	Aug. 2017	1	*MG788984

	<i>Oocatochus rufodorsatus</i>	SR2–SR3, SR111–SR116, SR125–SR128	Hunan, Zhejiang	Oct. 2013, May. 2014	12	KR045866–KR045867, KR045971–KR045976, KR045984–KR045986, MH153645
	<i>Oreocryptophis porphyraceus</i>	H-66, ZHJS01	Hunan	Nov. 2016, Aug. 2017	2	MH153692, MG788987
	<i>Ptyas dhumnades</i>	SR9–SR11, SR25–SR30, SR73–SR78, SR188–SR198, SR263–SR265, SR129–SR132, WSS1–WSS4	Hubei, Hunan, Jiangxi, Zhejiang	Sep. 2013, Oct. 2013, May. 2014, June. 2014, March. 2015, Oct. 2015	37	KR045889–KR045894, KR045873–KR045875, KR045935–KR045940, KR046022–KR046031, KR045987–KR045990, KR046083–KR046085, MH153653, MH153673– MH153676
	<i>Ptyas korros</i>	SR61–SR66, SR182–SR185, H-10–H-14	Hunan, Jiangxi	May. 2014, Nov. 2016	15	KR045924–KR045929, KR046020–KR046021, MH153651–MH153652, MH153680–MH153684
	<i>Ptyas mucosa</i>	SR154, SR156	Jiangxi	May. 2014	2	MH153647–MH153648
	<i>Rhabdophis tigrinus</i>	SR53–54, SR233–234	Hunan, Jiangxi	May. 2014	4	KR045916–KR045917, KR046056–KR046057
	<i>Sinonatrix annularis</i>	SR4, SR12– SR18, SR21–SR24, SR138–142, SR238–241, CLHYS1– CLHYS3	Jiangxi, Zhejiang	Oct. 2013, May. 2014, March. 2015, Oct. 2015	24	KR045868, KR045885– KR045888, KR045876–KR045882, KR045994–KR045998, KR046060–KR046063, MH153677–MH153679
	<i>Sinonatrix percarinata</i>	SR244, HYS01	Hunan, Jiangxi	May. 2014, Aug. 2017	2	MH153654, MH185203
	<i>Xenochrophis flavipunctatus</i>	SR50–SR52	Hunan	May. 2014	3	KR046089, KR045914– KR045915
Elapidae	<i>Bungarus multicinctus</i>	SR68–SR72	Hunan	May. 2014	5	KR045930–KR045934
	<i>Naja atra</i>	SR80–SR84	Hunan	May. 2014	5	KR045941–KR045945
	<i>Ophiophagus hannah</i>	SR254, SR256	Jiangxi	May. 2014	2	KR046075, MH153655
	<i>Sinomicrurus kelloggi</i>	FJHSHS01	Hunan	Aug. 2017	1	*MG788980
	<i>Sinomicrurus maccllellandi</i>	ZHSHS01	Hunan	Aug. 2017	1	MG788982
Viperidae	<i>Deinagkistrodon</i>	SR31–SR34,	Hunan,	May.	12	KR045895–KR045898,

<i>acutus</i>	SR246–SR253	Jiangxi	2014		KR046067–KR046074
<i>Gloydius brevicaudus</i>	SR149–SR153, DWF1–DWF4	Zhejiang	May. 2014, Aug. 2015, Oct. 2015	9	KR046005–KR046009, MH153661–MH153664
<i>Protobothrops mucrosquamatus</i>	H-70	Hunan	Nov. 2016	1	MH153694
<i>Trimeresurus stejnegeri</i>	SR99–SR104, SR199–SR206	Hunan, Jiangxi	May. 2014	14	KR045960–KR045965, KR046032–KR046039

a. *GenBank accession number: COI barcodes of these snake species (658 bp) were firstly reported and deposited in GenBank by this study

b. *D. acutus* was recorded in Chinese Pharmacopoeia 2020 as *Agkistrodon acutus* (Wubushe in Chinese)

Table 2 The fresh gallbladder bile specimens and their GenBank accession numbers used in this study

Family	Species	Locality	Date of collection	Quantity	Voucher Number	GenBank accession number
Colubridae	<i>Cyclophiops major</i>	Hunan	Sep. 2018, Oct. 2019	4	BS01, BS15–BS17	MZ031430, MZ031444–MZ031446
	<i>Elaphe carinata</i>	Hunan	Sep. 2018, Oct. 2019	6	BS02–BS04, BS18–BS20	MZ031431–MZ031433, MZ031447–MZ031449
	<i>Elaphe taeniura</i>	Hunan	Oct. 2019	1	BS21	MZ031450
	<i>Euprepiophis mandarinus</i>	Hunan	Sep. 2018, Oct. 2019	4	BS05, BS22–BS24	MZ031434, MZ031451–MZ031453
	<i>Lycodon flavozonatus</i>	Hunan	Oct. 2019	3	BS25–BS27	MZ031454–MZ031456
	<i>Lycodon rufozonatus</i>	Hunan	Sep. 2018, Oct. 2019	4	BS06, BS28–BS30	MZ031435, MZ031457–MZ031459
	<i>Myrophis chinensis</i>	Hunan	Oct. 2019	3	BS31–BS33	MZ031460–MZ031462
	<i>Oreocryptophis porphyraceus</i>	Hunan	Sep. 2018,	2	BS07–BS08	MZ031436–MZ031437
	<i>Ptyas dhumnades</i>	Hunan	Sep. 2018, Oct. 2019	4	BS09, BS34–BS36	MZ031438, MZ031463–MZ031465
	<i>Ptyas korros</i>	Hunan	Sep. 2018, Oct. 2019	4	BS10, BS37–BS39	MZ031439, MZ031466–MZ031468
	<i>Ptyas mucosa</i>	Hunan	Oct. 2019	1	BS40	MZ031469
	<i>Rhabdophis tigrinus</i>	Hunan	Sep. 2018, Oct. 2019	2	BS11, BS41	MZ031440, MZ031470
Elapidae	<i>Bungarus multicinctus</i>	Hunan	Sep. 2018,	1	BS12	MZ031441
	<i>Naja atra</i>	Hunan	Sep. 2018, Oct. 2019	4	BS13, BS42–BS44	MZ031442, MZ031471–MZ031473
Viperidae	<i>Deinagkistrodon acutus</i>	Hunan	Oct. 2019	4	BS14, BS45–BS47	MZ031443, MZ031474–MZ031476
	<i>Protobothrops mucrosquamatus</i>	Hunan	Oct. 2019	1	BS48	MZ031477
	<i>Trimeresurus stejnegeri</i>	Hunan	Oct. 2019	3	BS49–BS51	MZ031478–MZ031480
Phasianidae	<i>Gallus gallus domesticus</i>	Hubei	Nov. 2019	3	JiBS01–JiBS03	MZ031481–MZ031483

Anatidae	<i>Anas platyrhynchos domestica</i>	Hubei	Nov. 2019	3	YaBS01– YaBS03	/
Suidae	<i>Sus scrofa f. domestica</i>	Hubei	Nov. 2019	5	ZhuBS01– ZhuBS01	MZ031484– MZ031488
Bovidae	<i>Bos taurus</i>	Hubei	Nov. 2019	3	NiuBS01– NiuBS03	MZ031489– MZ031491
	<i>Ovis aries</i>	Hubei	Nov. 2019	3	YangBS01– YangBS03	MZ031492– MZ031493

/: no data was available through DNA barcoding

Table 3 The identification results of market Shedan medicinal materials and their GenBank accession numbers

No.	Batch number	Collecting place	Quantity (Sum)	Quantity (Detected)	Quantity (Identified)	Original source (number)	GenBank accession number
1	ZJ20100321	Deqing Moganshan Snakes Industrial Co., Ltd.	57	19	19	<i>Elaphe carinata</i> (n=18), <i>Naja atra</i> (n=1)	MH220587– MH220605
2	JX20100601	Nanchang Xinjian Snake Faring Cooperatives	64	21	21	<i>Lycodon rufozonatus</i> (n=4), <i>Ptyas dhumnades</i> (n=13), <i>Ptyas korros</i> (n=2), <i>Deinagkistrodon acutus</i> (n=2)	MH220606– MH220626
3	ZJ20100701	Deqing Moganshan Snakes Industrial Co., Ltd.	10	3	3	<i>Elaphe carinata</i> (n=2), <i>Elaphe taeniura</i> (n=1)	MH220627– MH220629
4	AH20100801	Bozhou herb market	34	11	11	<i>Elaphe carinata</i> (n=1), <i>Elaphe taeniura</i> (n=1), <i>Ptyas dhumnades</i> (n=4), <i>Ptyas korros</i> (n=1), <i>Ptyas mucosa</i> (n=2), <i>Naja atra</i> (n=2)	MH220630– MH220640
5	ZJ20100801	Deqing Moganshan Snakes Industrial Co., Ltd.	18	6	6	<i>Elaphe taeniura</i> (n=4), <i>Lycodon rufozonatus</i> (n=1), <i>Ptyas dhumnades</i> (n=1)	MH220641– MH220646
6	ZJ20121101	Deqing Moganshan Snakes Industrial Co., Ltd.	18	6	6	<i>Elaphe carinata</i> (n=3), <i>Elaphe taeniura</i> (n=2), <i>Lycodon rufozonatus</i> (n=1)	MH220647– MH220652
7	ZJ20121201	Deqing Moganshan Snakes Industrial Co., Ltd.	27	9	9	<i>Coelognathus radiatus</i> (n=2), <i>Ptyas korros</i> (n=1), <i>Elaphe carinata</i> (n=5), <i>Ptyas dhumnades</i> (n=1)	MH220653– MH220661
8	ZJ20130502	Deqing Moganshan Snakes Industrial Co., Ltd.	25	8	8	<i>Coelognathus radiatus</i> (n=1), <i>Elaphe carinata</i> (n=1), <i>Elaphe taeniura</i> (n=2), <i>Lycodon rufozonatus</i> (n=1), <i>Ptyas dhumnades</i> (n=1), <i>Deinagkistrodon acutus</i> (n=2)	MH220662– MH220669
9	ZJ20140301	Deqing Moganshan Snakes Industrial Co., Ltd.	10	3	3	<i>Elaphe carinata</i> (n=3)	MH220670– MH220672

10	HN20141201	Yongzhou Yishe Faring Cooperatives	36	12	12	<i>Elaphe carinata</i> (n=3), <i>Elaphe taeniura</i> (n=1), <i>Lycodon</i> <i>rufozonatus</i> (n=5), <i>Ptyas</i> <i>dhumnades</i> (n=2), <i>Deinagkistrodon</i> <i>acutus</i> (n=1)	MH220673– MH220684
11	GD20151009	Guangzhou Baiyunshan PanGao Shou Pharmaceutical Co., Ltd	28	9	9	<i>Lycodon rufozonatus</i> (n=9)	MH220685– MH220693
12	HB20161012	Hubei Yujindan Pharmaceutical Co., Ltd	42	14	14	<i>Amphiesma</i> <i>stolatum</i> (n=1), <i>Elaphe</i> <i>carinata</i> (n=7), <i>Elaphe</i> <i>taeniura</i> (n=2), <i>Ptyas</i> <i>dhumnades</i> (n=1), <i>Ptyas korros</i> (n=1), <i>Gloydus</i> <i>brevicaudus</i> (n=1), <i>Xenopeltis</i> <i>unicolor</i> (n=1)	MH220694– MH220707
13	HB20161020	Hubei Taizi Pharmaceutical Co., Ltd	40	13	13	<i>Lycodon</i> <i>rufozonatus</i> (n=1), <i>Ptyas</i> <i>dhumnades</i> (n=6), <i>Ptyas mucosa</i> (n=2), <i>Gloydus</i> <i>brevicaudus</i> (n=1), <i>Protobothrops</i> <i>mucrosquamatus</i> (n=1), <i>Xenopeltis</i> <i>unicolor</i> (n=2)	MH220708– MH220720
14	ZJ20130401	Deqing Moganshan Snakes Industrial Co., Ltd.	40	13	5	<i>Elaphe carinata</i> (n=2), <i>Naja atra</i> (n=1), <i>Gallus</i> <i>gallus</i> (n=2)	MZ045931– MZ045935
15	ZJ20150520	Hubei Taizi Pharmaceutical Co., Ltd	16	5	3	<i>Naja atra</i> (n=1), <i>Gallus</i> <i>gallus</i> (n=2)	MZ045936– MZ045938
16	GD20150801	Guangzhou Baiyunshan PanGao Shou Pharmaceutical Co., Ltd	8	3	0	/	/
17	AH20181024	Bozhou herb market	80	27	18	<i>Coelognathus</i> <i>radiatus</i> (n=2), <i>Elaphe</i> <i>carinata</i> (n=1), <i>Ptyas</i> <i>mucosa</i> (n=5), <i>Xenopeltis</i> <i>unicolor</i> (n=10)	MZ045946– MZ045947, MZ045950, MZ045954, MZ045957, MZ045966– MZ045968, MZ045970– MZ045972, MZ045974, MZ045978, MZ045987,

							MZ045991, MZ045995, MZ045998– MZ045999
18	ZJ20191026	ShishengYiShe Farming cooperatives	16	13	5	<i>Gallus gallus</i> (n=5)	MZ046000– MZ046005

/: no data was available through DNA barcoding

Table 4 Summary of genetic divergences (K2P model) among the snake species identified based on COI barcode sequences

Species	Quantity	Intraspecies genetic distance (mean)	Interspecies genetic distance (mean)
<i>Amphiesma stolatum</i>	2	0	0.1691–0.2479 (0.2011)
<i>Boiga kraepelini</i>	1	/	0.1417–0.2370 (0.1936)
<i>Calamaria septentrionalis</i>	1	/	0.1904–0.2638 (0.2225)
<i>Cyclophiops major</i>	5	0–0.0030 (0.0018)	0.1093–0.2339 (0.1847)
<i>Elaphe carinata</i>	24	0–0.0314 (0.0142)	0.1528–0.2403 (0.1976)
<i>Elaphe taeniura</i>	24	0–0.0848 (0.0320)	0.1409–0.2291 (0.1878)
<i>Euprepriophis mandarinus</i>	9	0.0015–0.0493 (0.0291)	0.1181–0.2146 (0.1850)
<i>Lycodon flavozonatus</i>	4	0–0.0361 (0.0191)	0.0986–0.2515 (0.1989)
<i>Lycodon futsingensis</i>	1	/	0.0910–0.2408 (0.1921)
<i>Lycodon rufozonatus</i>	23	0–0.0378 (0.0201)	0.0910–0.2385 (0.1985)
<i>Myrrophis chinensis</i>	6	0–0.0123 (0.0041)	0.1574–0.2446 (0.2104)
<i>Oligodon chinensis</i>	1	/	0.1795–0.2376 (0.2113)
<i>Opisthotropis cheni</i>	1	/	0.1762–0.2318 (0.2031)
<i>Oreocryptophis porphyraceus</i>	2	0.0015–0.0015 (0.0015)	0.1277–0.2450 (0.1937)
<i>Oocatochus rufodorsatus</i>	12	0–0.0139 (0.0070)	0.1181–0.2055 (0.1716)
<i>Ptyas dhumnades</i>	37	0–0.0523 (0.0144)	0.1093–0.2412 (0.1889)
<i>Ptyas korros</i>	15	0–0.0092 (0.0036)	0.1476–0.2286 (0.1946)
<i>Ptyas mucosa</i>	2	0	0.1356–0.2396 (0.1947)
<i>Rhabdophis tigrinus</i>	4	0	0.1619–0.2383 (0.2020)
<i>Sinonatrix annularis</i>	24	0–0.0249 (0.0073)	0.1252–0.2438 (0.2055)
<i>Sinonatrix percarinata</i>	2	0–0.0076 (0.0076)	0.1252–0.2529 (0.2105)
<i>Xenochrophis flavipunctatus</i>	3	0–0.0015 (0.0010)	0.1816–0.2698 (0.2119)
<i>Bungarus multicinctus</i>	5	0–0.0030 (0.0012)	0.1692–0.2557 (0.2087)
<i>Naja atra</i>	5	0–0.0030 (0.0018)	0.1748–0.2551 (0.2127)
<i>Ophiophagus hannah</i>	2	0	0.1704–0.2487 (0.1976)
<i>Sinomicrurus kelloggi</i>	1	/	0.1452–0.2570 (0.2087)
<i>Sinomicrurus macclellandi</i>	1	/	0.1452–0.2474 (0.2131)
<i>Deinagkistrodon acutus</i>	12	0–0.0092 (0.0025)	0.1555–0.2698 (0.2299)
<i>Gloydus brevicaudus</i>	9	0–0.0345 (0.0076)	0.1696–0.2638 (0.2256)
<i>Protobothrops mucrosquamatus</i>	1	/	0.1580–0.2529 (0.2143)
<i>Trimeresurus stejnegeri</i>	14	0–0.0491 (0.0253)	0.1555–0.2438 (0.2117)

/: no data was available

Figures

Figure 1

The NJ tree based on COI barcode sequences (Bootstrap value $\geq 50\%$). *Acrochordus javanicus* was used as the outgroup. HB, HN, JX and ZJ represented Hubei province, Hunan province, Jiangxi province and Zhejiang province, respectively. GB represented these sequences were downloaded from GenBank. The background highlighted in orange, green, purple and blue represented Colubridae, Elapidae, Viperidae and the outgroup, respectively

Figure 2

Amplification of the fresh snake bile specimens from 17 snake species with COI specific primers COISNFF/COISNFR. 1: *C. major*, 2: *E. carinata*, 3: *E. taeniura*, 4: *E. mandarinus*, 5: *L. flavozonatus*, 6: *L. rufozonatus*, 7: *M. chinensis*, 8: *O. porphyraceus*, 9: *P. dhumnades*, 10: *P. korros*, 11: *P. mucosa*, 12: *R. tigrine*, 13: *B. multicinctus*, 14: *N. atra*, 15: *D. acutus*, 16: *P. mucrosquamatus*, 17: *T. stejnegeri*, and CK: negative control

Figure 3

Amplification of the 17 fresh bile samples from five other animal species with COI specific primers COISNFF/COISNFR. 1–3: *Gallus gallus domesticus* (chicken), 4–6: *Anas platyrhynchos domestica* (duck), 7–11: *Sus scrofa f. domestica* (pig), 12–14: *Bos taurus* (cattle), 15–17: *Ovis aries* (sheep), and CK: negative control

Figure 4

The sensitivity of amplification with COI specific primers COISNFF/COISNFR in Shedan. 1–3: 100 ng/μL, 4–6: 10 ng/μL, 7–9: 1 ng/μL, 10–12: 100 pg/μL, 13–15: 10 pg/μL, 16–18: 1 pg/μL, and CK: negative control

Supplementary Files

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