Next-generation sequencing and targeted quantitative real-time polymerase chain reaction for detection of *Akebiae Caulis* in the traditional Chinese medical formula Longdan Xiegan Wan

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Background: *Akebiae Caulis* (Mu Tong) is commonly misused by *Aristolochiae Manshuriensis Caulis* (Guan Mutong) and *Clematidis Armandii Caulis* (Chuan Mutong), which are nephrotoxic and carcinogenic. However, in the Pharmacopoeia of the People's Republic of China (2015 Edition), the method for determining *Akebiae Caulis* remains undefined.

Methods: We used DNA barcode-based next-generation sequencing (NGS) combined with quantitative real-time polymerase chain reaction (qPCR) to detect *Akebiae Caulis* in Longdan Xiegan Wan (LDXGW) for the first time. Compared with chromatographic studies, NGS enables better evaluation of the ingredient components of traditional Chinese medicine (TCM) preparations. The feasibility of qPCR using species-specific primers to determine the authenticity of species has been validated. In this study, the constituents of *Akebiae Caulis* in LDXGW from three different manufacturers were scanned by NGS. The independently developed qPCR detection primers of *Akebiae Caulis*, *Aristolochiae Manshuriensis Caulis*, and *Clematidis Armandii Caulis* were specifically used to analyze the LDXGW mentioned above.

Results: The results showed that qPCR detected *Clematidis Armandii Caulis* in all commercial samples. Meanwhile, NGS detected the counterfeit species *Clematis peterae* (Tie-Xian Lian) in all samples. We found that qPCR shows a difference in detecting *Akebiae Caulis*, but it was not able to identify the unknown additives and adulterants for the primer pairs of *Clematidis Armandii Caulis*.

Conclusions: Hence, it is sensitive and rapid, qPCR is not suitable for detection alone. The NGS approaches offer important novel insights that complement the qPCR method. The combination of NGS and qPCR will be a powerful complement to traditional identification methods of TCM substances.

Keywords: Next-generation sequencing (NGS); DNA barcoding; quantitative real-time polymerase chain reaction (qPCR); traditional Chinese medicine (TCM)

Submitted Apr 13, 2022. Accepted for publication Jun 20, 2022.
doi: 10.21037/atm-22-2415
View this article at: https://dx.doi.org/10.21037/atm-22-2415

Introduction

Traditional Chinese Medicine (TCM) was included in the 11th revision of the World Health Organization (WHO) International Statistical Classification of Diseases and Related Health Problems (ICD) which came into effect on 1 January 2022 for the first time. This inclusion suggests that TCM will become more affordable and readily available than Western medicine in some countries.

Traditional medicine, dietary supplements, and natural herbal products containing herbal blends have flooded the markets in many Western countries (1). The public relies on drug labels for drug information (2). Regulators require product labels to include all product information. However, Chinese herbal medicinal products may be adulterated with impurities or contaminants during cultivation and processing (3). The general methods which can detect specific compounds in TCM are recorded in the Pharmacopoeia of the People's Republic of China (2015 Edition) (4), and are based on morphology, microscopic identification, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC). However, these methods cannot detect all labeled and unlabeled species simultaneously in multi-ingredient TCMs (4,5). A study showed that only 68% of *Hypericum perforatum* herbal products contained the target species, and differences between ingredient species and those listed on the label were detected in all products (6). To improve the safety and effectiveness of TCM, regulatory agencies should seek more effective and comprehensive detection methods.

Next-generation sequencing (NGS) based on DNA barcoding has been successfully used to identify components in mixed samples of animals and plants (7-9). There have also been cases of TCM component identification based on quantitative real-time polymerase chain reaction (qPCR) (8). However, the DNA of species in TCM compete with DNA barcode primers in the PCR process, where the more common templates are more easily amplified. This results in false negatives for low-abundance species (10,11). However, qPCR primers only specifically bind to the target gene, providing the possibility of detecting low-abundance species. To avoid the possible false-negative results of NGS, we also applied qPCR to identify the *Akebiae Caulis* (Mu Tong) in Longdan Xiegan Wan (LDXGW), which provided a method for the detection of *Akebiae Caulis* in the compound.

In the present study, we attempted to detect TCM that contain *Akebiae Caulis*. *Aristolochiae Manshuriensis Caulis* (Guan Mutong) and *Clematidis Armandii Caulis* (Chuan Mutong), which are nephrotoxic and carcinogenic, are often misused as *Akebiae Caulis* (12,13). However, the 2015 edition of the Chinese Pharmacopoeia did not describe a detection method of *Akebiae Caulis* in the TCM compounds (3). The misuse of the *Akebiae Caulis*, *Clematidis Armandii Caulis*, and *Aristolochiae Manshuriensis Caulis* will cause serious safety issues (14-16).

We performed DNA barcoding using NGS and targeted qPCR for *Akebiae Caulis* detection. Three primer pairs of qPCR which are specific for *Akebiae Caulis*, *Clematidis Armandii Caulis*, and *Aristolochiae Manshuriensis Caulis* were used in our analysis. A single primer pair of internal transcribed spacer 2 (ITS2) was employed for DNA barcoding, as ITS2 is a standard molecular marker with a high sensitivity for species identification (17-20). We selected LDXGW randomly, which should include *Akebiae Caulis*, as the research object. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2415/rc).

Methods

Sample collection

Six dry stem specimens of the botanical species source of *Clematidis Armandii caulis* (Chuan Mutong), *Akebiae Caulis* (Mu Tong), and *Aristolochiae Manshuriensis Caulis* (Guan Mutong) were collected from the Yunnan, Guangxi, Chongqing, Hubei, and Liaoning provinces to obtain the reference ITS2 sequences to design qPCR primers (*Table 1*). Commercial LDXGW specimens were randomly purchased from Beijing Tong Ren Tang Drugstore (Lot No. 17082007; Luohe, Henan, China), and the commercial product was marked as LDXGW139. The other LDXGW samples were purchased from Tianyi Qinkun Pharmaceutical Co., Ltd. (Lot No. 180801; Xi'an, China) and Lanzhou Foci Pharmaceutical Co., Ltd. (Lot No. 18I33; Lanzhou, China) were marked as LDXGW138 and LDXGW137, respectively.

NGS and qPCR analysis

The methods for NGS and qPCR analysis were described in previous study (6). In brief, DNA extraction, amplification, and library construction were performed using commercial kits. Ion S5 sequencing platform and Ion 530 chip were used

Table 1 Specimens of the botanical species source of *Clematidis Armandii Caulis* (Chuan Mutong), *Akebiae Caulis* (Mu Tong), and *Aristolochiae Manshuriensis Caulis* (Guan Mutong)

Herbal material	Species	Location	ITS2
Chuan Mutong	*Clematis armandii*	Yunnan	MK558851
	Clematis montana	Guangxi	MK558852
Mu Tong	*Akebia quinata*	Chongqing	MK558847
	Akebia trifoliata	Hubei	MK558848
	Akebia trifoliata var. australis	Hubei	MK558849
Guan Mutong	*Aristolochia manshuriensis*	Liaoning	MK558850

ITS2, internal transcribed spacer 2.

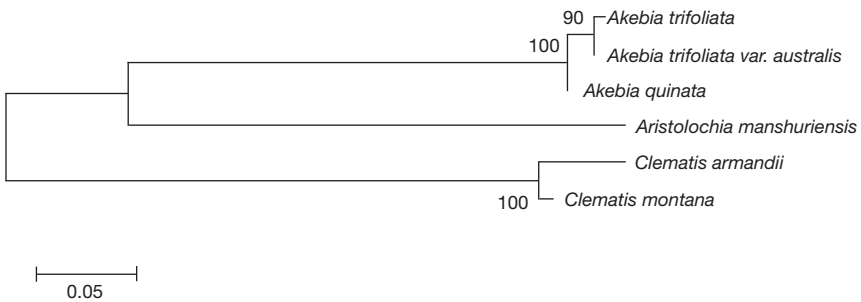

Figure 1 The neighbor-joining tree of Chuan Mutong, Mu Tong, and Guan Mutong constructed using ITS2 regions. ITS2, internal transcri bed spacer 2.

to generate high-quality (HQ) reads of targeted amplicons. We performed qPCR using the ViiA7 Real-Time System (Applied Biosystems®), whereby a standard qPCR protocol was employed. Details are provided in the supplementary methods.

Results

Specimen authentication, ITS2 sequence variation, and qPCR primer design

The ITS2 regions of specimens were all successfully amplified and bi-directionally sequenced using Sanger sequencing technology. Specimen authentications were implemented in the DNA barcoding system of Chinese herbal materials (21). The results confirmed that the samples were of the species recorded in the Chinese Pharmacopoeia 2015 edition (Chuan Mutong, Mu Tong) and 1990 edition (Guan Mutong). The sequence lengths of Chuan Mutong, Mu Tong, and Guan Mutong were 218, 216, and 274 bp, respectively. There are 123 variable position sites and 76 Parsim-info position sites among Chuan Mutong, Mu Tong, and Guan Mutong, so there are plenty of options to design qPCR primers to detect these three herbal materials. In addition, the phylogenetic tree strongly supported the potential discrimination of Chuan Mutong, Mu Tong, and Guan Mutong using ITS2 regions (*Figure 1*).

Targeted qPCR detections

Targeted qPCR detected *Akebiae Caulis* (Mu Tong) in three LDXGW samples, and one brand of LDXGW was not detected for *Akebiae Caulis*. *Clematidis Armandii Caulis* (Chuan Mutong) was detected in all commercial samples, while *Aristolochiae Manshuriensis Caulis* (Guan Mutong) was negative in all commercial samples (*Figure 2*). The information of the qPCR primer pairs is summarized in *Table 2*.

NGS results of complex matrix

Based on the Ion Torrent S5 sequencing platform, three

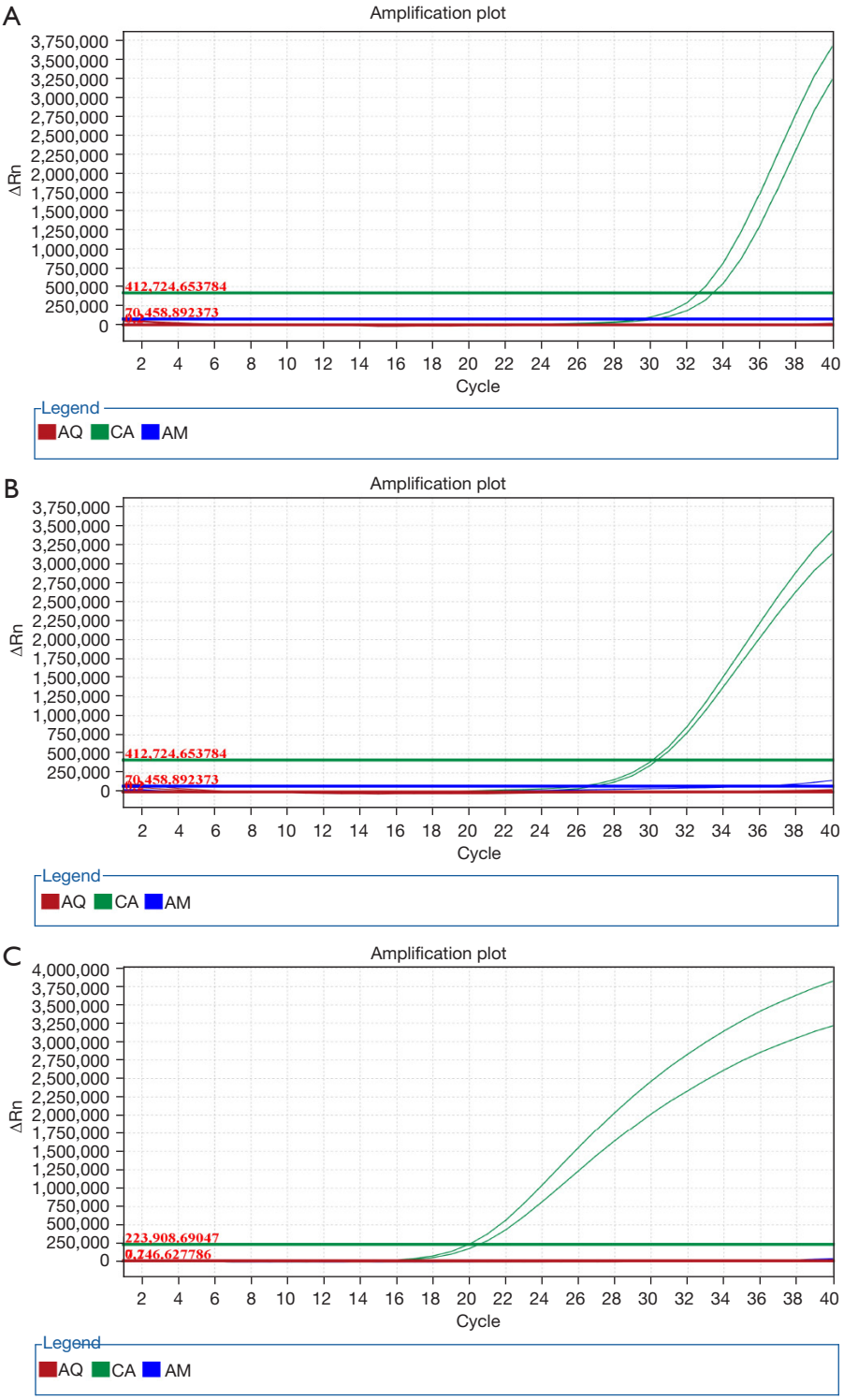

Figure 2 qPCR amplification plots of *Clematidis Armandii Caulis* (Chuan Mutong) in LDXGW samples of different brands [(A) LDXGW137, (B) LDXGW138, (C) LDXGW139]. Green amplification plot represents PCR from the amplification of Chuan Mutong with CA primer, the red amplification plot represents the Mu Tong amplification with AQ primer and the blue amplification plot represents the Guan Mutong amplification with AM primer. AQ, *Akebiae Caulis*; CA, *Clematidis Armandii Caulis*; AM, *Aristolochiae Manshuriensis Caulis*; qPCR, quantitative real-time polymerase chain reaction; LDXGW, Longdan Xiegan Wan.

Table 2 qPCR primer pairs for each species

No.	Species	Forward primer (5' to 3')	Reverse primer (5' to 3')
1	*Akebiae Caulis*	TGGTGGTTGACGTGCCTCTT	CACCGACAAGGTCCGCGT
2	*Clematidis Armandii Caulis*	GCGGTCAGCGGTGGTTGT	CGCGTCGTTTCTGTCTTTGG
3	*Aristolochiae Manshuriensis Caulis*	TCGGGTGCGGTTGGCT	GGAGGCGAACGGTTAGGGTC

qPCR, quantitative real-time polymerase chain reaction.

Table 3 High-throughput sequencing versus qPCR for *Akebiae* Caulis and adulterants detection in the LDXGW samples based on the ITS2 regions

Herbal material	Species	LDXGW137		LDXGW138		LDXGW139	
		qPCR	NGS	qPCR	NGS	qPCR	NGS
Mu Tong	*Akebia quinata*						
	Akebia trifoliata						
	Akebia trifoliata var. australis						
Chuan Mutong	*Clematis armandii*	√		√		√	√
	Clematis montana	√		√		√	√
Guan Mutong	*Aristolochia manshuriensis*						
Tie-Xian Lian	*Clematis peterae*		√		√		√

qPCR, quantitative real-time polymerase chain reaction; LDXGW, Longdan Xiegan Wan; ITS2, internal transcribed spacer 2; NGS, next-generation sequencing.

batches of LDXGW samples (LDXGW137, LDXGW138, and LDXGW139) were used for NGS of ITS2 molecular tags in this study, and a total of 3,035,227 reads were obtained. A total of 1,957,902 HQ reads were obtained after filtering adaptors, low-quality sequences [quality value (QV) < quality score of 20 (Q20)], and short reads (length <300 bp). The filtered data were subjected to TCM V1.0 plug of Ion Torrent S5-in analysis, and *Clematis peterae* (Tie-Xian Lian) was detected in all commercial samples (LDXGW137, LDXGW138, LDXGW139) based on the alignment of ITS2 sequences. In contrast, *Akebiae Caulis* was not found in any of the three samples. The non-prescribed species, *Clematis armandii*, and *Clematis montana*, were detected in LDXGW139.

NGS versus qPCR for Akebiae Caulis and adulterants detection

Although there are 15 kinds of TCM compounds containing *Akebiae Caulis* in the 2015 edition of the Chinese Pharmacopoeia, an identification method of *Akebiae Caulis* is not specified (3). Aristolochic acid (AA), which is found in many Aristolochiaceae species, is nephrotoxic (20). *Aristolochiae Manshuriensis Caulis* contains AA, but *Akebiae Caulis* does not. It is not easy to distinguish the two by appearance alone. In addition, other TCMs, including *Clematidis Armandii Caulis*, are often misused as *Akebiae Caulis*, which may also have potential issues. This study utilized the two different methods of NGS and qPCR for detection. The NGS and qPCR methods differed significantly in the detection of *Akebiae Caulis* and adulterants. The main difference is that only *Clematidis Armandii Caulis* was detected in the compound by qPCR. However, NGS found that *Clematis peterae* was used in the commercial samples (*Table 3*). This raised the question of why qPCR and NGS yielded different results. We checked the ITS2 sequences of *Clematis peterae* and *Clematidis Armandii Caulis* and found a high homology of ITS2 sequences between the two species. The primer pairs of *Clematidis Armandii Caulis* would not mark off both of them (*Figure 3*), which led to the difference in identification results between qPCR and NGS. An identical positive correlation was observed between qPCR cycle threshold (Ct) values and the *Clematidis Armandii Caulis* NGS reads

Figure 3 High homology of ITS2 sequences between *Clematis peterae* (Tie-Xian Lian) and *Clematidis Armandii Caulis* (Chuan Mutong). (A) Highlighted region is the forward primer sequence of Chuan Mutong. (B) Highlighted region is the reverse primer sequence of Chuan Mutong. ITS2, internal transcribed spacer 2.

obtained from samples of LDXGW137-139. Samples with lower qPCR Ct values generally yielded more sequencing reads.

Discussion

The NGS results of this study show that the advantage of NGS lies in the discovery of unknown added components or unknown fakes. The NGS method can provide identification of elements based on any amplifiable DNA molecular tag sequence. Nevertheless, the processing or manufacture of compound Chinese medicinal materials will lead to the degradation of DNA in the restorative materials (2,22), resulting in no molecular tags being amplified and the occurrence of false negatives. On the contrary, shorter PCR primers can solve the false negative detection due to DNA degradation (23). Due to the difference in technical principles, NGS is more expensive. It takes longer to detect DNA molecular tags than qPCR technology, but NGS becomes more cost-effective as more samples are processed (24,25). As NGS is an emerging technology, and its sequencing cost is decreasing year by year, higher sample throughput and greater affordability will be possible in the future. Therefore, in the long run, NGS will become more cost-effective, provide more data at a lower cost, and approach the detection sensitivity of qPCR in compounds without processed medicinal materials. Each authentication method has limitations. The NGS methods have different sequencing error rates, depending on the platform: about 1% for Ion-Torrent™ Personal Genome Machines (PGM; Thermo Fisher, Waltham, MA, USA), about 1% for Illumina platforms (Illumina, San Diego, CA, USA), and about 15% for Pacific Biosciences (Menlo Park, CA, USA) (26). Sequencing errors appear similar to those of DNA mutations and can complicate accurate species identification. To identify TCM compound ingredients, NGS technology and qPCR technology should complement each other, not replace. The NGS approach can quickly scan the components in the sample and find unknown added or faulty features. Fluorescence qPCR achieves the purpose of fast, fixed-point detection with its quick, simple, and economic characteristics.

In the 2015 edition of the Chinese Pharmacopoeia, there are 15 TCM compounds containing Mu Tong, but there is no description of the identification method of Mu Tong in the compound form. AAs exist in many plants of the Aristolochiaceae family, which pose a severe threat to human life and health (27). The "Longdan Xiegan Pill Incident" in the 1990s resulted in a painful TCM safety incident due to the lack of effective means to detect Mu Tong medicinal materials (28). There have been many reports of instances where Chuan Mutong has also replaced Mu Tong (29). If there is a continued lack of effective detection methods and methods, potential danger will remain a threat to the safety of TCM. This study is a supplement for detecting compound Mu Tong in TCM products.

Acknowledgments

Funding: This work was supported by the Special Project of the National Clinical Research Base of Traditional Chinese Medicine (No. 2021JDZX2090).

Footnote

Reporting Checklist: The authors have completed the MDAR

reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2415/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2415/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2415/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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(English Language Editor: J. Jones)

Cite this article as: Song Y, Yang Z, Wang P, Song K, Zhang S, Fan T. Next-generation sequencing and targeted quantitative real-time polymerase chain reaction for detection of *Akebiae Caulis* in the traditional Chinese medical formula Longdan Xiegan Wan. Ann Transl Med 2022;10(12):706. doi: 10.21037/atm-22-2415

