Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Rapid Identification of Medicinal Leech by Species-Specific Polymerase Chain Reaction Technology

Yang Zheng, Beibei Lu, Yaya Yang, Zhaoqun Jiao, Liqun Chen, Pingtian Yu, Yuping Shen, Huan Yang

Department of Chinese Medicine and Pharmacy, School of Pharmacy, Jiangsu University, Zhenjiang 212013, China

Submitted: 27-11-2018 Revised: 14-01-2019 Published: 16-05-2019

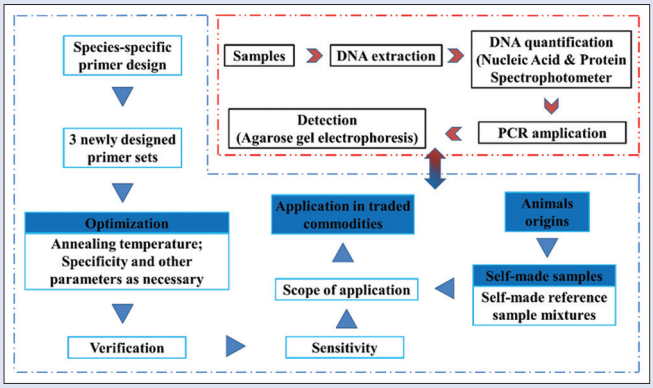
ABSTRACT

Background: Leech is a well-known animal-derived Chinese medicine; intentionally or unintentionally, interfusing fake products have exposed patients to high risk. **Objective:** In this study, we would like to find a rapid and accurate technology to identify medicinal leech. **Materials and Methods:** Three sets of species-specific primer were designed, and polymerase chain reaction (PCR) conditions were optimized for the four species analyzed. Specificity and sensitivity test were carried out, and the reference sample mixtures were analyzed. Finally, the developed method was used to assess the authenticity of commercially available products. **Results:** Three sets of species-specific primer were confirmed to have high interspecies specificity and good intraspecies stability. The limit of detection was estimated to be 1 ng for all of four assayed species. Subsequent validation demonstrated that the raw material, processed, and even highly processed products of leech can be conveniently authenticated with good sensitivity and precision by this newly established PCR-based method. **Conclusion:** These designed novel primer sets have shown distinct performance on the identification of adulterate products. The new technology needs no procedure for sequencing and has been successfully employed for the reliable authentication of raw material of leech and even their processed and highly processed products.

Key words: Authentication, deoxyribonucleic acid barcoding, leech, polymerase chain reaction, species-specific primer

SUMMARY

- A novel polymerase chain reaction technology was developed to determine leech species based on the difference in mitochondrial genome. Three species-specific primers were designed and confirmed to have a high level of interspecies specificity and good intraspecies stability. The limit of detection was estimated to be 1 ng of genomes for all of four assays. The raw material and processed products can be conveniently authenticated and with both good sensitivity and precision by this newly proposed approach. Especially, these primer sets have still performed well when the samples are adulterated.



Abbreviations used: DNA: Deoxyribonucleic acid; COI: Mitochondrial cytochrome c oxidase subunit 1; PCR: Polymerase chain reaction; bp: Base pair.

Correspondence:

Prof. Yuping Shen,
School of Pharmacy, Jiangsu University,
Xuefu Road 301, Zhenjiang 212013, Jiangsu Province, China.
E-mail: syp131@ujs.edu.cn
Prof. Huan Yang,
School of Pharmacy, Jiangsu University,
Xuefu Road 301, Zhenjiang 212013, Jiangsu Province, China.
E-mail: yanghuan1980@ujs.edu.cn

DOI: 10.4103/pm.pm_596_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Leech, a well-known animal-derived Chinese medicine, is the dry product of the whole body of *Whitmania pigra* Whitman, *Hirudo nipponica* Whitman, or *Whitmania acranulata* Whitman. Medically, it has been widely used as an anticoagulation and antithrombosis agent in clinics for a very long time.[1] In addition, it is also considered as promising drug targets for anticancer and anti-inflammation therapy.[2,3] Medicinal leeches appear to provide methods of reducing blood coagulation and reliving venous pressure from pooling blood, especially after plastic and organ transplantation surgery.[4,5] However, the abundance requirement and high price of leech in market have caused serious intentional fraudulent modification of this medicine, and the similar morphological characteristics in different species of closer phylogenetic relationship and lack of professional experience can usually lead to unintentionally interfusing fake products. For instance, *Poecilobdella manillensis* Lesson was misused as medicinal leech quite often, and study had shown that it has different effects on thrombin and coagulation pathways with medicinal leech.[6] Owing to accurate authentication of herbal medicines which is strictly a legal requirement worldwide and a prerequisite to deliver quality products that meet consumers' expectations on their efficacy,[7] highly selected and sensitive methods for leech accurate identification are vital.

Cite this article as: Zheng Y, Lu B, Yang Y, Jiao Z, Chen L, Yu P, *et al*. Rapid identification of medicinal leech by species-specific polymerase chain reaction technology. Phcog Mag 2019;15:410-5.

Conventional methods for leech identification are based on the morphological characteristics of the intact adult leech which is not applicable for the larval and incomplete individuals yet. Several current analysis methods are mostly relying on chromatographic and mass spectrometer (MS)-based techniques which require high cost, complicated, and expensive instrument.[8-10] For instance, high-performance liquid chromatography fingerprint and gas chromatography (GC)/MS were popularly employed for detection of characteristic small molecule, and electrophoresis-based or biological MS-based technologies were often used to monitor signature proteins or specific peptide fragments.[11-15] However, similar chemical properties always make accurate identification of a mixture difficult. In addition, complicated procedures have to be performed while using these technologies and expensive equipment is usually needed. At present, deoxyribonucleic acid (DNA) molecular identification that can differentiate species correctly in close phylogenetic relationship groups for herbal medicines has been well recognized and become more and more popular.[16-21] It always undergoes a sequencing procedure of amplicon after polymerase chain reaction (PCR) amplification using universal primers, and the obtained sequence is compared with that in database to identify species. For instance, the DNA barcodes system was established for the reliable authentication of Chinese medicinal plants.[22] However, this method requires high purity of DNA sample and not suitable for adulterants. Furthermore, the broad applications of DNA barcoding are limited as the original DNA is probably degraded into very short fragments after Chinese medicines have been highly processed. Alternatively, species-specific primers have been designed to bind to highly conserved gene regions to aid the amplification of this gene region in species where universal primers fail.[23] Up to date, they have been successfully used to identify *Agkistrodon acutus*, *Zaocys dhumnades*, and so on to demonstrate their good prospects for the identification of Chinese animal medicines.[24-30] However, there are currently no correlative studies on the molecular identification of leech species using species-specific PCR.

In this study, a reliable PCR-based approach was established for the identification of leech species for the first time. Species-specific primers were designed and evaluated by DNAMAN, Oligo and online NCBI Primer-BLAST, followed by optimization of amplification conditions and validation for specificity and sensitivity [Figure 1]. The newly developed method can be used for specific and rapid authentication of leech products for their form of raw material, the processed, and even the highly processed.

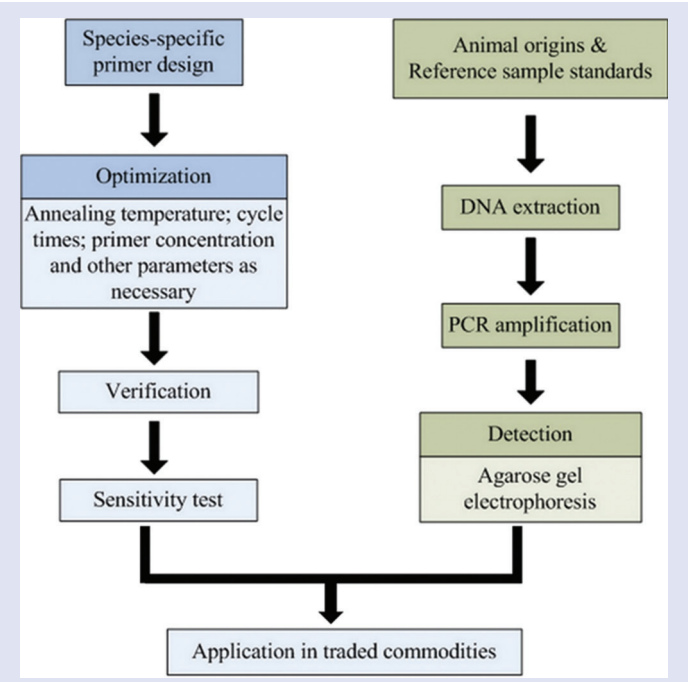


Figure 1: Flow chart for the establishment of the novel strategy

MATERIALS AND METHODS

Samples

Three leech species including *W. pigra*, *H. nipponica*, or *W. acranulata* and a fake leech species *P. manillensis* were used in this study. Collection locations of these animal origins were listed in Table 1, and all of them used cytochrome c oxidase subunit I barcoding for verification and authenticated by Professor Jun Chen (Pharmacognosy Research Facility, School of Pharmacy, Jiangsu University). The samples were then handled to prepare the raw materials and processed products of leech according to the relevant protocols recorded in the prevailing China Pharmacopoeia (Ch. P., 2015 Ed, Vol. 1) as illustrated in Figure 2. In addition, a certified reference substance (raw material of *W. pigra*; B/N: 121061-201305) was purchased from National Institutes for Food and Drug Control, PRC.

Primers' design

Mitochondrial complete gene sequences from four species of leech were incorporated to develop an accurate and convenient method for their identifications. All the primers were designed in accordance with the principles including length between 18 base pair (bp) and 25 bp, GC% >40%, and no hairpin structure and dimers. Species-specific primer sets were designed according to both intraspecific homology and interspecific variation in mitochondrial genome of leeches and their similar species using Oligo software (Version 7.60). All of them were evaluated by Oligo and online NCBI Primer-BLAST [Figure 3] and synthesized by Sangon Biotech (Shanghai) Co., Ltd. As two species, namely *W. pigra* and *W. acranulata* have a similarity of 95% in genome, no specific primers can be employed to distinguish them. Eventually, they shared one primer set in this study, and the length of its target product is 63 bp. In addition, two pairs of primers were selected for *H. nipponica* and *P. manillensis,* respectively, and the expected amplicons for each species were 102 bp and 75 bp. The three species-specific primer sets were listed in Table 2. Specificity testing for each primer set in the PCR assays was performed on the four selected species.

Deoxyribonucleic acid extraction and polymerase chain reaction amplification

All solid samples after complete lyophilization were grounded into their fine powder and then subjected to genomic DNA extraction by SDS-based protocols according to the procedures provided by Yang *et al.*[7] Nucleic acid and protein spectrophotometer (Bio Spec-mini, Shimadzu) were used to quantify the purity and concentration of the extracted DNA. These DNA samples extracted from raw materials, processed, and highly processed products were diluted to 100 ng/μL as template in further PCR assays. PCR amplification was performed in a final reaction volume of 25 μL composed of buffer, Mg^{2+}, dNTP, primers, Taq polymerase, DNA template, and H_2O. During the amplification, several factors including the concentration of template and primer, the type and amount of polymerase, annealing temperature, and time and cycle times may affect the output. Based on the result of preliminary experiments, the annealing temperature of the primers was optimized. The PCR cycler conditions used were an initial denaturation at 95°C for 3 min, followed by 30–35 cycles of 95°C for 30 s, 60°C~64°C for 30 s, and

Table 1: Sources of Leech species used in the study

Code	Species	Collection site	Collection date	Code	Species	Collection site	Collection date
WP1	*W. pigra*	Yangzhou, Jiangsu	January 5, 2017	HN2	*H. nipponica*	Chongqing	April 9, 2017
WP2	*W. pigra*	Suqian, Jiangsu	March 4, 2017	HN3	*H. nipponica*	Jinan, Shandong	June 14, 2017
WP3	*W. pigra*	Nanning, Guangxi	June 11, 2017	HN4	*H. nipponica*	Xiangfan, Hunan	June 24, 2017
WP4	*W. pigra*	Wenzhou, Zhejiang	June 12, 2017	P1	*P. manillensis*	Shantou, Guangdong	January 6, 2017
WP5	*W. pigra*	Linyi, Shangdong	July 8, 2017	P2	*P. manillensis*	Yulin, Guangxi	April 7, 2017
WP6	*W. pigra*	Changde, Hunan	March 16, 2018	P3	*P. manillensis*	Yulin, Guangxi	June 23, 2017
WP7	*W. pigra*	Nanchang, Jiangxi	March 5, 2018	P4	*P. manillensis*	Guangzhou, Guangdong	January 16, 2018
WA	*W. acranulata*	Bozhou, Anhui	April 18, 2017	P5	*P. manillensis*	Maoming, Guangdong	March 14, 2018
HN1	*H. nipponica*	Dalian, Liaoning	November 8, 2016	P6	*P. manillensis*	Zhongshan, Guangdong	March 27, 2018

W. pigra: Whitmania pigra, W. acranulata: Whitmania *acranulata, H. nipponica: Hirudo nipponica, P. manillensis: Poecilobdella manillensis*

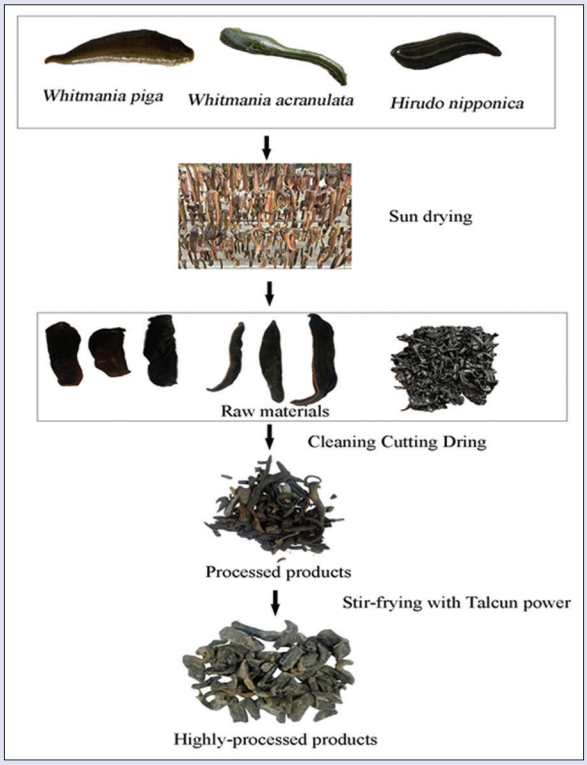


Figure 2: Procedures for assessing raw materials, processed, and highly processed products of leech

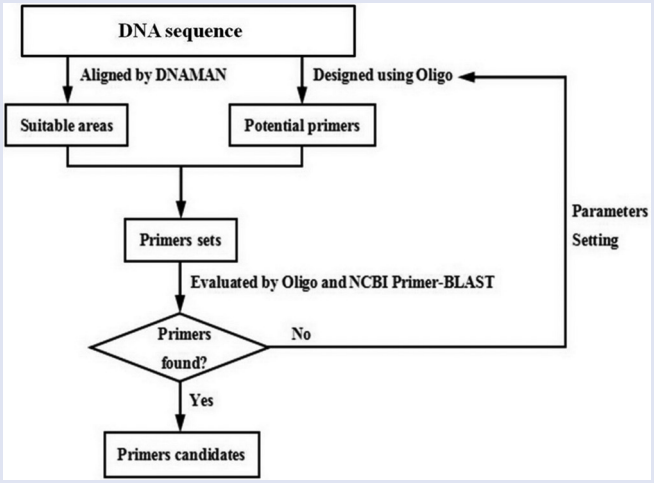


Figure 3: Flow chart for the design of species-specific primer sets

72°C for 1 min with a final extension at 72°C for 5 min. After resolution of the resulting amplicons by electrophoresis on 2%~3% agarose gel and staining in ethidium bromide, the gel was put under ultraviolet light for visualization of bands.

Specificity and sensitivity

The specificity test was carried out under the optimum conditions for different batches of leech. Sensitivity of the selected species-specific primers was determined in a concurrent PCR run with DNA template of a series of concentrations (0.001 ng/μL, 0.01 ng/μL, 0.1 ng/μL, 1 ng/μL, 10 ng/μL, and 100 ng/μL) while the concentration of the primer remained unchanged.

Analysis of reference sample mixtures

Before making various reference mixtures, sample of different leech species was collected to undergo DNA extraction and serve as a positive control. Three reference mixtures (WP:P, WA:P, and HN:P) were prepared in five proportions (9:1, 7:3, 1:1, 3:7, and 1:9) of one species mixed with the second species, with a total weight of 20 mg per sample. Individual sample was homogenized with 1 mL of the extraction buffer for DNA extraction by SDS-based protocols as aforementioned. The selected primer sets and the optimized PCR conditions were then applied to these reference sample mixtures.

Application of polymerase chain reaction assay to commercial leech products

Finally, the developed method was used to assess the authenticity of commercial products listed in Table 3, for the identification of animal origins and the verification of labeling compliance. Ten milligram of each of these samples was individually subjected to DNA extraction. The optimized PCR conditions for each species were then applied to the DNA samples.

RESULTS

Optimized polymerase chain reaction conditions

As shown in Figure 4, under the conditions where the cycle number was 35, species-specific primers could not only amplify the corresponding species but also other species when the annealing temperature is low. Primers achieved a good specificity when the temperature was raised to the appropriate level, and the optimal annealing temperature of the PW, PHN, and PP was 62°C, 60°C, and 61°C, respectively. The PCR conditions employed were summarized in Table 4.

Specificity and sensitivity

Many samples were used to verify the specificity of these primers. As shown in Figure 5, the extracted DNA from *W. pigra* and *W. acranulata* has been amplified against the same primer (PW) with the product at 63 bp while the amplification of the DNA from *H. nipponica* and *P. manillensis* produced two bands at 102 bp and 75 bp. The verification results of different batches of these four-leech species adequately demonstrated that each primer set can produce a species-specific band without any other bands for all of the negative controls. These primer sets were successfully used to specifically identify different leech species regardless of life stage or morphology. Sensitivity of a selected specific primer set for each of the four species was determined on one batch of the collected sample of each species, and DNAs within the range from 1 pg to 100 ng were subjected to the PCR amplification. The resulting bands after amplification of DNAs from these leech species are shown in Figure 6. The brightness of the strip gradually decreased with the decline of DNA concentration. Finally, it was found that the detection limits were 10 ng for *W. acranulata* and 1 ng for *W. pigra*, *H. nipponica,* and *P. manillensis*, indicating that the detection of four leeches by these primers has a fairly high sensitivity.

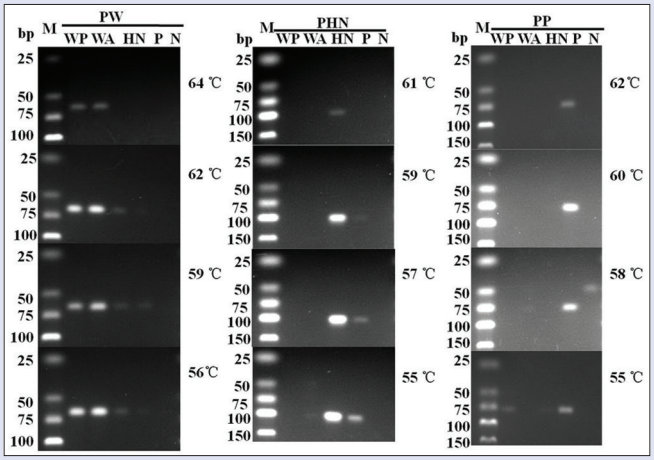


Figure 4: Optimized polymerase chain reaction conditions for four species (M: Low ladder, SN127)

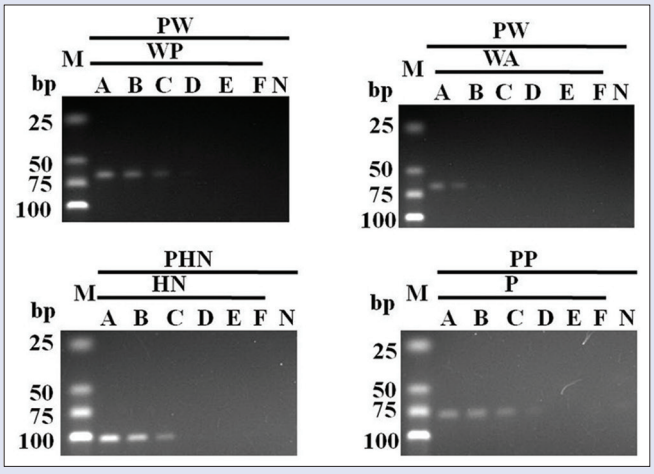


Figure 6: Sensitivity test for three specific primer sets. The concentration of template deoxyribonucleic acid from lane A to lane F was 100, 10, 1, 0.1, 0.01, and 0.001 ng/μL

Analysis of reference sample mixtures

To investigate whether the three primers were applicable for adulterated products, 15 reference samples with known ingredient compositions were assayed. As shown in Figure 7, the DNA mixtures have been amplified, and each species in all of the 15 mixed samples was accurately detected without interference. The specificity of the primers was not affected while being applied to the identification of the adulterated leeches, and the specific primers only amplified the corresponding

Table 2: Primer sets used for polymerase chain reaction assay in this study

Species	Code	Sequence (5′-3′)	Length
W. pigra	PW		
W. acranulata	F	AAGATTCCTTGGAGACGACC	63 bp
	R	ATTATAACCAACCCATGAGCC	
H. nipponica	PHN		
	F	CCAGGTAKATTTCTAGGGGAT	102 bp
	R	CCCRCCAATCAAAATAGGTA	
P. manillensis	PP		
	F	CTTTAATTACTGCACATGGAC	75 bp
	R	AATTACCAAACCCACCGAT	

W. pigra: Whitmania pigra; W. acranulata: Whitmania *acranulata; H. nipponica: Hirudo nipponica; P. manillensis: Poecilobdella manillensis*

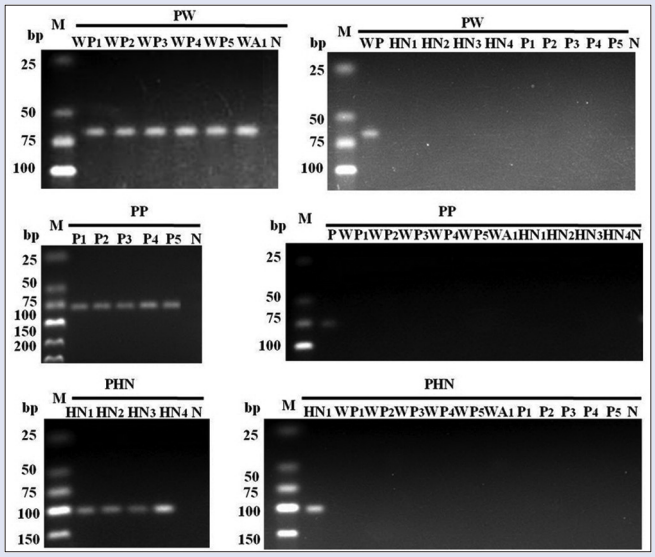


Figure 5: Specificity test for three specific primer sets

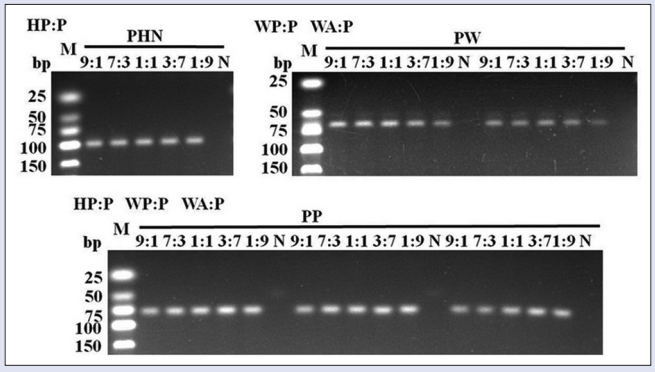


Figure 7: Analysis of reference sample mixtures by polymerase chain reaction

Table 3: Traded leech products from different sources tested in the study

Code	Products	Sources	Batch number
L1	Raw leech	Bozhou, Anhui	171002
L2	Raw leech	Linyi, Shandong	171028
L3	Raw leech	Bozhou, Anhui	171227
L4	Raw leech	Yulin, Guangxi	180103
L5	Raw leech	Yulin, Guangxi	180113
L6	Processed leech	Hefei, Anhui	171225
L7	Processed leech	Bozhou, Anhui	180103
L8	Highly processed leech	Bozhou, Anhui	171215
L9	Highly processed leech	Suqian, Jiangsu	171226
L10	Highly processed leech	Bozhou, Anhui	150920
L11	Highly processed leech	Wuxi, Jiangsu	160114
L12	Highly processed leech	Bozhou, Anhui	160301
L13	Highly processed leech	Taizhou, Jiangsu	160706
L14	Highly processed leech	Wenzhou, Zhejiang	111609018
L15	Highly processed leech	Bozhou, Anhui	161226
L16	Highly processed leech	Yichang, Hubei	160926
L17	Highly processed leech	Bozhou, Anhui	170201
L18	Highly processed leech	Kunming, Yunnan	170701
L19	Highly processed leech	Bozhou, Anhui	170801

Table 4: Optimized polymerase chain reaction conditions for three primers analyzed in this study

Program step	Primers		
	PW	PHN	PP
Amplification			
Initial denaturation	95°C (3 min)[a]		
Denaturation	95°C (30 s)[a]		
Annealing	64°C (30 s)	60°C (30 s)	61°C (30 s)
Extension	72°C (1 min)[a]		
Cycle number	35[a]		
Final extension	72°C (7 min)[a]		

[a]These conditions were the same for all primers

species in the mixed DNAs. The brightness of the strip was also obvious even though the adulteration ratio was 10%, and it is consistent with the results of previous sensitivity test. These results indicated the good feasibility and suitability of the primers for the detection of both leech species and adulterated leech products by the developed technology.

Application of polymerase chain reaction assay to commercial products

The selected primers were applied to commercial products including raw processed and highly processed leech. As shown in Figure 8, all of the raw and processed leeches were successfully identified using the novel nonsequencing approach. Twelve batches of commercial samples were authenticated as true leech products made from *W. pigra* or *W. acranulata*, while another three batches were counterfeited by *P. manillensis*. None of *H. nipponica* was found in commercial leech products. This could be due to the lack of natural resources and higher price. In addition, three batches (L12, L13, and L14) of these products did not show positive reaction against any of three primer sets (PW, PHN, and PP), indicating that they were fake products made from other animal origins rather than *P. manillensis*. Meanwhile, one batch (L15) was amplified by both PW and PP, and it has been considered as an adulterant product. It can be seen that the situation of counterfeiting or misusing is very serious as the percentage of fake or adulterate products detected was higher than one-third, and it warned the public to be vigilant while they consume leech products.

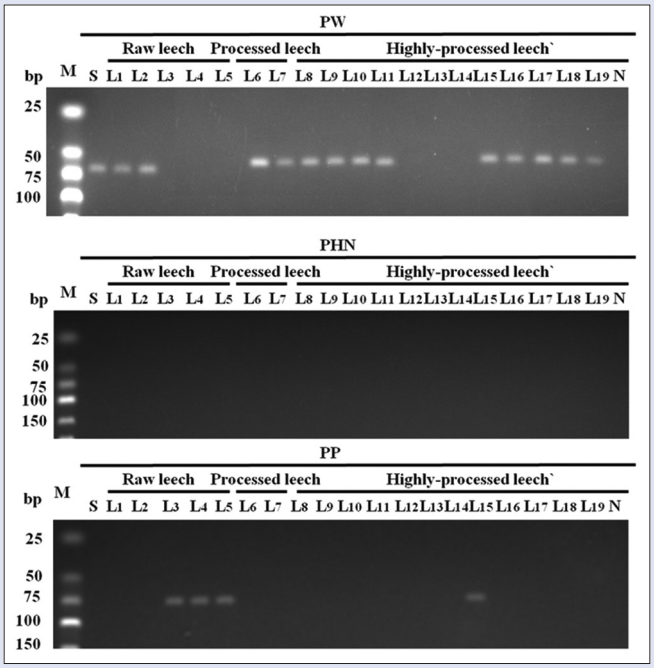


Figure 8: Analysis of commercial product of leech by polymerase chain reaction

DISCUSSIONS

In this study, three specific primers were designed for rapid identification of four leech species. In the optimized PCR conditions, the result of amplification of each species is consistent with the target DNA fragment. Moreover, all DNA samples at the concentration of 10 ng/μL have resulted in single band of strong intensity and demonstrated that the primers have good specificity and high sensitivity. In addition, it can accurately identify not only individual species but also adulterated leeches, which was verified while being applied to commercial products. The fake or adulterate leech products were more than one-third of all tested samples, indicating the importance of accurate authentication and market regulation of commercial leech products. In our study, the result showed that species-specific primers can usefully, rapidly, and accurately identify the leech species. Compared with the DNA barcoding technology, it saves a lot of time and cost; with an unknown species DNA, we can confirm it within 3 h. Moreover, conservative areas of the species sometimes are not necessarily conserved in different individuals of the same species and that has been leading to the failure of PCR amplification in some applications, it can be avoided by using species-specific primer. However, in this study, *W. pigra* Whitman and *W. acranulata* Whitman were not differentiated; however, it does not negatively affect the authentication as both of them are genuine. In the future study, we would focus on looking for specie-specific primers based on other DNA barcode such as the internally transcribed spacer or other biological technologies such as proteomics.

CONCLUSION

This established novel approach is convenient, low cost, accurate, and sensitive, although a validation step for amplicon sequencing may be needed to ensure accuracy in practice. Our technique could be important from an economic point of view in terms of fair trade and consumer rights and will be very useful for the inspection of edibility and medicinal value of leech.

Acknowledgements

We would like to thank Dr. Mingqi Wang, School of Pharmacy, Jiangsu University, for his valuable advice on manuscript preparation.

Financial support and sponsorship

This study was financially supported by the National Natural Science Foundation of China (81773855), the China Postdoctoral Science Foundation (2017T100338), Six Talent Peaks Project in Jiangsu (YY-010), and Qing Lan Project (2016).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Bott JA. Leech Protein having Anticoagulant and Antimetastatic Properties. US: 5194589A. US; 1993.
2. Ammar AE, Hassona MH, Chin MY, Helaluddin AB, Mohammed AA, Mohamed A, *et al*. Assessment of the antitumor activity of leech (huridinaria manillensis) saliva extract in prostate cancer. J Clin Oncol 2015;33:e16120.
3. Borisch B, Jahn G, Scholl BC, Filger-Brillinger J, Heymer B, Fleckenstein B, *et al*. Inhibition of lung tumor colonization by leech salivary gland extracts from *Haementeria ghilianii*. Cancer Res 1983;43:1633-5.
4. Aslan G, Terzioglu A, Tuncali D. The re-usable medicinal leech. Plast Reconstr Surg 2003;111:1358-9.
5. Cho BH, Ahn HB. Microsurgical replantation of a partial ear, with leech therapy. Ann Plast Surg 1999;43:427-9.
6. Ding YZ, Duan TX, Shan Y, Wang XF, Yang H, Yuan RJ. Comparative studies on anti-thrombin activity and anticoagulant mechanism between *Whitmania pigra* Whitman and Hirudinaria Manillensis. China Pharm 2016;19:1621-4.
7. Yang H, Yu P, Lu Y, Jiao Z, Chen L, Zhou Y, *et al*. A novel non-sequencing approach for rapid authentication of testudinis carapax et plastrum and trionycis carapax by species-specific primers. R Soc Open Sci 2018;5:172140.
8. Huang BM, Chen TB, Xiao SY, Zha QL, Luo P, Wang YP, *et al*. A new approach for authentication of four ginseng herbs and their related products based on the simultaneous quantification of 19 ginseng saponins by UHPLC-TOF/MS coupled with OPLS-DA. RSC Adv 2017;7:46839-51.
9. Chen Q, Pan XD, Huang BF. Authentication of shrimp muscle in complex foodstuff by in-solution digestion and high-resolution mass spectrometry. RSC Adv 2017;7:32903-8.
10. Nemati M, Oveisi MR, Abdollahi H, Sabzevari O. Differentiation of bovine and porcine gelatins using principal component analysis. J Pharm Biomed Anal 2004;34:485-92.
11. Yang H, Zheng J, Wang HY, Li N, Yang YY, Shen YP. Comparative proteomic analysis of three gelatinous Chinese medicines and their authentications by tryptic-digested peptides profiling using matrix-assisted laser desorption/Ionization-time of flight/Time of flight mass spectrometry. Pharmacogn Mag 2017;13:663-7.
12. Yang H, Shen Y, Xu Y, Maqueda AS, Zheng J, Wu Q, *et al*. A novel strategy for the discrimination of gelatinous Chinese medicines based on enzymatic digestion followed by nano-flow liquid chromatography in tandem with orbitrap mass spectrum detection. Int J Nanomedicine 2015;10:4947-55.
13. Sun X, Zhang L, Cao Y, Gu Q, Yang H, Tam JP. Quantitative analysis and comparison of four major flavonol glycosides in the leaves of *Toona sinensis* (A. Juss.) roemer (Chinese toon) from various origins by high-performance liquid chromatography-diode array detector and hierarchical clustering analysis. Pharmacogn Mag 2016;12:S270-6.
14. Guohua X, Pan R, Bao R, Ge Y, Zhou C, Shen Y. Rapid quantitative analysis of naringenin in the fruit bodies of *Inonotus vaninii* by two-phase acid hydrolysis followed by reversed phase-high performance liquid chromatography-ultra violet. Pharmacogn Mag 2017;13:659-62.
15. Yang H, Chen B, Wang XB, Chue PW, Shen YP, Xia GH, *et al*. Rapid quantitative analysis of diosgenin in the tubers of *Dioscorea zingiberensis* C.H. Wright by coupling cellulose enzymolysis and two-phase acid hydrolysis in tandem with HPLC-UV. Nat Prod Res 2013;27:1933-5.
16. Hebert PD, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc Biol Sci 2003;270:313-21.
17. Lockley AK, Bardsley RG. DNA-based methods for food authentication. Trends Food Sci Technol 2000;11:67-77.
18. Schuenemann VJ, Peltzer A, Welte B, van Pelt WP, Molak M, Wang CC, *et al*. Ancient Egyptian mummy genomes suggest an increase of sub-Saharan African ancestry in post-roman periods. Nat Commun 2017;8:15694.
19. Kerr KC, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, Hebert PD. Comprehensive DNA barcode coverage of North American birds. Mol Ecol Notes 2007;7:535-43.
20. Li Q, Yan H, Lin D, Wang Y, He M, Zhang W, *et al*. Molecular identification of three *Aquilaria* (*Thymelaeaceae*) species through DNA barcoding. Biol Pharm Bull 2018;41:967-71.
21. Zhou J, Wang W, Liu M, Liu Z. Molecular authentication of the traditional medicinal plant *Peucedanum praeruptorum* and its substitutes and adulterants by DNA – Barcoding technique. Pharmacogn Mag 2014;10:385-90.
22. Chen S, Yao H, Han J, Liu C, Song J, Shi L, *et al*. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS One 2010;5:e8613.
23. Mehrdad H, Malex S, Daniel J, Josephinej R, Jamesb W, Pauldn H. A minimalist barcode can identify a specimen whose DNA is degraded. Mol Ecol Resour 2006;6:959-64.
24. Tang XJ, Feng CQ, Huang LQ, Qian ZZ, Cui GH, Zhang J. High specific PCR identification of *Agkistrodon acutus* Güenther and its adulterants. Chin J Pharm Anal 2006;26:152-5.
25. Tang XJ, Feng CQ, Huang LQ, Qian ZZ, Cui GH, Zhang J. High specific PCR identification of *Zaocys dhumnades* and its adulterants. Chin Pharmacol J 2007;5:333-6.
26. Bai GB, Zhang LY, Liu CS, Cheng W, Chen DX. Analysis of DNA sequence of Chinese medicinal materials deers and PCR identification of *Cervus elaphus* and *C. nippon*. Chin Tradit Herbal Drugs 2006;37:1566-9.
27. Jiang C, Zhao Q, Jin Y, Yuan Y, Zhao YY. Molecular authentication of Gecko and its adulterants by rapid specific PCR. Mod Chin Med 2017;19:21-5.
28. Yang Y, Zheng Y, Lu B, Jiao Z, Chen L, Gblinwon RT, *et al*. Rapid identification of *Cervus* antlers by species-specific PCR assay. Nat Prod Res 2019;33:1-5. DOI is 10.1080/14786419.2018.1560285.
29. Yang H, Zhou Y, Yu P, Yang Y, Jiao Z, Tam JP, *et al*. A novel PCR-based technology for rapid and non-sequencing authentication of *Bombyx* batryticatus using species-specific primers. Nat Prod Res 2018;32:1-6.
30. Zong Y, Wang Y, Li H, Li N, Zhang H, Sun J, *et al*. Simultaneous quantification and splenocyte-proliferating activities of nucleosides and bases in Cervi Cornu Pantotrichum. Pharmacogn Mag 2014;10:391-7.