Recombinase polymerase amplification combined with fast DNA extraction for on–spot identification of *Deinagkistrodon acutus*, a threatened species

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Abstract

Recombinase polymerase amplification combined with fast DNA extraction for on–spot identification of Deinagkistrodon acutus*, a threatened species.* This study addresses the use of recombinase polymerase amplification combined with fast DNA extraction for on–spot identification of *Deinagkistrodon acutus*, a snake species threatened due to over–exploitation and habitat destruction. For its conservation, an efficient species identification method is urgently neededto fight against illegal capture and trade. Fourteen individuals representing 12 snake species (including *D. acutus* and other snake species) were collected from mountainous regions in Southern China. Genomic DNA was extracted within five minutes by a modified alkaline lysis method. Species–specific primers for recombinase polymerase amplification (RPA) were designed based on the sequences of cytochrome C oxidase subunit I (COI) barcode region, and an optimized RPA assay system was set up. Specificity and sensitivity of the assay were checked, and the assay was validated by identifying 10 commercial Qi She crude drug samples derived from *D. acutus*. Under optimized RPA conditions, a distinct single band of 354 bp was amplified only for *D. acutus* but not for the related snake species. The entire procedure can be completed in 30 min at room temperature. Commercial Qi She crude drug identification validated effectiveness of the established assay system. Using a recombinase polymerase amplification (*RPA*) assay with rapid DNA extraction, we established an on–spot *D. acutus* identification method with good specificity and sensitivity. This method could become an efficient tool for rigorous supervision of illegal *D. acutus* capture and trade.

Key words: *Deinagkistrodon acutus*, Recombinase polymerase amplification, Rapid DNA extraction, On–spot identification, Conservation

Resumen

La amplificación por recombinasa y polimerasa combinada con la extracción rápida de ADN para la identificación sobre el terreno de Deinagkistrodon acutus*, una especie en peligro de extinción*. En este estudio se aborda la utilización de la amplificación por recombinasa y polimerasa (RPA en su sigla en inglés) en combinación con la extracción rápida de ADN para identificar sobre el terreno a *Deinagkistrodon acutus*, una especie de serpiente que se encuentra en peligro de extinción debido a la sobreexplotación y la destrucción del hábitat. Con vistas a su conservación, es muy conveniente disponer de un método eficiente de identificación que permita combatir la captura y el comercio ilegales de la especie. Se capturaron 14 ejemplares de 12 especies de serpiente (incluida *D. acutus*) en las regiones montañosas del sureste de China. Se extrajo el ADN genómico con un método de lisis alcalina que llevó cinco minutos. Se diseñaron cebadores para la RPA, específicos de la especie, a partir de las secuencias de la región del código de barras de la subunidad I de la citocromo c oxidasa, y se estableció un sistema optimizado de análisis mediante RPA. Se comprobaron la especificidad y sensibilidad del ensayo, que se validó mediante la identificación de 10 muestras comerciales de la sustancia sin elaborar conocida como Qi She derivada de *D. acutus*. En condiciones de RPA optimizadas, se amplificó una banda única de 354 pb solo para *D. acutus*, pero no para las especies de serpiente relacionadas. El proceso se puede llevar a cabo en 30 minutos a temperatura ambiente. La identificación de la sustancia comercial sin tratar Qi She permitió validar la eficacia del sistema de análisis establecido. Al combinar el análisis mediante RPA con la extracción rápida de ADN, establecimos un método de identificación de *D. acutus* sobre el terreno

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con buena especificidad y sensibilidad. Podría convertirse en un instrumento eficiente para llevar a cabo la supervisión rigurosa de la captura y el comercio ilegales de *D. acutus*.

Palabras clave: *Deinagkistrodon acutus*, Amplificación por recombinasa y polimerasa, Extracción rápida de ADN, Identificación sobre el terreno, Conservación

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Introduction

Deinagkistrodon acutus (Günther, 1888), the sharp–snouted pit viper, is the only representative of the monotypic genus *Deinagkistrodon*. More than 90 % of the populations of this species in the world occur in the Chinese provinces south of the Yangtse River, i.e., Zhejiang, Anhui, Jiangxi, Hubei, Hunan, Fujian, Taiwan, Guangdong, Guangxi, Chongqing, and Guizhou (Huang et al., 2007; Uetz et al., 2010; Roskov et al., 2019). This highly venomous species is well known in traditional chinese medicine for the effectiveness of its dried body (Agkistrodon, known as Qi She in Chinese, QS) in treating rheumatoid arthritis, stroke symptoms (such as limb numbness and spasm, eye and mouth drooping, facial paralysis, and hemiplegia), clonic convulsions (Chinese Pharmacopoeia Commission, 2015) and, of particular note, for treating hepatocellular carcinoma (Xu et al., 2020).

In recent years, there has been an ever–increasing demand for the QS crude drug, leading to over–exploitation of wild *D. acutus* (Zhou and Jiang, 2004). As the species has a narrow distribution, specialized habitat requirements, and low fecundity, *D. acutus* is facing both serious social–economical pressure and natural pressure. It has been estimated that the population was reduced by at least 30 % in the decade before 2004 (Huang et al., 2007). As a result, *D. acutus* was listed in China Species Red Book as 'Endangered' (Zhao, 1998). A later study further confirmed its vulnerability and assessed it as at high–risk (Zhou and Jiang, 2005). In the Biodiversity Red List of China: Vertebrates published in May 2015, it was again evaluated as 'Endangered' (Ministry of Environmental Protection and Academy of Sciences of China, 2015). Conservation and protection of *D. acutus* is therefore of increasing concern. More recently, the National Forestry and Grassland Administration proposed to recognize *D. acutus* as a second class national–level protected animal (National Forestry and Grassland Administration of China, 2019). Efforts should be strengthened to combat its illegal capture, trafficking, and trade (Gong et al., 2020).

In the practice of law enforcement struggling against the illegal trade, officers often encounter lawbreakers who argue that what they are selling is not *D. acutus* but other snake species with similar body size and appearance, including related vipers such as *Gloydius halys*, *G. intermedius*, *Ovophis monticola*, *Daboia siamensis*, and even some rat snakes or elapid snakes with larger body size, such as *Orthriophis moellendorffi*, *Euprepiophis mandarinus*, *Ptyas mucosus*, and *Naja atra* (Su et al., 2016). It is especially difficult for the enforcement personnel to identify *D. acutus* once the snakes have been eviscerated and dried, and the spots and color pattern on the skin have disapeared. An accurate and fast on spot identification of species would provide the indispensable technical support for *D. acutus* conservation.

Conventionally, the identification of *D. acutus* is largely based on morphology or histology, which rely heavily on the experience of examiners and the result is often subjective (He, 1991; Xu and Gao, 1992; Wang, 1995). In recent years, DNA–based identification methods such as species–specific PCR (Tang and Feng, 2006) and DNA barcoding (Liao et al., 2013) have been developed for its identification, and the Chinese Pharmacopoeia has documented the use of a highly specific PCR method (State Pharmacopoeia Committee, 2010). A recent article by Jiang et al. (2015) described a homogeneous fluorescent specific PCR method using cationic conjugated polymer for the identification of medicinal snakes including *D. acutus*. Nevertheless, all these molecular approaches for *D. acutus* identification do not allow efficient or convenient on–spot identification (Huang et al., 2014). Chen et al. (2014) recently reported a rapid PCR method to identify medicinal snake species, but this approach is still based on the classic PCR and requires the use of a PCR thermoclycler with high denaturation and annealing temperatures. Ideally, a rapid on–spot assay is expected to allow identification at room temperature within a short period of time and without requiring special equipment.

As one of the latest isothermal nucleic acid amplification techniques, recombinase polymerase amplification (RPA) shows much potential for application in on–spot identification of the species of medicinal snakes (Qin et al., 2017). RPA works with three core enzymes (a recombinase, a single–stranded DNA–binding protein (SSB) and a strand–displacing polymerase), and the reaction can take place over a wide range of ambient temperatures (Piepenburg et al., 2006). RPA shows some advantages over other isothermal nucleic acid amplification techniques (Liang et al., 2017). For example, loop–mediated isothermal amplification (LAMP) requires four primers aiming at six specific DNA binding sites in the target gene, so the primer design for LAMP is complicated; it is difficult to acquire species specific primers for differentiating close–related species with high sequence similarity (Notomi et al., 2000; Wu et al., 2016; Lee, 2017). In comparison, only one pair of primers is needed for RPA, and the design is almost as simple as conventional PCR. Like PCR amplification, RPA assay also has a high specificity, but it is much faster; in some cases, the result can be generated in 3–10 min (Daher et al., 2016). In addition, an RPA assay using a commercial kit does not require the use of an expensive thermocycler or any additional equipment or reagents (Fan et al., 2016). These advantages make RPA an ideal option for on–spot inspection in clinical diagnosis, laboratory medicine, forensic science, and the food industry (Gao et al., 2016; Ammour et al., 2017; Raja et al., 2017). Tian et al. (2017) have successfully extended its application to the field of medicinal plant identification.

To the best of our knowledge, no other report or publication has been available to describe the use of RPA for identification and conservation in threatened or endangered animal species. In this study, we established an RPA–based method combined with fast DNA extraction for on–spot *D. acutus* identification that can be completed in 30 min at 37 ºC.

Material and methods

Materials

Fourteen specimens representing 12 snake species, including three specimens of *Deinagkistrodon acutus* (A1–A3) and 11 specimens of other snake species (A4–A14) (table 1), were collected from the mountainous regions in Guangdong, Hunan, Jinagxi and Guangxi in South China with the approval of the National Natural Science Foundation of China (NSFC No. 81573540). Vouchers were identified by Dr. Liang Zhang from Guangdong Institute of Applied Biological Resources and deposited in School of Traditional Chinese Medicine, Southern Medical University (Guangzhou, China). The snakes were euthanized through rapid cooling and freezing (Lillywhite et al., 2017). All these specimens were preserved in 75% ethanol. In addition, 10 crude drug samples of QS (S1–S10) were purchased from local pharmacy or crude drug market in various cities of China (table 2). The TwistAmp® Basic RPA kit, a product of TwistDx™ Limited (Cambridge, UK), was used in this study.

RPA primer design

The species–specific RPA primers for *D. acutus* were designed with the aid of Primer Premier 5.0 software. The primers targeted at cytochrome C oxidase subunit I (COI) barcode region, which provides abundant variation sites to distinguish *D. acutus* from other snake species. We had sequenced the COI region of each specimen, except for that of *Daboia siamensis*, which was downloaded from GenBank. The accession numbers are listed in table 1. The primers were synthesized by Invitrogen Biotechnology (Shanghai) Co., Ltd.

DNA extraction

For each sample, about 30 mg of muscular tissue was dissected from the dorsal muscle. The total genomic DNA was extracted from 30 mg of tissue sample with a modified alkaline lysis method (Jiang et al., 2013). In brief, the homogenate tissues and 20 µL of extraction buffer (containing 0.5 mol/L NaOH, 1% polyvinyl polypyrrolidone, and 1% Triton X 100) were added to a 200–µL PCR tube and vortexed for 10–15 s, followed by incubation in a boiling water bath for 10–15 s; 80 *µ*L of Tris–HCl (0.1 mol/L, pH 8.0) was added to the cooled–down mixture, which was gently vortexed and then centrifuged for 5 min at 300xg. The supernatant containing the DNA was collected and the quality and concentration of the DNA solution were assessed using 1.5% agarose gel electrophoresis and a NanoDrop 1000 UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA), respectively.

Recombinase Polymerase Amplification (RPA)

Following the instruction manual of TwistAmp® Basic (TwistDx Ltd., Babraham, UK) kit, RPA was performed with a total reaction volume of 50 µL containing the forward and reverse RPA primers (10 µM, 2.4 µL each), rehydration buffer (29.5 µL), magnesium acetate (280 mM, 2.5 µL), genomic DNA

Table. 1. Information about the species and sites where snake samples were collected: No, sample number; Genbank No, Genbank accession number.

Tabla 1. Información sobre la especie y el lugar de recogida de las muestras de las serpientes: No, número de la muestra; Genbank No, numero de acceso de Genbank.

No	Species	Voucher	Site of collection	Genbank No.
A1	*Deinagkistrodon acutus*	TP–13	Hunan	JQ658433
A2	*D. acutus*	TP–8	Guangxi	JQ658432
A3	*D. acutus*	TP–7	Jiangxi	JQ658431
A4	*Protobothrops mucrosquamatus*	CH–1	Conghua, Guangdong	JX233625
A5	*Gloydius brevicaudus*	ZB–9	Yongzhou, Hunan	KC841453
A6	*Trimeresurus stejnegeri*	ZS–13	Zhongshan, Guangdong	JX233623
A7	*Daboia siamensis*	DS–1609	Zhongshan, Guangdong	KP772294
A8	*Naja atra*	FC–2	Taishan, Guangdong	JN833603
A9	*Bungarus fasciatus*	TP–1	Guangxi	JN833615
A10	*Orthriophis moellendorffi*	TP–9	Guangxi	JN833617
A11	*Ptyas dhumnades*	TP–11	Conghua, Guangdong	JX233651
A12	*Ptyas mucosa*	DS–11	Zhongshan, Guangdong	JX233645
A13	*Lycodon rufozonatus*	ZS–3	Zhongshan, Guangdong	JN833598
A14	*Lycodon ruhstrati*	HB–Lr–1109171	Conghua, Guangdong	JX233634

Table. 2. Details of the commercial Qi She crude drug samples identification: + positive amplification; – negative amplification.

Tabla 2. Información detallada sobre la identificación de las muestras comerciales de la sustancia sin elaborar Qi She: + amplificación positiva; – amplificación negativa.

Sample No.	Location of acquisition	RPA results	BOLD identification results
S1	Jian Qi Pharmacy	–	*Euprepiophis mandarinus* (= *Elaphe mandarinus*)
S2	Oriental Pharmacy	+	*Deinagkistrodon acutus*
S3	Min Xin Pharmacy	+	*Deinagkistrodon acutus*
S4	Bao Zhi Lin Pharmacy	–	*Euprepiophis mandarinus*
S5	Ping Shan Tang Pharmacy	–	*Naja kaouthia*
S6	Zhangshu Crude Drug Market	+	*Deinagkistrodon acutus*
S7	Qingping Crude Drug Market	–	*Lycodon rufozonatus*
S8	Ji Shi Tang Dispensary	+	*Deinagkistrodon acutus*
S9	Bozhou Crude Drug Market	–	*Daboia russelii*
S10	Changcheng Pharmacy	–	*Lycodon rufozonatus*

(3.2 µL), and ddH_2O (10.0 µL). With an initial incubation at 37º for 5 min, the reaction tube was taken out and shaken for 8–10 times at room temperature, followed by further incubation at 37º for 20 min. The RPA products were detected using 1.5 % agarose gel electrophoresis.

We tested different temperatures to optimize the amplification of the genomic DNA of a *D. acutus* sample (A1). Following the protocols for RPA reaction described above, temperature gradients of 37, 32, 27, and 22 ºC were tested for amplification for 20 min. The amplification time was optimized with a gradient of 10, 15, 20, 30, and 40 min at the optimal temperature using the same sample.

The specificity of RPA assay was evaluated using genomic DNA templates from *D. acutus* samples (A1–A3) and the other species (A4–A14). With the specific primers, the amplification protocols were identical to those described above. The primers were considered specific when the amplification signals were observed only in *D. acutus* samples but not in the related snake species.

For sensitivity assessment of the RPA assay, serially dilutions (100, 10, 1, 0.1, and 0.01 ng/µL) of the genomic DNA of *D. acutus* sample A1 were prepared to test the minimum detection limit of the assay with identical amplification protocols.

Identification of Qi She crude drug samples by RPA

Ten commercial QS crude drug samples (S1–S10; table 2) were collected from the pharmacy stores or crude drug market in Guangzhou, Xingning (Guangdong), Zhangshu (Jiangxi), and Bozhou (Anhui). RPA amplification was performed with the *D. acutus* –specific primers under the optimized temperature and reaction time.

To verify the accuracy of RPA assay, the zoological origin of the samples was identified by COI barcode. The genomic DNA from those samples was amplified with COI barcode universal primers LCO1490 and HCO2198 (Folmer et al., 1994) or primers for Viperidae species DK1–CO1 and DK1–CO2 (Li et al., 2015; Cai et al., 2016). The PCR reaction mixture consisted of 12.5 µL of 2× Tap PCR MasterMix (with dye) (Tiangen, Beijing), 1 µL of each primer (10 µM), 2 µL of DNA template and 8.5 µL of ddH_2O. Amplification was performed with an initial holding at 94 ºC for 5 min followed by 35 thermal cycles of 94 ºC for 30 s, 53 ºC for 1 min, and 72 ºC for 1 min, with a final extension at 72 ºC for 7 min. The PCR products were then sequenced. Species were identified using BOLD (Barcoding of Life Database) system V4.

Results

Template DNA quality

The concentrations of genomic DNA exracted from the samples were between 56 to 81 ng/µL, and the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) ranged from 1.69 to 2.01.

Design and specificity assessment of RPA primers

Successful specific amplification for *D. acutus* was obtained using the primer pair below:

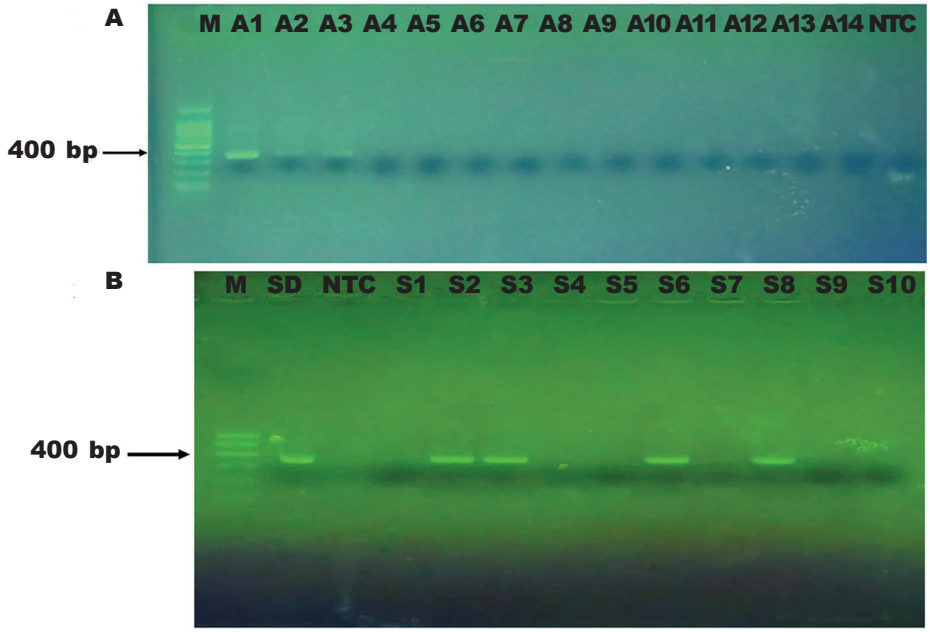

Fig. 1. Electrophoretogram of RPA products: A: RPA of *D. acutus* and related snake species; A1–A3, *D. acutus*; A4–A14, other snake species; NTC, negative control (distilled water); M, 100 bp DNA ladder. B: RPA identification of 10 commercial QS samples; S1–S10, QS samples; M, 100 bp DNA Ladder I; SD, positive control; NTC, blank control.

Fig. 1. Electroforetograma de los productos de la RPA: A: RPA de D. acutus *y las especies de serpiente relacionadas; A1–A3,* D. acutus*; A4–A14, otras especies de serpiente; NTC, control negativo (agua destilada); M, marcador de peso molecular de ADN de 100 pb. B: Identificación mediante RPA de 10 muestras comerciales de QS; S1–S10, muestras de QS; M, marcador de peso molecular de ADN de 100 pb; SD, control positivo; NTC, control.*

QSCOAII: 5'–TTACTCCTATTACTATCCTCCTCC-TACATC–3'

QSCOF1130A: 5'–GCCCCCTCCGCTCGGATCA-AAGAAGGTGGTGTTAAG–3'

The binding sites of the forward and reverse primers were located at 12–50 nt and 335–355 nt of the COI region, respectively. Under optimized RPA conditions, a distinct single band of 354 bp was amplified using the primer pair only for *D. acutus* but not for the other snake species (fig. 1A), demonstrating the high specificity of the primers.

RPA optimization and sensitivity

The results showed that the target fragment could be amplified at a temperature range of 27–42 °C, and the optimal temperature range was 37 to 42 °C (fig. 2A). From a practical point of view, we chose 37 °C as the optimal reaction temperature for convenience of on–spot RPA. At this temperature, the reaction time was optimized and the results showed that amplifications for 15 to 40 min all yielded single and clear bands, whereas a reaction time of 10 min resulted in a rather weak signal (fig. 2B). We thus chose 15 min as the optimal reaction time for RPA.

The sensitivity of RPA was assessed using the optimized reaction temperature and time setting. The target fragment could be successfully amplified from the serially diluted genomic DNA template with concentrations ranging from 100 to 0.1 ng/μL; the target fragment failed to be amplified when the template was further tenfold diluted to 0.01 ng/μL (fig. 2C). This result suggests that the lower detection limit of RPA was 0.1 ng/μL for *D. acutus* genomic DNA.

Identification of commercial QS crude drug samples

RPA of the 10 commercial QS samples with the optimized settings yielded a band of 354 bp for samples S2, S3, S6, and S8, and no amplification product was obtained for the other samples (table 2). These results suggested that samples S2, S3, S6, and S8 were authentic crude drug of *D. acutus*, while the others were the related species (fig. 1B).

DNA barcoding confirmed the results of diagnostic RPA with samples S2, S3, S6, and S8 derived from *D. acutus*. The identities of other samples were determined by DNA barcoding, and the results are listed in table 2.

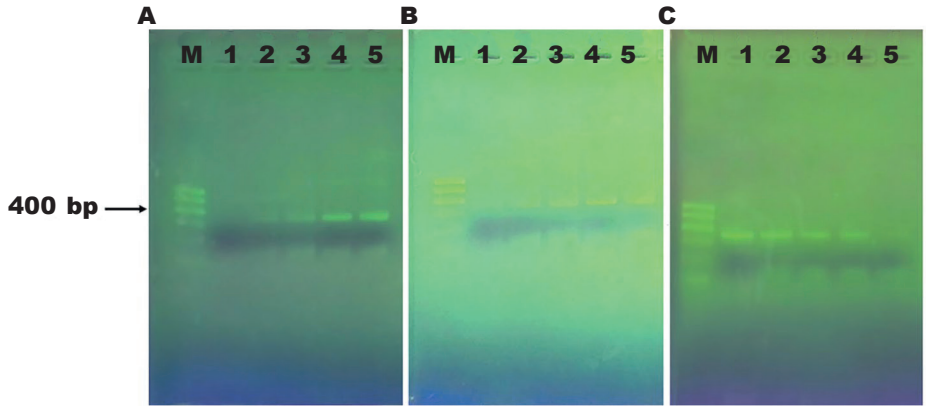

Fig. 2. Agarose gel electrophoresis of the RPA product for amplification of the COI region: A, RPA reaction temperature optimization (1, 22 °C; 2, 27 °C; 3, 32 °C; 4, 37 °C; 5, 42 °C; M, 100 bp DNA ladder). B, optimization of RPA reaction time (1, 10 min; 2, 15 min; 3, 20 min; 4, 30 min; 5, 40 min; M, 100 bp DNA ladder). C, sensitivity of RPA assay for serially diluted genomic DNA of *D. acutus*. (Lanes 1–5, genomic DNA of 100, 10, 1, 0.1, 0.01 ng/μL, respectively; M, 100 bp DNA ladder).

Fig. 2. Electroforesis en gel de agarosa del producto de la RPA para la amplificación de la región COI. A, optimización de la temperatura de reacción de la RPA (1, 22 ºC; 2, 27 ºC; 3, 32 ºC; 4, 37 ºC; 5, 42 ºC; M, marcador de peso molecular de ADN de 100 pb). B, optimización del tiempo de reacción de la RPA (1, 10 min; 2, 15 min; 3, 20 min; 4, 30 min; 5, 40 min; M, marcador de peso molecular de ADN de 100 pb). C, sensibilidad del análisis mediante RPA del ADN genómico diluido en serie de D. acutus*. (Carriles 1–5, ADN genómico de 100, 10, 1, 0,1 y 0,01 ng/μl, respectivamente; M, marcador de peso molecular de ADN de 100 pb).*

Discussion

Over–exploitation led by increasing demands for *D. acutus* as a medicinal product has imposed great pressure on survival of this species (Yin et al., 2015). In addition, habitat destruction has worsened its populations (Hu et al., 2013). In pace with its listing as a threatened species, more powerful and effective measures should be taken to ensure its conservation. Species identification is a prerequisite for the enforcement of conservation and effective trade monitoring on regulated or protected animals and plants (Palumbi and Cipriano, 1998; Hsieh et al., 2001; Chapman et al., 2003; Wei and Szmidt, 2013). A rapid and accurate identification method will serve as a technical aid to help conserve *D. acutus* (Huang et al., 2014).

In this study, we established an RPA system combined with fast DNA extraction for rapid on–spot identification of the highly valued crude drug Qi She, the most common *D. acutus* product. The amplification results of the genomic DNA from *D. acutus* and other snake species showed high species specificity of the designed primers (fig. 2A). The optimal reaction temperature for RPA was 37 ºC, but successful amplification also occurred at a temperature as low as 27 ºC when the reaction time was extended, suggesting that an RPA assay could be conducted at room temperature or using only a heater when the environmental temperature is below 27 ºC. A visible target band could be generated after a reaction time of 10 min, and amplification for 15 min resulted in a clearer band for effective detection. As the entire amplification can be completed in 15 min at room temperature without using any sophisticated instrument, this RPA assay provides an efficient and highly specific means for on–spot QS crude drug identification.

The manufacturer of the commercial RPA kit (TwistDx) provides three options for detection using agarose gel electrophoresis (TwistAmp® Basic), real–time fluorescent probes (TwistAmp® exo), or lateral flow strips (TwistAmp® nfo) (Piepenburg et al., 2013). Real–time fluorescent detection requires a probe and a real–time fluorescence analyzer as high–resolution melting (HRM) analysis does (Maslin et al., 2015; Wei et al., 2017). Lateral flow detection requires additional lateral flow strips, which increase the cost of the assay. The low cost and easy accessibility of gel electrophoresis is more appropriate for on–spot detection.

Jiang et al. (2014) reported a rapid PCR identification of Flos Lonicerae (flower bud of *Lonicera japonica*), in which fluorescent dye SYBR Green I was used to detect the amplified product. They added SYBR Green I dye after PCR amplification, and the genuine crude drugs generated a positive amplification signal by emitting green fluorescence when irradiated with 365 nm UV light, while the fake drugs did not produce any fluorescence due to non–amplification. Detection with the fluorescent dye was simple and fast, but our attempts at using SYBR Green I all failed, that is, of

all the QS samples, either authentic or fake, the RPA products gave strong fluorescence signals. This might be attributed to the formation of dimers between the long RPA primers, which could be stained by the highly sensitive and nonspecific fluorescent dye (Li et al., 2019, 2020). In contrast, gel electrophoresis detection does not suffer so much from dimers.

A practical on–spot molecular identification method for crude drugs also needs to consider the time spent in DNA extraction. Conventional DNA extraction involves a complex procedure that includes several rounds of precipitation and centrifugation, which can be time–consuming. DNA extraction can take more than four hours with the CTAB method (Cota–Sánchez et al., 2006) and the SDS method (Yang et al., 2008), and 1–2 h with the commonly used silica gel column method (Abdel–Latif and Osman, 2017). Chelex®100 resin–based extraction is a simple and rapid method for genomic DNA extraction from animal tissues. It can be performed in 30 min–1 hour, and shows excellent performance in difficult samples such as forensic samples (Li et al., 2019; Simon et al., 2020). However, alkaline lysis has also proved to be a quick and inexpensive single–tube method for extracting DNA (Klintschar and Neuhuber, 2000). The protocol developed in recent years contains only two steps: lysis and neutralization, and it can be completed in about five minutes (Jiang et al., 2013; Chen et al., 2014). For this reason we used the alkaline lysis method in this study for efficient DNA extraction. The extracted genomic DNA had a concentration above 50 ng/μL with the A260/A280 values ranging from 1.69–2.01, suggesting that both the DNA quantity and quality met RPA requirements (Lucena–Aguilar et al., 2016).

We tested 10 commercial QS crude drug samples following the established on–spot identification assay system. The results were confirmed by COI barcode identification, suggesting the validity of this RPA–based method for QS identification. The successful identification of commodities on the market suggests that the RPA method can be a practical means to combat illegal trade and to strengthen the protection and sustainable utilization of *D. acutus* (Liu and Ding, 2019).

Conclusions

In conclusion, we established an efficient on–spot RPA assay coupled with rapid DNA extraction for QS identification. With a good detection specificity and sensitivity, this assay allows on–spot QS identification within 30 min at room temperature, and is thus a potential powerful means for *D. acutus* conservation and utilization.

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