Bar-cas12a, a Novel and Rapid Method for Plant Species Authentication: A Case of *Phyllanthus* Species

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Abstract

The rapid and accurate species diagnosis accelerates the performance to investigate various biology fields and its relevant, perhaps but morphology-based species taxonomy/identification hamper. DNA barcodes (Bar) has been employed extensively for plant species identification. Recently, CRISPR-cas system can be applied for diagnostic tool to detect pathogen's DNA based on the collateral activity of cas12a or cas13. Here, we developed barcode-hyphenated with cas12a assay, "Bar-cas12a" for species authentication using *Phyllanthus amarus* as a model. The gRNAs were designed from *trnL* region, namely gRNA-A and gRNA-B. As a result, gRNA-A was highly specific to *P. amarus* amplified by RPA in contrast to gRNA-B even in contaminated condition. Apart from the large variation of gRNA-A binding in DNA target, cas12a- specific PAM's gRNA-A as TTTN can be found only in *P. amarus*. PAM site may be recognized one of the potential regions for increasing specificity to authenticate species. In addition, the sensitivity of Bar-cas12a using both gRNAs gave the same detection limit at 0.8 fg and it was 1,000 times more sensitive compared to agarose gel electrophoresis. Overall, Bar-cas12a using *trnL*-designed gRNA offer a highly specific, sensitive, speed, and simple approach for plant species authentication and is likely to implement point-of-care testing.

Introduction

Species authentication/discrimination is an essential task in various areas in biology systematics, ecology, evolution, forensics, food science, medical as well as even herbal and cosmetic industries, leading to correct species exploitation regarding their purposes¹⁻⁸. Traditional species taxonomy has been performed using the external morphological features or microanatomy which tightly requires the complete flower features or complete significant characteristics for species identification by an expert⁹. Perhaps, in several areas, the specimens obtained have been incomplete forms, immature stage, or modified/processed samples without key characters to identify, contributing to difficulty/impossibility in species identification and hampering the advance of investigation or research⁹. In several decades ago, advanced molecular approaches e.g., hybridization, DNA fingerprint, DNA barcodes, high resolution melting (HRM) have been used widely and extensively for facilitating species authentication in various organisms^{2-6, 10-16}. Certainly, these molecular approaches enable species identification despite the specimens with completely damaged but DNA existing, especially DNA barcodes (Bar) which there are many regions exhibiting a successful species discrimination for plant species (e.g., *rbcl*, *matK*, *trnL*, and ITS)¹⁵⁻¹⁶. However, they are relatively complex, time and cost-consuming because they require expensive equipment (e.g., thermal cycler, realtime PCR, sequencer machine).

Currently, nucleic acid isothermal amplification (e.g., RCA, LAMP and RPA) has been emerging and gaining attention for RNA/DNA amplification, in particular pathogen detection as they require only heat box or water bath, leading to adaptation for point-of-care testing¹⁷⁻¹⁹. RPA is one of isothermal amplification based on enzymatic activities relating to DNA replication process and the reaction can be

performed at constant temperature in range of 30–45°C for DNA amplification (optimal temperature at 37°C)^{18–19}, mycoplasma²⁰, and virus/viroid RNA in plant²¹.

Recently, CRISPR-cas systems exhibited the high potential for genome editing with accuracy and precise in the specific DNA target and included the adaptation for pathogen diagnostic with high sensitivity, specificity, simplicity, and speed, for instances, HPV-16 and 18^{22–23} and shrimp pathogens e.g., white spot syndrome virus (WSSV)²⁴. The cas12a can be applied as diagnostic tool because it has the collateral activity or trans-activity for cleavage of non-target single stranded (ss) DNA once forming a tertiary complex (cas12a-gRNA-target)²⁵. ssDNA is designed as reporter based on fluorescence resonance energy transfer (FRET) between fluorescence and its quencher or antigen-antibody interaction by lateral flow dipstick readout^{22–24, 26}.

Herein, we would like to establish a novel method for plant species authentication using the hyphenation of plant DNA barcode, *trnL* and cas12a, namely “Bar-cas12a”. In this study, *Phyllanthus* species including *Phyllanthus amarus*, *Phyllanthus urinaria*, *Phyllanthus debilis*, *Phyllanthus virgatus*, were used as a model to validate Bar-cas12a for species authentication of *P. amarus* because they have similar morphological features and have been used as herbal commercialized products.

Results

Condition optimization of cas12a assay

In this study, we presented two gRNAs designed from *trnL* region which were specific to *P. amarus*. The gRNA-A were designed in the opposite direction to gRNA-B and the variation nucleotide in binding site of gRNA-A was more diverse than that of gRNA-B which existed only single point (Fig. 1A). Both of two gRNAs for cas12a assay were successfully produced by *in vitro* transcript which duplex DNAs for two gRNAs were used as the templates for gRNA synthesis as depicted in Fig. 1A and 1B. The scheme illustration for the principle of *in vitro* digestion of cas12a were shown in Fig. 1C. In addition, the concentration of cas12a and gRNA gave the highest fluorescence from the cleavage of ssDNA reporter at 37°C for an hour was at 100 nM : 100 nM whereas there was no fluorescence in control (without DNA target) (Fig. 1D).

Species authentication performance of Bar-cas12a

To evaluate the performance of Bar-cas12a assay for species authentication of *P. amarus*. For specificity determination, *trnL* region of different four *Phyllanthus* species were amplified by RPA using modified universal *trnL* primer, yielding about 600 bp (Fig. 2A) and RPA products of four species were used for cas12a using gRNA-A and gRNA-B. Our results displayed that gRNA-A gave the fluorescence signal with specific PA while gRNA-B was positive fluorescence signal for all species tested (Fig. 2B). In addition, the sensitivity assay was done using the different starting amount of DNA's *P. amarus* for DNA amplification by RPA in range of 0–80 ng. For RPA amplification under agarose gel electrophoresis, we found that the

limit of detection (LOD) was at 0.8 ng which gave DNA band as depicted in Fig. 2C-D. Meanwhile Bar-cas12a using gRNA-A and gRNA-B was observed for positive fluorescence signal in range of 0.8 pg – 80 ng for both gRNAs, indicating significantly more sensitivity than the agarose gel electrophoresis-visualized RPA assay (Fig. 2C-D).

In addition, species authentication by Bar-cas12a was validated using the admixture between *P. amarus* and *P. urinaria* with different amount of DNA proportion. Indeed, all admixtures gave the positive for DNA amplification by RPA (Fig. 3A). However, Bar-cas12a with gRNA-A demonstrated greater capability of species authentication with high specificity than gRNA-B because Bar-cas12a with gRNA-B produced positive results (100%) for *P. urinaria* but not with gRNA-A (Fig. 3B-E). Moreover, we found that Bar-cas12a using gRNA-A produced positive results even with a relatively small amount of *P. amarus* DNA (only 2%) in the admixture condition (Fig. 3B-C).

Discussion

CRISPR-cas system has not been only accomplished for genome editing in the certain target for various organisms but it can also be adapted for efficient diagnostic tool with high sensitivity and specificity to detect the pathogens^{20–24, 26}. In addition, this approach offers high potential for applying point-of-care testing as of short-time process without sophisticated equipment^{20–24, 26}. This was the first report to apparently exhibit the feasibility of hyphenating DNA barcode and cas12a assay to authenticate plant species as *P. amarus*. Our significant findings demonstrated that Bar-cas12a using gRNA-A of *trnL* barcode based on RPA enabled to specifically authenticate *P. amarus* with high sensitivity of LOD at 0.8 fg which was three orders of magnitude more sensitive than RPA visualized by agarose gel electrophoresis. Additionally, Bar-cas12a using gRNA-A enables species authentication of *P. amarus* even after contaminated with *P. urinaria*.

In the present study, gRNA-A and gRNA-B were designed based on the *trnL* sequences derived from the different four species including *P. amarus*, *P. urinaria*, *P. debilis* and *P. virgatus* because of the high variation sequences^{27–28}. Although all of them share common morphological features, *P. amarus* is a single species existing bioactive compound of phyllanthin and hypophyllanthin responsible for hepatoprotection²⁹. *P. amarus* has been commercialized as various product forms e.g., tea infusion, capsule, and tablets⁶. Thus, *P. amarus* was employed as a plant models to authenticate by Bar-cas12a assay. For cas12a condition, the sufficient and suitable concentration ratio of cas12a and gRNA to form binary complex and trigger the activity for positive fluorescence within an hour was 100 nM: 100 nM. Several reports revealed that the optimal concentration between cas12a and gRNA for detecting the target were used in various concentrations and ratios from 200 nM : 500 nM²⁰ to the least at 30 nM : 36 nM²².

The specificity and sensitivity of Bar-cas12a using either gRNA-A or gRNA-B for *P. amarus* authentication were determined. Obviously, the specificity of gRNA-A was the *P. amarus*- specific marker due to other species without the positive fluorescence whereas gRNA-B gave the positive for all species. Furthermore,

we verified the species authentication ability of Bar-cas12a in contaminated conditions with unwanted species by admixing different amounts of *P. amarus* and *P. urinaria* DNA. Our findings demonstrated that Bar-cas12a using gRNA-A is highly species-specific to *P. amarus* rather than using gRNA-B. With these regards, it was unsurprising that Bar-cas12a using gRNA-A have more specificity than gRNA-B as the gRNA-A was designed based on PAM site of cas12a (TTTG) in front of the DNA target which it existed only in *P. amarus* while the other species without PAM sites because they are TCTG. In contrast, gRNA-B contain only a single variable site although a variable site (A > G) was in the seed regions (1–5 first base next to PAM site)²⁵. This might suggest that the presence of a single variable site at seed region may be insufficient to distinguish the different nucleotides or SNP within DNA target. The variation of PAM site might be one of the efficient targets to discriminate difference in nucleotide for SNP genotyping or species discrimination/authentication. For sensitivity assay, Bar-cas12a using both gRNA-A and gRNA-B provide the LOD at 0.8 fg which was three orders of magnitude more sensitive than agarose gel electrophoresis-visualized RPA. This indicated that the hyphenation of cas12a enable to increase the sensitivity via the signal amplification of DNase activity of cas12a triggered by specific DNA target. Cas12a coupled with nucleic acid amplification such as LAMP or RPA has been achieved for detecting plant RNA viruses²¹, HPV16 and HPV18^{22–23} and bacterial contamination in food³⁰, with high specificity and sensitivity.

Here, we describe the feasibility of implementing cas12a combined with isothermal amplification to facilitate species authentication of *P. amarus*. However, this approach still having inherent limitation for being highly specific, sensitive, speed and simple tool for diagnostics is that it requires an amplification process to increase a large amount of DNA target to activate the collateral activity of cas12a. Given that direct DNA extracts can be used as template to be performed by cas12a without DNA amplification, we strongly believe that the approach would be near the ideal method for rapidity. Hence, we purpose a concept to reduce DNA amplification step by use of the multiple gRNAs to bind in different but specific DNA target, contributing to increasing amount of activated cas12a in the reaction.

In summary, our findings demonstrated that Bar-cas12a serve as immensely promising tool with highly specificity, sensitivity, speed, and simplicity for species discrimination/authentication in plant species especially in genus *Phyllanthus*. We proposed that this approach is a new shed of light in accommodating species discrimination/authentication for point-of-care testing which make us identify or distinguish plant species/commercial product in fields without the sophisticated equipment in two hours.

Methods

Specimens and DNA extraction

Phyllanthus species including *P. amarus*, *P. urinaria*, *P. debilis* and *P. virgatus* were collected around Naresuan University, Phitsanulok, Thailand and these species were identified through a key from Flora of Thailand *Euphorbiaceae* (<http://www.nationaalherbarium.nl/ThaiEuph/ThPspecies/ThPhyllanthusT.htm>). The experiment and

collection of plants complied with guidelines of Department of Biology, Faculty of Sciences, Naresuan University. In this study, *P. amarus* was used a plant model to authenticate by cas12a assay because it has been extensively used for medical purpose. Leaves were used for DNA extraction by Genomic DNA isolation kit (PureDireX, Taiwan). The quality and quantity of DNA obtained were measured by Nanodrop (Thermo Scientific, USA) and 1% agarose gel electrophoresis. DNA were diluted as 20 ng/ul and stored at -20°C for further use.

Design and synthesis of guide RNA for cas12a

To generate suitable gRNAs for cas12a assay, *trnL* region of the four species was conducted for multiple alignment by MEGA X. There were two significant points as guideline for gRNA design to species differentiation: (i) searching for protospacer adjacent motif (TTTV (V = A, G or C)) and (ii) the sequences for DNA targets having variation among four species in the seed sequences (1–5 first bases next to PAM)²⁵, given as gRNA-A and gRNA-B for specific *P. amarus* (Fig. 1A). The synthesis of gRNA was done by *in vitro* transcription (IVT) under double stranded (ds) DNA as a template. The dsDNA was constructed and synthesized from Integrated DNA technologies (IDT, USA) which consisted of three parts as (i) T7 promoter regions, (ii) tracrRNA to incorporate with cas12a to form binary complex and (iii) crRNA to bind with DNA target, forming a tertiary complex. These dsDNAs were used as template for RNA synthesis via *in vitro* transcription (IVT) by HiScribe™ T7 Quick (#E2050S, NEB, US). The synthetic gRNAs were purified to remove the impurities by the Monarch RNA Cleanup Kit (50 µg) (NEB, US). The synthetic gRNA products were measured for amount and purity by Nanodrop and 2% agarose gel electrophoresis and then adjusted for concentration as 10 µM for further study.

In vitro cas12a assay

In this experiment, the concentration ratio of cas12a or cpf1 (#M0653T, NEB, US) and gRNA were varied to find out the suitable condition of *in vitro* digestion of cas12a. The ratio of cas12a and gRNA was constantly done at 1 : 1 but the concentrations were varied from 12.5 nM : 12.5 nM to 100 nM : 100 nM. Firstly, the binary complex between cas12a (cpf1) and gRNA was formed under admixture of 1X 2.1 NEB buffer, 100 nM cpf1, 100 nM gRNA-A or -B and then incubated at 37°C for 10 minutes. Subsequently, 1 µl of 50 µM single stranded DNA reporter (ssDNA reporter) (FAM/TTATT/3IABkFQ) and 5 µl of DNA targets (RPA products) were added. Finally, the nuclease-free water was added to 24 µl and incubated at 37°C for an hour. The cleavage of ssDNA report was determined under LED transilluminator to visualize the florescence signal by visible eye.

Specificity and sensitivity determination of Bar-cas12a assay

To assess the specificity of cas12a assay for species authentication of *P. amarus*, gRNA-A and gRNA-B were compared by PCR products which were amplified from DNA's different *Phyllanthus* species including *P. amarus*, *P. urinaria*, *P. debilis* and *P. virgatus*. DNA amplification of these species were

performed by RPA. A reaction of 25- μ l volume were consisted of 1X reaction buffer, 1X probe E-mix, 1.8 mM, 0.48 μ M forward primer (CGAAATCGGTAGACGCTACG), 0.48 μ M reverse primer (GGGGATAGAGGGACTTGAAC), 1X core reaction mix, 20 ng of DNA template or plant extract and 14 mM magnesium acetate on the lid of PCR tube and added nuclease free water to 25 μ l. The reaction was performed under heating box at 37°C for 40 minutes. The RPA products were checked by 1.5% agarose gel electrophoresis. Afterward, RPA products of different species amplified were used as DNA targets for assessing the specificity of Bar-cas12 assay using gRNA-A or gRNA-B.

To evaluate the sensitivity of Bar-cas12a assay using gRNA-A and gRNA-B, the diluted DNA's *P. amarus* were varied for 0.8 fg to 80 ng to amplify by RPA. Subsequently, RPA products were detected for *P. amarus* by Bar-cas12a. The fluorescence which indicates the presence of DNA target was recorded every minute for 2 hours of incubation under realtime PCR and after completing the reaction of cas12a assay, the tubes was determined under LED transilluminator to visualize the florescence signal by visible eyes.

Species authentication by Bar-cas12a

To test the species authentication performance of Bar-cas12a assay using gRNA-A and gRNA-B, the admixture between two species of *P. amarus* and *P. urinaria* was done in different amount percentage proportion, given as 100%:0%, 50%:50%, 25%:75%, 10%:90%, 2%:98% and 0%:100%, respectively. The initial DNA concentration of each species used to create an admixture was 10 ng/ μ l. Subsequently, these admixtures were employed to authenticate *P. amarus* by the Bar-cas12a using gRNA-A and gRNA-B with realtime PCR to acquire the fluorescence signal over time, and then PCR tubes were observed under LED transilluminator.

Declarations

Author contributions

Conceptualization, K.B.; Methodology, K.B., W.P. and S.C.; Sample preparation and Experimental work, S.P., N.S.; Figures, K.B., S.P., and N.S.; Results analysis, Writing Manuscript, K.B., Y.M. and S.C.; Review, all authors; and Project administration, K.B. and S.C.

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Figures

Figure 1

The preparation of cas12a condition for species authentication. (A) gRNA-A and gRNA-B design based on the multiple alignment of trnL loci and the construction of double stranded DNA as templates for gRNA synthesis. (B) The synthesis of gRNA-A and gRNA-B by in vitro transcription with T7 RNA polymerase and the synthesized gRNAs were detected by agarose gel electrophoresis. (C) The schematic illustrates the mechanism of cas12a to form binary and tertiary complex to cleave the reporter single stranded DNA as result of the fluorescence signal. (D) The condition optimization of in vitro digestion of cas12a by varying the concentration ratio of cas12a and gRNA at 1: 1 to produce the fluorescence at 37°C for an hour. Phyllanthus species were studied including Phyllanthus amarus (PA), Phyllanthus urinaria (PU), Phyllanthus debilis (PD), Phyllanthus virgatus (PV). Raw figure of agarose gel electrophoresis was in Figure S1.

Figure 2

Specificity and sensitivity of Bar-cas12a for *P. amarus* authentication. (A) DNA amplification for the four species including *P. amarus* (PA), *P. urinaria* (PU), *P. debilis* (PD) and *P. virgatus* (PV) by RPA using universal trnL primer. (B) Specificity test by Bar-cas12a using gRNA-A and gRNA-B. (C-D) Sensitivity test by PCR and Bar-cas12a using gRNA-A and gRNA-B and the fluorescence signal were monitored for cleavage of ssDNA reporters by realtime PCR within two hours. Raw figures of agarose gel electrophoresis for specificity and sensitivity were in Figure S2 and S3, respectively.

Figure 3

Species authentication by Bar-cas12a in admixture samples. (A) DNA amplification of DNA admixture in different proportions by RPA using trnL loci. DNA admixture between *P. amarus* (PA) and *P. urinaria* (PU) were done in different amount proportions as 100%, 50%, 25%, 10%, 5%, 2% and 0% of PA contaminated with PU which stock concentration of each species used was 10 ng/μl. (B,D) In vitro digestion of cas12a using gRNA A and gRNA B and observed under LED transilluminator. (C,E) the acquisition of fluorescence signal by realtime PCR over two hours. Raw figure of agarose gel electrophoresis was in Figure S4.

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