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Convenient, Sensitive and High-Throughput Method for Screening Botanic Origin

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In this work, a rapid (within 4–5 h), sensitive and visible new method for assessing botanic origin is developed by combining loop-mediated isothermal amplification with cationic conjugated polymers. The two Chinese medicinal materials (Jin-Yin-Hua and Shan-Yin-Hua) with similar morphology and chemical composition were clearly distinguished by gene SNP genotyping assays. The identification of plant species in Patented Chinese drugs containing *Lonicera* buds is successfully performed using this detection system. The method is also robust enough to be used in high-throughput screening. This new method is very helpful to identify herbal materials, and is beneficial for detecting safety and quality of botanic products.

An increasing number of functional foods, medicines, and cosmetics containing botanical components have been gaining popularity worldwide. However, fresh and dried organs of some plants exhibit similar morphology, making them difficult to distinguish and creating safety issues with their use. It can be even more difficult to know with certainty the botanical origin of processed powders and other finished products. For example, components of two Chinese medicines, Jin-Yin-Hua and Shan-Yin-Hua, belong to the genus *Lonicera*. Jin-Yin-Hua includes *Lonicera japonica* buds, while Shan-Yin-Hua includes buds of *L. hypoglauca*, *L. macranthoides*, *L. confusa*, and *L. fulvotomentosa*¹.

Because it is a highly important antiviral and antibacterial herbal medicine, the price of Jin-Yin-Hua is much higher than that of Shan-Yin-Hua. The two materials have similar morphology and chemical composition but different medicinal usages; thus, correct identification of plant species is extremely important. Traditional methods for identifying plant components include high-performance liquid chromatography (HPLC)², gas chromatograph-mass spectrometry (GC-MS)³, and thin-layer chromatography (TLC)⁴. However, very slight differences in chemical compounds have been identified in relative species, and chemical composition is also affected by environmental conditions, plant development and processing⁵.

To improve the precision of identifying components of interest, genetic techniques, such as polymerase chain reaction (PCR) for genotyping single-nucleotide polymorphisms (SNP)^{6–10} have been used to differentiate plants at the species level^{11–15}. Using sequencing analysis, we previously identified a C/A SNP site in *trnL-trnF* between Jin-Yin-Hua and Shan-Yin-Hua and we developed an allele-specific PCR (AS-PCR) system for identifying the two medicinal materials¹⁶. Since only trace amounts of botanical materials are included in processed products, a simple, rapid, and sensitive method for identifying these materials is required to reach the extent of visual resolution.

In recent years, we have taken advantage of the excellent light-harvesting ability of conjugated polymers to enhance the sensitivity of DNA detection^{17,18}. Electrostatic complexes of cationic conjugated polymers with DNA have been designed for homogeneous, sensitive, and simple fluorescence assays for SNP genotyping^{19–22}. Furthermore, loop-mediated isothermal amplification (LAMP), which operates at a consistent temperature and only use a heating equipment, has been described as an easy and rapid tool for amplification of target DNA^{23–26}. Here, we demonstrate a simple, rapid, sensitive, and high-throughput system that combines LAMP with cationic conjugated polymers for screening botanical origin in multi-color and one-tube SNP genotyping assays. The identification of plant species in patented Chinese drugs containing *Lonicera* buds is successfully performed using this detection system.



Results

Figure 1 illustrates the principles for screening botanical origin. A sequence in *Lonicera trnL-trnF* that contains a polymorphic site is used as a DNA target in which nucleotide C in the wild type (Jin-Yin-Hua) is replaced by nucleotide A in the mutant target (Shan-Yin-Hua). Three SNP genotypes are thus possible: homozygous C (Jin-Yin-Hua), homozygous A (Shan-Yin-Hua), and heterozygous C/A (mixture). Two pairs of LAMP primers (F3/B3, FIP/BIP) and Bst DNA polymerase were used for amplification of the target DNA. A water-soluble cationic conjugated polymer, poly[(9,9-bis(6'-N,N,N-triethylammonium) hexyl) fluorenylene phenylene] (PFP), was used as a donor in fluorescence resonance energy transfer (FRET) experiments. Fluorescein-labeled ddUTP (ddUTP-FI) and cyanine 3 (Cy3)-labeled deoxyguanosine triphosphate (dGTP) were chosen as acceptors. PFP acts as the donor for fluorescein and Cy3; fluorescein acts as the acceptor for PFP and the donor for Cy3 to satisfy the overlap integral requirement for FRET. The primer is complementary to the wild target and to the mutant target immediately upstream of the polymorphic site. Taq DNA polymerase, ddUTP-FI, and dGTP-Cy3 were used for primer-extension reactions. For the homozygous C genotype, only dGTP-Cy3 was incorporated into the primer; upon addition of PFP, strong electrostatic interactions caused attraction between negatively charged DNA and cationic PFP, and efficient FRET from PFP to Cy3 occurred. For the homozygous A genotype, only ddUTP-FI was incorporated into the primer, and efficient FRET occurred from PFP to FI. For the heterozygous C/A genotype, ddUTP-FI and dGTP-Cy3 were incorporated into the primers for the mutant and wild targets, respectively. In this case, upon exciting PFP at 380 nm, multi-step FRET occurred.

Decisions of the origin of Jin-Yin-Hua and Shan-Yin-Hua was performed using LAMP and CCP-based method; the three genotypes could be clearly distinguished by different colors. The maximum emission of PFP in buffer solution appeared at approximately 425 nm, and no emission of fluorescein at 530 nm or of Cy3 at 574 nm was observed (Figure 2a and 2b). For homozygous A (Shan-Yin-Hua), efficient FRET from PFP to fluorescein led to a

significant quenching of PFP emission at 425 nm and appearance of the fluorescein emission at 530 nm (Figure 2c). Images of the extension products with PFP dropped on a glass slide under 300 nm UV light with transmission mode are presented in Figure 2d. A band-pass optical filter was used to filter the emission of the excitation source and PFP. The solution exhibited a green emission color (Figure 2a and 2d). Emission of Cy3 was observed for homozygous C (Jin-Yin-Hua) and the solution emitted a purplish red color (Figure 2d). Emission of both fluorescein and Cy3 was observed for heterozygous C/A (mixture), and this solution was pinky-white in color (Figure 2b and 2d).

Three shold values used to estimate botanical origin were plotted according to Equation (1):

$$RC_{\text{FRET}} = \text{FRET}/\text{FRET}_0 \quad (1)$$

Where RC_{FRET} represents the relative change of FRET ratio; and “FRET₀” and “FRET” are the initial and target-added ratios of $I_{574 \text{ nm}}/I_{425 \text{ nm}}$ or $I_{530 \text{ nm}}/I_{425 \text{ nm}}$, respectively. Threshold values of Jin-Yin-Hua were determined as $RC_{\text{FRET}} (I_{530 \text{ nm}}/I_{425 \text{ nm}}) \leq 2.3$, and $RC_{\text{FRET}} (I_{574 \text{ nm}}/I_{425 \text{ nm}}) \geq 3.5$ (Figure 3a and 3b). All (192/192) of Shan-Yin-Hua and 92.7% (47/48) of the mixture were separated from Jin-Yin-Hua.

To investigate the dynamic range of the target concentration, the emission spectra of a series of extension products containing various concentrations of heterozygous C/A (equal DNA concentration of Jin-Yin-Hua and Shan-Yin-Hua) were measured with excitation wavelength 380 nm after adding PFP (Figure 3c). The dynamic range at which the mixed DNA was separate from the DNA of Jin-Yin-Hua was 0.05 to 50 ng μl^{-1} (Figure 3d). To further investigate the dynamic range of the mixture, the emission spectra of a series of extension products containing various percentages of Jin-Yin-Hua and Shan-Yin-Hua were measured at excitation wavelength 380 nm after adding PFP (Figure 4a, Figure S4). The limit of detection (LOD) of this method was obtained from seven independent measurements using Equation (2):

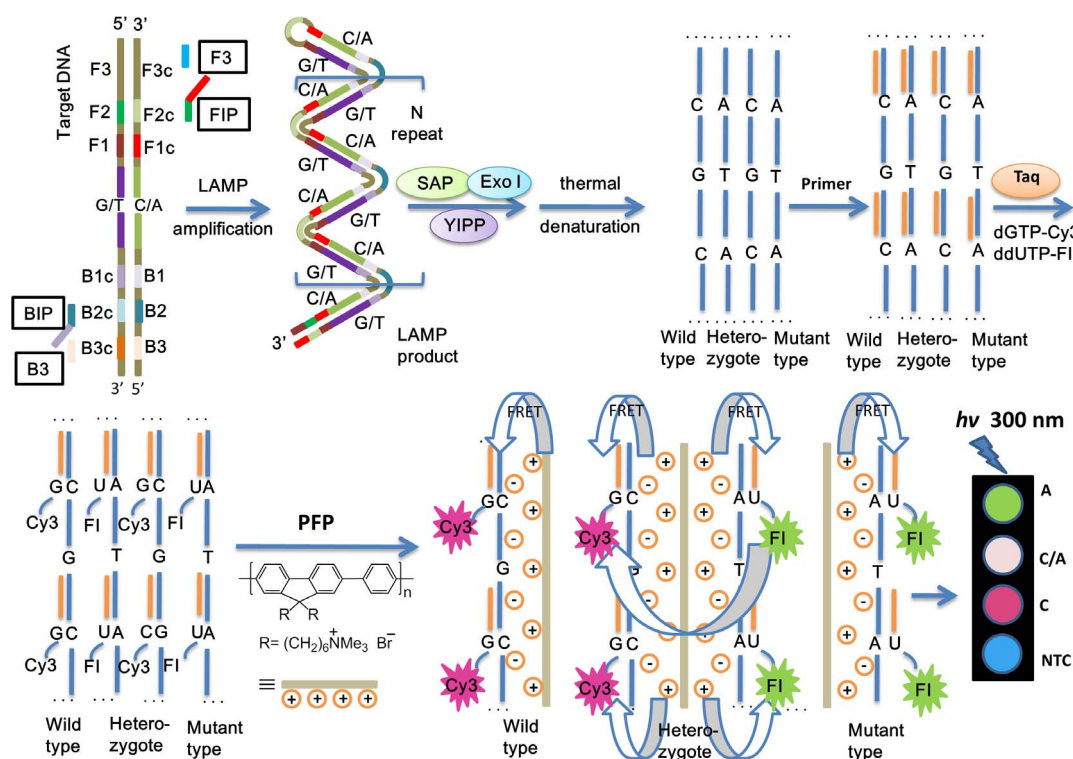


Figure 1 | Schematic principle of conjugated polymer-based assay method for screening botanical origin.

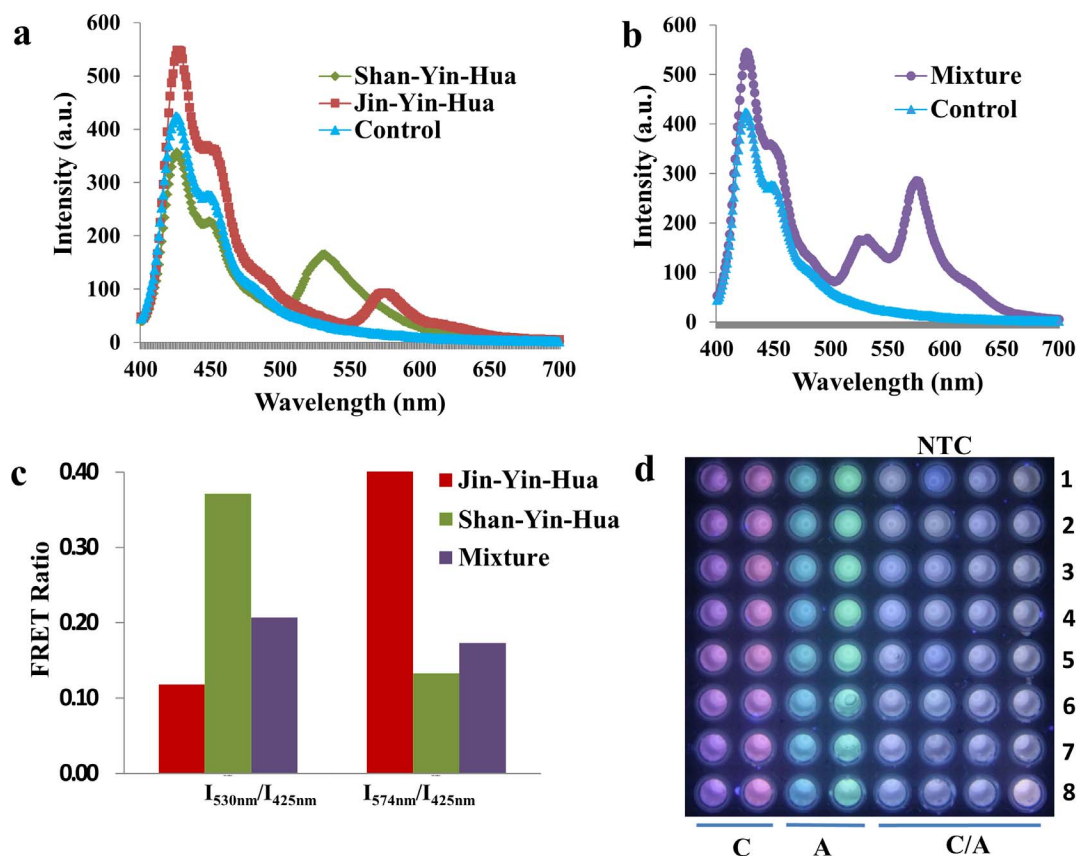


Figure 2 | (a) Emission spectra of PFP in the presence of Jin-Yin-Hua and Shan-Yin-Hua. (b) Emission spectra of PFP in mixture. (c) Integrated emission intensities. (d) A photograph of fluorescence pattern on a microplate corresponding to Jin-Yin-Hua, Shan-Yin-Hua and their mixture. The solutions contain PFP and extension products of homozygous C (Jin-Yin-Hua), heterozygous C/A (Mixture) and homozygous A (Shan-Yin-Hua). A no-template control (NTC) was used as the blank.

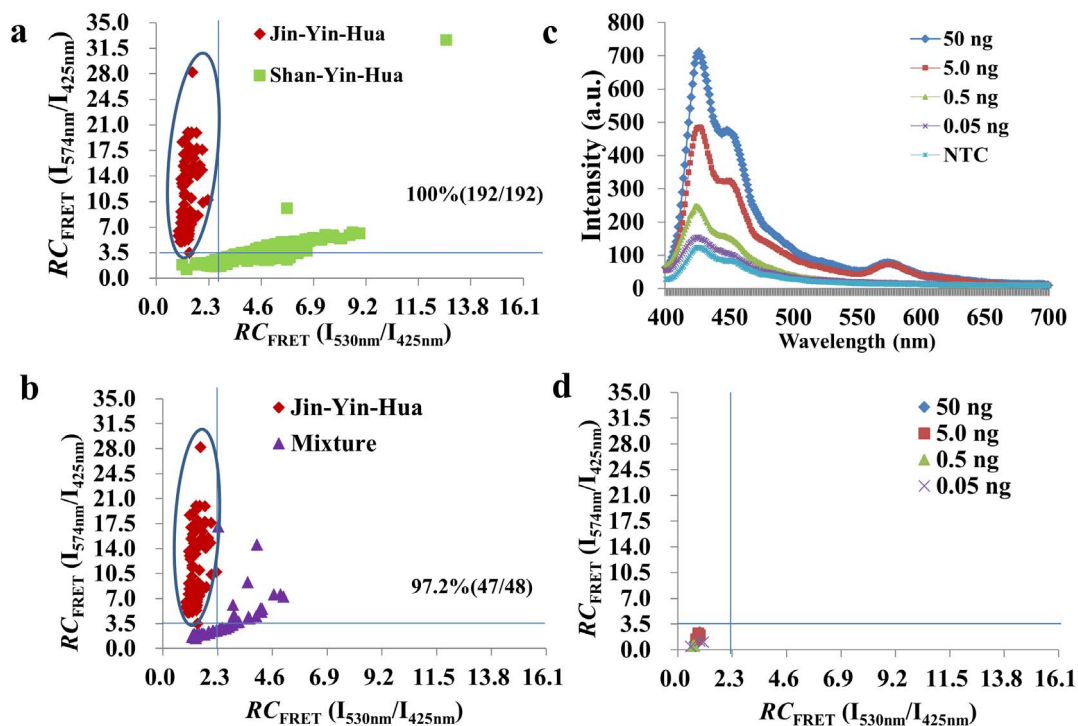


Figure 3 | (a) Threshold values of Jin-Yin-Hua (96/96) and Shan-Yin-Hua (192/192). (b) Threshold values of Jin-Yin-Hua (96/96) and mixture (47/48) with equal DNA concentration of Jin-Yin-Hua and Shan-Yin-Hua. Emission spectra (c) and threshold values (d) from solutions containing PFP and extension products in the presence of varying concentrations of heterozygous C/A (mixture with equal DNA concentration of Jin-Yin-Hua and Shan-Yin-Hua). No-template control (NTC) was used as the blank.

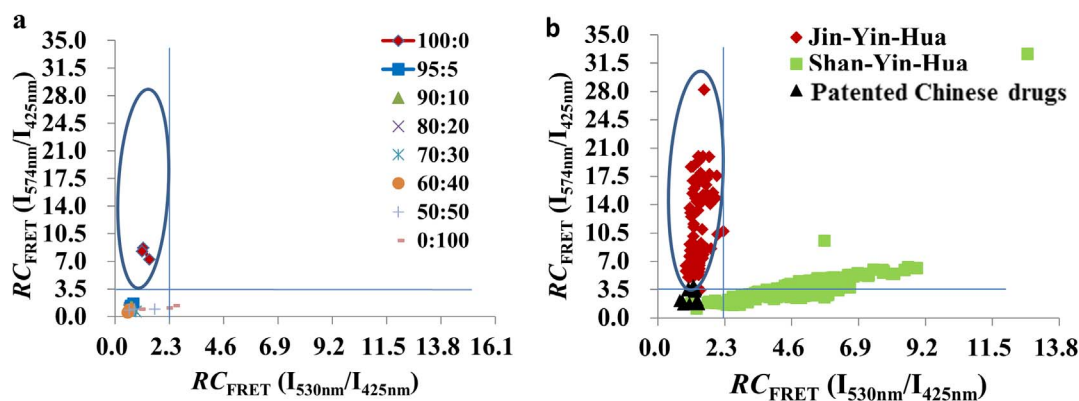


Figure 4 | (a) Threshold values of mixture containing various ratios of Jin-Yin-Hua and Shan-Yin-Hua, including 100:0 (only Jin-Yin-Hua), 95:5 (5% of Shan-Yin-Hua), 90:10 (10% of Shan-Yin-Hua), 80:20 (20% of Shan-Yin-Hua), 70:30 (30% of Shan-Yin-Hua), 60:40 (40% of Shan-Yin-Hua), 50:50 (50% of Shan-Yin-Hua), and 0:100 (only Shan-Yin-Hua). (b) Threshold values of botanical origin in Patented Chinese drugs.

$$\text{LOD} = 3 \times S_0 / S \quad (2)$$

Where S_0 is the standard deviation of the background and S is the sensitivity. The relative standard deviation for seven measurements of the blank was 6.2%, and a LOD for Shan-Yin-Hua of 0.84% was achieved with the proposed method. Thus, this method afforded high sensitivity for an SNP genotyping assay.

We selected twelve patented Chinese drugs (Qin Re An Chuang Wan, Lin Yan Jie Du Ke Li, Jian Nao Bu Shen Wan, Jin Sang San Jie Wan, Niu Huang Qing Gong Wan, Xiao Er Yan Bian Ke Li, Gan Mao Zhi Ke Ke Li, Lian Qiao Bai Du Wan, Zhi Zi Jin Hua Wan, Qing Guo Wan, Fu Fang Zhen Zhu An Chuang Pian, and Xiao Er Gan Mao Ning Tang Jiang) that contained *Lonicera* buds and screened their botanical origin using this detection system. The $\text{RC}_{\text{FRET}}(I_{530\text{ nm}}/I_{425\text{ nm}})$ and $\text{RC}_{\text{FRET}}(I_{574\text{ nm}}/I_{425\text{ nm}})$ for Qin Re An Chuang Wan, Lin Yan Jie Du Ke Li, and Jian Nao Bu Shen Wan fell within the threshold values of those of Jin-Yin-Hua (Figure 4b), suggesting that the plant materials shared an origin in *L. japonica* (Table 1).

Discussion

Combining loop-mediated isothermal amplification with cationic conjugated polymers, we have performed a rapid (4–5 h), sensitive, and visible new method for assessing botanical origin from DNA extraction, LAMP amplification and single-base extension to detection of botanical origin. Water-soluble cationic conjugated polymer PFP, was used as a donor in fluorescence resonance energy transfer (FRET) experiments. Fluorescein-labeled ddUTP and Cy3-labeled dGTP were chosen as acceptors. PFP acts as the donor for fluorescein and Cy3; fluorescein acts as the acceptor for PFP and the donor for Cy3 to satisfy the overlap integral requirement for FRET. By triggering a shift in emission color or a change in the emission intensity of PFP, fluorescein, or Cy3, it was possible to assay the three SNP genotypes in one extension reaction. Thus, Jin-Yin-Hua, Shan-Yin-Hua, and their mixture could be simultaneously differentiated and maximum visual resolution could be achieved by using this method.

Two Chinese medicinal materials (Jin-Yin-Hua and Shan-Yin-Hua) with similar morphology and chemical composition were clearly distinguished by SNP genotyping assays. The identification of plant species in patented Chinese drugs containing *Lonicera* buds was performed successfully using this method, which is robust enough to be used in high-throughput screening. Further, no complicated instrumentation or expensive reagents are utilized in the assay, making the method cost effective. This new method will be advantageous for identifying herbal materials and for determining the safety and quality of botanical products.

Methods

Allele-specific PCR detection of samples at SNP site C/A. A C/A SNP site in *trnL-trnF* between Jin-Yin-Hua and Shan-Yin-Hua by sequencing analysis (Supporting Information Figure S1). Genomic DNA was isolated from samples (25 mg each) using the modified CTAB method²⁷. Total genomic DNA was used in the allele-specific PCR according to the protocol established by Jiang et al.¹⁶. PCR was performed by adding 2.5 μl $10\times$ rTaq Taq buffer, 2 μl 10 mM dNTPs, 0.5 U rTaq (Takara, China), 0.2 pmol of each forward *trnL.F* and reverse primer *trnL.R* (Supporting Information Table S3), and 10 ng DNA per reaction volume. Total genomic DNA was also used to amplify *trnL-trnF* fragment and from Jin-Yin-Hua and Shan-Yin-Hua, respectively. The PCR results showed that all of the Jin-Yin-Hua samples contained allele C and all of the Shan-Yin-Hua samples contained allele A (Supporting Information Figure S2).

Loop-mediated isothermal amplification (LAMP). Fresh *Lonicera genus* plants, dried buds (medicinal materials), and twelve patented Chinese drugs were collected (Supporting Information Table S1, S2). The LAMP reaction mixture included $1\times$ ThermoPol[®] reaction buffer, 0.32 U μl^{-1} large-fragment Bst DNA polymerase, 0.2 mM dNTPs, 1.6 μM FIP and BIP primers, 0.2 μM F3 and B3 primers, and ddH₂O. Twenty-four microliters of reaction mixture was pipetted into each well of a 0.2-ml, 96-well PCR plate on ice. Then, 1 μl of each genomic DNA sample was added individually to the appropriate wells. The LAMP reactions were performed by incubating the samples at 63 °C for 1 h, heating at 80 °C for 10 min to inactivate the reactions, and then hold at 4 °C. Three negative control reactions (using ddH₂O instead of genomic DNA) were performed simultaneously to ensure that the LAMP products were not the result of contamination of reagents with the DNA (Supporting Information Figure S3).

Primer design for genomic LAMP. The online software Primer Explorer version 4.0 (<http://primerexplorer.jp/elamp4.0.0/index.html>) was used to design LAMP primers. Parameter conditions were as follows: (1) the length of F1c/B1c was 18–23 bp and the length of F2/B2 and F3/B3 was 18–22 bp; (2) the melting temperature (T_m) for F1c/B1c was 60–65 °C and T_m for F2/B2 and F3/B3 was 58–61 °C; (3) The distance between F2 and B2 was 50–150 bp and the distance between F1c and B1c was 0–

Table 1 | Results of relative change of FRET ratio in patented Chinese drugs

Patented Chinese drugs	$\text{RC}_{\text{FRET}}(I_{530\text{ nm}}/I_{425\text{ nm}})$	$\text{RC}_{\text{FRET}}(I_{574\text{ nm}}/I_{425\text{ nm}})$
Qing Re An Chuang Wan	1.30	3.51
Li Yan Jie Du Ke Li	1.05	3.63
Jian Nao Bu Shen Wan	1.22	4.00
Jin Sang San Jie Wan	0.83	2.13
Niu Huang Qing Gong Wan	1.27	3.34
Xiao Er Yan Bian Ke Li	1.24	3.30
Gan Mao Zhi Ke Ke Li	0.87	1.71
Lian Qiao Bai Du Wan	1.35	2.23
Zhi Zi Jin Hua Wan	1.08	1.75
Qing Guo Wan	1.41	1.76
Fu Fang Zhen Zhu An Chuang Pian	1.05	3.41
Xiao Er Gan Mao Ning Tang Jiang	0.75	2.11



50 bp; (4) the single-nucleotide polymorphism (SNP) site was established in the middle of the LAMP primers F2/B2.

Primer design for single-base extension. The single-base extension primer (25–32 bp) was designed using Primer Premier Version 5.0 software (<http://www.premierbiosoft.com/crm/jsp/com/pbi/crm/client/ProductList.jsp>). The SNP site was located at the 3'-terminus of the allele-specific primer. The T_m of the primer was 60–65 °C and self-complementarity was avoided.

Digestion of LAMP product. Digestion mixtures (12 μ l) were prepared and included 1.2 μ l 10 \times SAP buffer, 1 U exonuclease I, 1 U SAP, 0.05 U pyrophosphatase, and 8.3 μ l LAMP product. The mixtures were prepared in a 0.2-ml, 96-well PCR plate on ice and were then transferred to a thermocycler and incubated at 37 °C for 60 min, heated at 80 °C for 10 min, and held at 4 °C.

Single-base primer extension. Single-base primer extension mixtures (15 μ l) were prepared and included 1.5 μ l 10 \times rTaq buffer, 8.4 pM fluorescein-12-ddUTP, 8.4 pM Cy3-dGTP, 10 μ M allele-specific primer, 1 U Taq DNA polymerase, and 8.2 μ l high-purity water. The mixtures were pipetted into adjacent columns of a 0.2-ml, 96-well PCR plate on ice. Two microliters of digested samples were added individually to the appropriate wells of the PCR plate. The reaction was performed in a 9700 GeneAmp[®] Thermocycler as follows: initial denaturation at 94 °C for 2 min; 3 cycles denaturation at 94 °C for 30 s, annealing and extension at 63 °C for 30 s, and holding at 4 °C. The SAP solution included 5 \times SAP buffer. SAP (0.5 U μ l⁻¹) was prepared and added to each well of the 96-well PCR plate containing the single-base primer-extension products. The plate was transferred to a thermocycler and incubated at 37 °C for 20 min, heated at 80 °C for 10 min, and held at 4 °C.

Fluorescence detection. For fluorescence detection by fluorescence resonance energy transfer (FRET) ratio, 558 μ l 25 mM HEPES buffer, 36 μ l 15 μ M PFP, and 6 μ l single-base primer extension products was added to a 1.5-ml plastic spectrofluorimeter cuvette. The mixture was vortexed vigorously for 5 s and the emission spectra or intensity of the solution was measured in the cuvette. The FRET ratios of PFP to fluorescein ($I_{530\text{ nm}}/I_{425\text{ nm}}$) and of PFP to Cy3 ($I_{574\text{ nm}}/I_{425\text{ nm}}$) of the allele-specific extension reactions were plotted pairwise in scatter plots. For visible detection of FRET, 24 μ l of PFP was added to the 96-well PCR plate containing single-base primer-extension products and mixed thoroughly by pipetting. The 96-well PCR plate was placed under a UV lamp (ZF-7A) and digital camera (Canon EOS 550D) was used to record the images; the contrast of the images was adjusted so that the inherent fluorescence of the PCR plate was not visible.

Controls. For LAMP, we recommend including three negative control reactions in which high-purity water is used instead of genomic DNA; this ensures that the LAMP products are not the result of contamination of reagents with the DNA. For SNP genotyping using FRET ratios ($I_{530\text{ nm}}/I_{425\text{ nm}}$ and $I_{574\text{ nm}}/I_{425\text{ nm}}$), “blank” single-base extension reactions should be carried out for each allele-specific primer in the absence of target DNA template. The FRET ratio of the blank is used to calculate threshold values.

Detection of botanical origin by fluorescence resonance energy transfer (FRET). LAMP products were digested by exonuclease I, shrimp alkaline phosphatase (SAP), and pyrophosphatase at 37 °C for 60 min, then heated at 80 °C for 10 min and held at 4 °C. Single-base primer extension was then conducted in a reaction mixture that included Cy3-dGTP and FI-ddUTP. The SAP solution (2 μ l) was added into each well of a 96-well PCR plate containing the single-base primer extension products; the PCR plate was then incubated at 37 °C for 20 min, 80 °C for 10 min and then held at 4 °C. The single-base primer-extension product (8 μ l) was diluted to 600 μ l with HEPES buffer in a 1.5-ml fluorimeter cuvette after which the emission spectrum was measured with excitation wavelength 380 nm. The spectrum was measured again after adding 36 μ l PFP (15 μ M). Changes in fluorescence emission intensity at 425, 530, and 574 nm were recorded to calculate the FRET ratios.

Mixture of Jin-Yin-Hua and Shan-Yin-Hua for screening of botanical origin. Equal amounts of DNA from Jin-Yin-Hua and Shan-Yin-Hua were mixed and 48 samples were prepared for emission measurements. To investigate the dynamic range of the target (Jin-Yin-Hua and Shan-Yin-Hua) DNA, the emission spectra of a series of extension products containing various proportions of each source were measured at excitation wavelength 380 nm after adding PFP.

Screening of patented Chinese drugs. Twelve patented Chinese drugs containing Lonicera buds were used in the study (Qin Re An Chuang Wan, Lin Yan Jie Du Ke Li, Jian Nao Bu Shen, Jin Sang San Jie Wan, Niu Huang Qing Gong Wan, Xiao Er Yan Bian Ke Li, Gan Mao Zhi Ke Ke Li, Lian Qiao Bai Du Wan, Zhi Zi Jin Hua Wan, Qing Guo Wan, Fu Fang Zhen Zhu An Chuang Pian, and Xiao Er Gan Mao Ning Tang Jiang). Total DNA was extracted from each plant according to Cai et al.^{27,28}. Upon adding PFP to the single-base primer-extension products of these DNA samples, FRET was measured for detection of botanical origin at excitation wavelength 380 nm.

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Author contributions

Y.Y. designed experiments and wrote the paper. C.J. conducted experiments and data analysis. S.Y. and Z.C. collected the majority of the samples provided the case information in this study. M.C. conducted experiments; S.W. designed experiments and revised the paper. L.L. synthesized the polymer CCP and conducted data analysis. S.L. conducted data analysis. L.H. designed experiments, performed data analysis and wrote the paper.



Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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