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Short communication

Rapid detection of two emerging viruses associated with necrotic symptoms in *Areca catechu* L. by reverse transcription loop-mediated isothermal amplification (RT-LAMP)

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ABSTRACT

Two reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assays were developed for the detection of areca palm necrotic ringspot virus (ANRSV) and areca palm necrotic spindle-spot virus (ANSSV), respectively. These two emerging viruses both induce necrotic symptoms in areca palms. The coat protein (CP) gene of ANRSV and the 9 K gene of ANSSV were used to design the respective RT-LAMP primers for the assays. Each set of four primers designed for each of these viruses was found to be highly specific in the detection of the respective targeted virus. The optimal incubation conditions for the RT-LAMP assays were 63 °C for 40 min for ANRSV and at 61 °C for 40 min for ANSSV. The sensitivity of the RT-LAMP method for each of these viruses was 10-fold greater than that of the corresponding conventional reverse-transcription polymerase chain reaction (RT-PCR). The RT-LAMP assays may be useful for the rapid early detection of ANSSV and ANRSV in commercial areca palm production.

Areca palm (*Areca catechu* L.) is mainly distributed in the tropical Asian and Pacific regions, and in parts of East Africa (Arjungi, 1976; Peng et al., 2015). Areca palm is grown for its economically valuable seed (i.e., areca nut), which represents the fourth most popular psychoactive substance following caffeine, nicotine, and alcohol (Zdrojewicz et al., 2015). In China, India and other South and Southeast Asian countries, the areca nut is used in traditional and multifunctional medicines for treating diseases, including parasitic diseases, digestive function disorders, and depression (Peng et al., 2015). Additionally, nut chewing is an important cultural and social custom in some areas of South and Southeast Asia and the Asia-Pacific region (Murphy and Herzog, 2015).

The production and consumption of areca nuts have recently increased rapidly in southern China, and the areca palm has become an important economic crop in the tropical regions of China (Yang et al., 2019). Recently, two new virus, areca palm necrotic ringspot virus (ANRSV) (Yang et al., 2019) and areca palm necrotic spindle-spot virus (ANSSV) (Yang et al., 2018), were identified in areca palms growing in Hainan, China. These two viruses, which are classified in a putative new genus (*Arepavirus*) of the family *Potyviridae*, are associated with the development of severe necrotic symptoms on infected leaves. Specifically, ANRSV infection induces necrotic ringspot symptoms on the areca palm leaves (Yang et al., 2019), whereas ANSSV infection results in necrotic spindle-spots on the infected leaves (Yang et al., 2018). A field survey revealed that ANRSV was highly endemic in the areca palm growing regions of Hainan, with an average incidence rate exceeding 40 % in some severely affected regions (Yang et al., 2019). In contrast, the incidence rate of ANSSV infection was much lower (under 1.0 %, unpublished data). Nevertheless, the emergence of ANRSV and ANSSV represents a serious threat to areca palm cultivation in China.

A comparison of the genomes of ANSSV and ANRSV indicated that they are similar in size (9434 versus 9437 nucleotides, respectively), excluding the 3′-poly(A) tail), and structure (HC-Pro1, HC-Pro2, P3, 7 K, CI, 9 K, NIa-VPg, NIa-Pro, NIb, and CP). The polyproteins of ANRSV and ANSSV share 72.5 % and 76.2 % identities at the nucleotide (nt) and amino acid sequence levels, respectively, which are below the levels of identity demarcating species in the family *Potyviridae*, but the nucleotide and amino acid sequence identities of their coat protein (CP)

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genes exceed the species demarcation threshold (Yang et al., 2018, 2019). Whether ANSSV and ANRSV are distinct species or isolates of the same species remains unclear.

The early and accurate diagnosis of viral diseases in the field are essential for determining the actions required to reduce disease spread and to minimize economic losses. The use of virus-free planting material is an agricultural strategy for efficient control of destructive viral diseases (Wang et al., 2018). Because areca palm is propagated from seed, detecting the casual viruses in seedlings before the appearance of symptoms is also important. To date, only a reverse-transcription polymerase chain reaction (RT-PCR) assay based on the ANRSV CP gene is available, and only for ANRSV detection (Yang et al., 2019). Thus, it is necessary to develop additional rapid, sensitive and reliable methods for the detection of both ANRSV and ANSSV. Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) is a diagnostic tool used to amplify a target nucleic acid within 60 min under a constant temperature of 60 − 65 °C (Notomi et al., 2000, 2015). RT-LAMP is widely used to diagnose many plant virus infections because of its advantages over conventional PCR-based methods. It does not require the precise thermal cycling used in conventional PCR assays (Notomi et al., 2015), and the time-consuming post-amplification visual detection with different dyes (Boonham et al., 2014; Wong et al., 2018). In this study, two specific, sensitive and rapid RT-LAMP assays were developed for detecting ANRSV and ANSSV, respectively, and their potential application for testing field areca palm samples was evaluated.

Previously, a multiple sequences alignment showed that the 843-nucleotide (nt)-long CP gene fragments of various ANRSV isolates (DA, MK, NH, NP, NY, SB, and XC) from different geographic origins shared 91.6–100 % identities among the isolates and 80.1–81.5 % identities with ANSSV, both at the nucleotide acid levels (Yang et al., 2019). To specifically detect multiple ANRSV isolates in a single RT-LAMP assay, the consensus sequence of the CP genes was obtained from a sequence alignment of the ANRSV isolates and used to design four RT-LAMP primers (ANRSV-F3/B3 and ANRSV-FIP/BIP) using the Primer Explorer program (https://primerexplorer.jp/e /index. html, version 5.0) (Table 1). To date, only one ANSSV isolate, HNBT, had been identified and sequenced (GenBank accession number: MH330686) (Yang et al., 2018). Among the 10 putative genes of ANSSV and ANRSV, the 9 K genes share the lowest identities between them at the nucleotide and amino acid levels (61.8 % and 60.2 %, respectively) (Yang et al., 2019). Therefore, the ANSSV 9 K gene was selected for designing the RT-LAMP primers (ANSSV-F3/B3 and ANSSV-FIP/BIP) (Table 1) to specifically detect ANSSV. The two RT-LAMP primer sets were designed to specifically amplify the 204-bp target sequence of the ANRSV CP gene and 193-bp target sequence of ANSSV 9 K gene, respectively. The multiple sequences alignments and target positions of the primers for the ANSSV and ANRSV RT-LAMP assays are shown in Supplementary Fig. 1.

Total RNA was extracted from previously stored areca palms leaf samples (100 mg) at − 80 °C, respectively infected with ANRSV isolate XC1 (GenBank accession number: MH395371) (Yang et al., 2019) and ANSSV isolate HNBT (Yang et al., 2018) using the RNAprep Pure Plant Kit (Tiangen Biotech, China). Based on previous methods of the RT-LAMP (Soliman and El-Matbouli, 2006; Shen et al., 2014), the RT-LAMP reactions were conducted in 25-μL reaction volumes containing 2.5 μL of 10× ThermoPol Buffer (containing 2 mM MgSO4), 8 U of Bst DNA polymerase (large fragment; New England Biolabs, USA), 5 U of M-MLV reverse transcriptase (TaKaRa, China), 0.2 μM of each outer primer for ANRSV(ANRSV-F3/ANRSV-B3) or ANSSV(ANSSV-F3/ANSSV-B3), 1.6 μM of each inner primer for ANRSV or ANSSV (ANRSV-FIP/ANRSV-BIP or ANSSV-FIP/ANSSV-BIP), 1.4 mM of dNTP mixture (TaKaRa), 6 mM MgSO4, 0.5 μL of RNA template and DNase/RNase-free dH_2O. The RT-LAMP products were analyzed by 2 % agarose gel electrophoresis with ethidium bromide staining and visualized by the addition of 0.2 μL of SYBR Green I (10×) to the reaction mixture (Invitrogen, USA). After the RT-LAMP reaction, virus-positive samples produced ladder-like bands of varying sizes in agarose gels (Notomi et al., 2000) and their reaction mixtures also turned green with the addition of SYBR Green I (Soliman and El-Matbouli, 2006). The respective RT-LAMP assay conditions for ANRSV and ANSSV were optimized for reaction temperatures and reaction times using a water bath. Six reaction temperatures (57, 59, 61, 63, 65 and 67 °C) were tested in a 60-min reaction. Analysis of the results by both agarose gel electrophoresis and visualization with SYBR Green I correspondingly confirmed that the range of temperatures tested all resulted in positive amplifications of ANRSV and ANSSV DNA in the respective virus-infected samples (Fig. 1A). The agarose gel electrophoresis results indicated that a maximum amount of amplified product was obtained at 63 °C for ANRSV and 61 °C for ANSSV based on the intensity of the bands (Fig. 1A). Thus, these optimal amplification temperatures were used to determine the optimal reaction times for the ANRSV and ANSSV RT-LAMP assays, by testing reaction times of 10, 20, 30, 40, 50, 60 and 70 min. Analysis of the resultant reactions showed that, for both viruses, the typical ladder-like bands of varying sizes were only observed after a 30-min reaction. Additionally, reaction times exceeding 40 min did not improve the amplification results (Fig. 1B). Therefore, the optimal incubation conditions for the RT-LAMP assays were 63 °C for 40 min for ANRSV and at 61 °C for 40 min for ANSSV. Furthermore, direct sequencing of the RT-LAMP products was performed to confirm the accuracy of the ANRSV and ANSSV RT-LAMP assays. The RT-LAMP products were purified with a PCR purification kit (Tiangen Biotech) and sequenced with inner primers. The sequences obtained were perfectly matched with the expected target sequences of the ANRSV CP gene and ANSSV 9 K gene, respectively (data not shown).

The specificity of the respective primer sets used in the ANRSV and ANSSV RT-LAMP assays was tested using total RNA extracted from areca palms infected with ANSSV isolate HNBT (Yang et al., 2018) and seven ANRSV isolates (DA, MK, NH, NP, NY, SB, and XC) (Yang et al., 2019). Four known potyvirid-infected plant samples from Hainan

Table 1
The primers used for the RT-LAMP assays for detecting ANRSV and ANSSV.

Primer name	Description	Length (nt)	Genome position(nt)[a]	Sequence (5′→3′)
ANRSV-F3	Forward outer	18	8989-9006	AGGAACACTCACGAGGTG
ANRSV-B3	Backward outer	21	9192-9172	TCATAACTTGTTCCCTGTGAT
ANRSV-FIP (F1c + F2)	Forward inner	45	9093-9069,9009-9029	ACAACTGCAGTAACTTTGTGATCAA-CAATTAAAAGGGGTATCCGTC
ANRSV-BIP (B1c + B2)	Backward inner	42	9098-9120,9169-9151	GAGATCCTAACTCAAGCAAAGGC-ATTGCCATCCGTAAGCATT
ANSSV-F3	Forward outer	21	5183-5203	AATCAAACACTCACAAAGGAA
ANSSV-B3	Backward outer	19	5375-5357	ACAACGTGCTAATTGTTGG
ANSSV-FIP (F1c + F2)	Forward inner	46	5272-5248,5205-5224	TCCCTCAACTATTGCCTTTGTTATT-AGGTGATAGAATTTTGCGAG
ANSSV-BIP (B1c + B2)	Backward inner	44	528 1-5 304,5356-5337	AACAGCACTTTGGGGATTATTTTG-TATGTGTTGCACACACTGAT

[a] Genome position according to the complete genome sequences of ANRSV (GenBank Accession No. MH395371) and ANSSV (GenBank Accession No. MH330686).

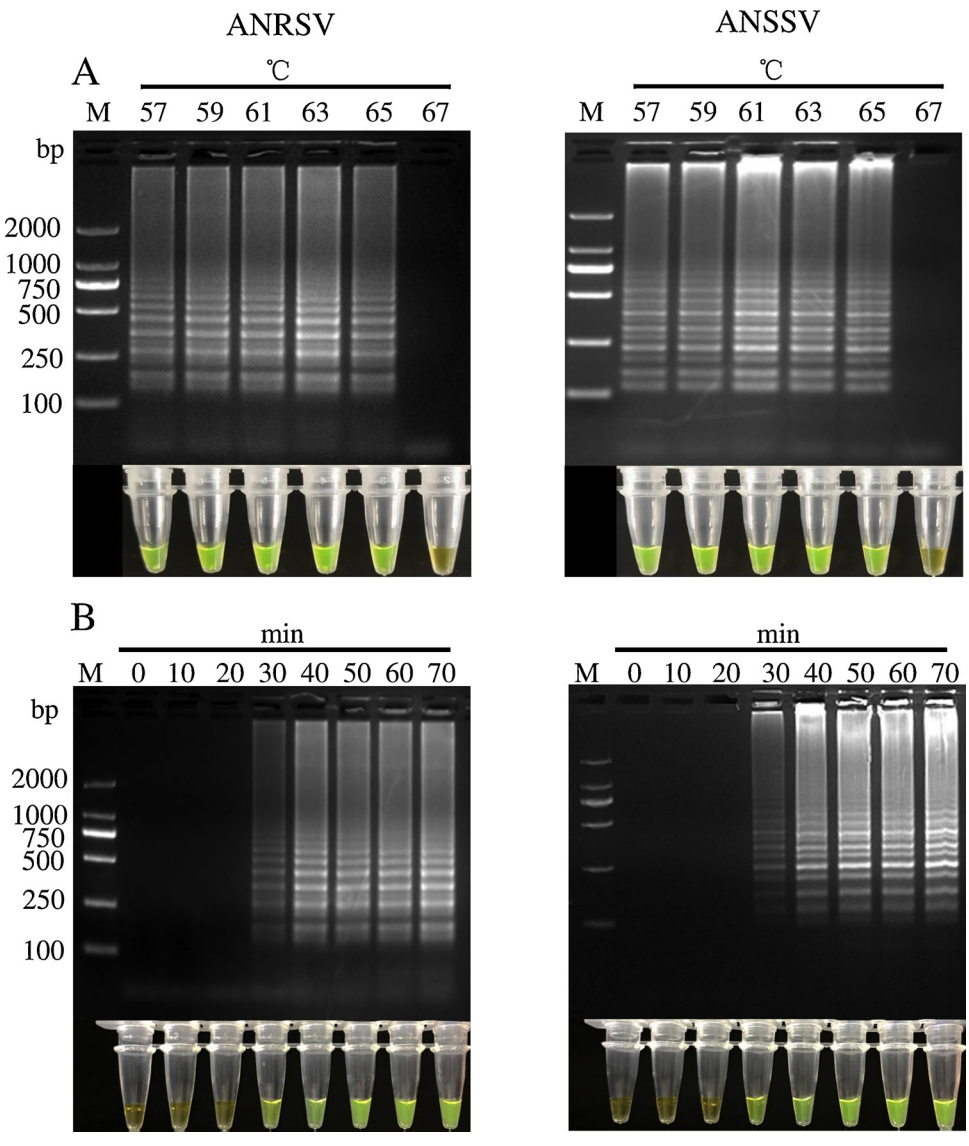

Fig. 1. Optimization of the RT-LAMP reaction conditions for the detection of the ANRSV CP gene fragment and the ANSSV 9 K gene fragment. (A) Determining the optimal temperatures for the ANRSV and ANSSV RT-LAMP reactions and analysis of the reactions by agarose gel electrophoresis (top) and by visual detection with the SYBR Green I (bottom). (B) Determining the optimal reaction times were tested for the ANRSV and ANSSV RT-LAMP reactions and analysis of the reactions by agarose gel electrophoresis (top) and visual detection with the addition of SYBR Green I (bottom). Lane M: DNA markers DL2000 with 2000,1000, 750, 500, 250, and 100 bp.

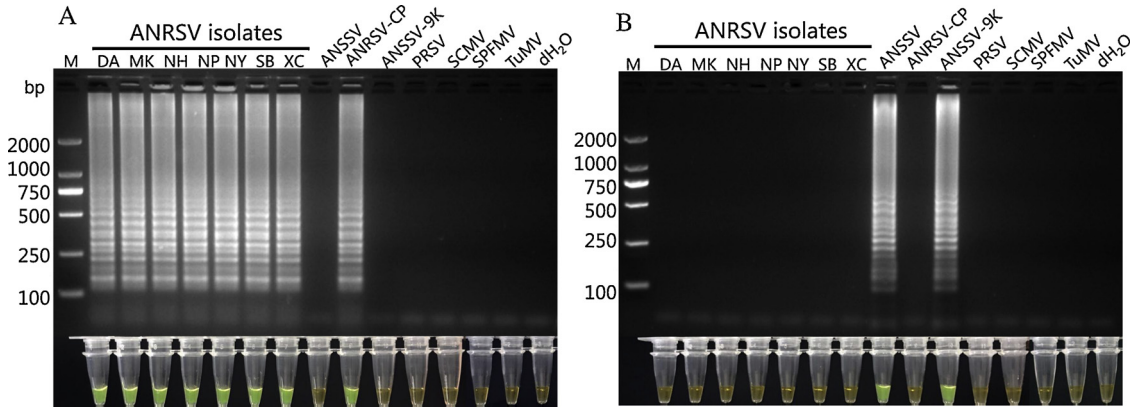

Fig. 2. Specificity of the ANRSV RT-LAMP assay (A) and the ANSSV RT-LAMP assay (B). For each of the virus assays, total RNA from areca palm respectively infected with each of the ANRSV isolates (DA, MK, NH, NP, NY, SB and XC) and the ANSSV isolate HNBT, plasmids containing the respective ANRSV CP gene (ANRSV CP) and ANSSV 9 K gene (ANSSV 9 K), and plants respectively infected with papaya ringspot virus (PRSV), sweet potato feathery mottle virus (SPFMV), sugarcane mosaic virus (SMV) and turnip mosaic virus (TuMV), and dH_2O, were used templates. The resultant RT-LAMP assay reactions were analysed by agarose gel electrophoresis (top) and visual detection with SYBR Green I (bottom). Lane M: DL2000 with 2000, 1000, 750, 500, 250, and 100 bp DNA markers.

[papaya, sweet potato, sugarcane and *Nicotiana benthamiana* infected with papaya ringspot virus (PRSV) isolate HNVb (Zhao et al., 2015), sweet potato feathery mottle virus (SPFMV, unpublished), sugarcane mosaic virus (SCMV unpublished) and turnip mosaic virus (TuMV, unpublished), respectively] were used to evaluate specificity and two plasmids containing the ANRSV CP gene and ANSSV 9 K gene,

respectively, were used as positive controls (Yang et al., 2018, 2019). All the RT-LAMP reactions were carried out using the optimal reaction temperatures and times for each of these viruses. The results showed that the RT-LAMP reactions produced the typical ladder-like bands in the agarose gel and the corresponding green color change with SYBR Green I only in samples infected with each of the ANRSV isolates and in the ANRSV CP plasmid positive control but not in the ANSSV infected sample or the ANSSV 9 K plasmid or any of the other potyvirid-infected plant samples (Fig. 2A). Likewise, the RT-LAMP assay for ANSSV produced positive reactions only for the sample infected with ANSSV and the 9 K plasmid positive control and not in any of the negative controls (Fig. 2B). These results suggested that the two RT-LAMP primer sets for ANRSV and ANSSV specifically amplified the respective targeted virus, while the RT-LAMP assay for ANRSV has a broad specificity for detecting multiple ANRSV isolates.

The relative sensitivities of the ANRSV and ANSSV RT-LAMP assays and their corresponding conventional RT-PCR assays in detecting the target viruses were assessed, using the total RNA of ANRSV isolate XC1-infected and ANSSV isolate HNBT-infected areca palms. The quantity and quality of each RNA preparation was determined using a NanoDrop spectrophotometers (Thermo Scientific, USA). The RNA was serially diluted in 10-fold dilutions in nuclease-free dH_2O and used as templates at 1.0–1.0 × 10^{-7} μg of RNA per RT-LAMP or RT-PCR reaction. The conventional RT-PCR assays were performed using the OneTaq® One-Step RT-PCR Kit (New England Biolabs) and the RT-LAMP outer primers according to the manufacturer's instructions. The 25-μL RT-PCR reaction mixture contained 12.5 μL of 2× OneTaq One-Step Reaction Mix, 1 μL of 25× OneTaq One-Step Enzyme Mix, 0.2 μM of each outer primer pair (ANRSV-F3/ANRSV-B3 or ANSSV-F3/ANSSV-B3), 0.5 μL of RNA template, and the required volumes of DNase/RNase-free dH_2O. The thermal cycling conditions were as follows: 48 °C for 30 min (reverse transcription reaction); 94 °C for 1 min; 30 cycles of 94 °C for 15 s, 53 °C for 30 s, and 68 °C for 15 s; 68 °C for 5 min. The RT-PCR products were examined by 2 % agarose gel electrophoresis. The expected amplicon sizes of the ANRSV CP and ANSSV 9 K fragments were 204 bp and 193 bp, respectively. Based on the sensitivity assays, the detection limits of the one-step RT-PCR assays were 1.0 × 10^{-5} μg total plant RNA per reaction for both ANRSV and ANSSV, while the detection limits for the RT-LAMP assays were 1.0 × 10^{-6} μg total plant RNA per reaction for both ANRSV and ANSSV (Fig. 3A, B). Therefore, the RT-LAMP assays for detecting ANRSV and ANSSV were 10-times more sensitive than the corresponding conventional RT-PCR assays, implying that the RT-LAMP assays were better at detecting low concentrations of ANRSV and ANSSV, which is relevant for virus detection during the early stages of ANRSV and/or ANSSV infections in the field.

The respective optimized RT-LAMP assays were applied on 60 asymptomatic and 25 symptomatic areca palm samples collected from diverse cultivation regions in Hainan, using colorimetric detection of ANRSV and ANSSV with SYBR Green I. The corresponding ANRSV and ANSSV RT-PCR assays were also performed on all these samples simultaneously, using gel electrophoresis to detect the products. Total RNA was extracted from the samples using the RNAprep Pure Plant Kit (Tiangen Biotech) and used as templates. Among the 25 symptomatic samples, 23 samples displayed necrotic ringspots and 2 samples showed necrotic spindle-spots. The test results showed that, out of the 60 asymptomatic samples, 4 samples were found to be positive for ANRSV by both the ANRSV RT-LAMP and RT-PCR assays, while another 3 samples that were positive for ANRSV by the RT-LAMP assay were negative for the virus by the RT-PCR assay (Table 2). This suggested that the ANRSV RT-LAMP was more sensitive than the ANRSV RT-PCR for the detection of low concentrations of the virus and would be suitable for early virus diagnosis. However, none of the asymptomatic samples were found to be positive for ANSSV by either the RT-LAMP or the RT-PCR assay (Table 2). In contrast, among the 25 symptomatic samples, all 23 samples showing necrotic ringspots were found to be positive for ANRSV and not for ANSSV by the respective RT-LAMP and RT-PCR assays, while the remaining two samples that showed necrotic spindle-spots both tested positive for ANSSV infections by both assays, and were negative for ANRSV (Table 2). This result suggested that ANRSV may be the main viral pathogen infecting areca palm, which is consistent with the findings of a previous field survey (Yang et al., 2018, 2019).

In summary, individual RT-LAMP assays were developed for the rapid detection of each of two viruses infecting areca palms, ANRSV and ANSSV. This is the first report describing the use of RT-LAMP assays for detecting ANRSV and ANSSV in areca palm. The positive

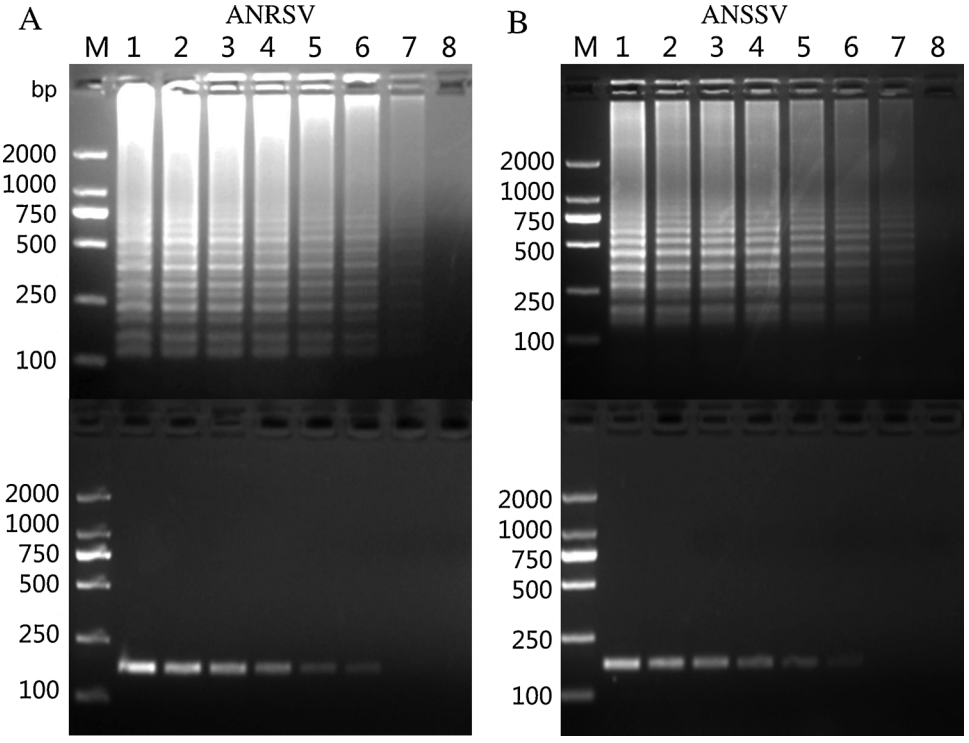

Fig. 3. Comparison the sensitivities of the respective of RT-LAMP and conventional RT-PCR assays for detecting ANRSV and ANSSV. (A) Analysis of the RT-LAMP assay (top) and conventional RT-PCR (bottom) assay products by agarose gel electrophoresis using a serial dilution of total RNA from areca palm infected with ANRSV. (B) Analysis of the RT-LAMP (top) and conventional RT-PCR (bottom) assay products in an agarose gel electrophoresis using a serial dilution of total RNA from areca palm infected with ANSSV. Lane M: DL2000 with 2000, 1000, 750, 500, 250, and 100 bp DNA markers; lanes 1-8: Reactions containing 1.0–1.0 × 10^{-7} μg of total RNA from areca palm infected with ANRSV(A) or ANSSV(B) per reaction.

Table 2
Detection of ANRSV and ANSSV in field samples by RT-LAMP and RT-PCR assays.

Number of samples	Disease symptoms	ANRSV		ANSSV	
		RT-LAMP positive[d]	RT-PCR positive[d]	RT-LAMP positive	RT-PCR positive
60	No[a]	7	4	0	0
23	Yes[b]	23	23	0	0
2	Yes[c]	0	0	2	2

[a] Symptomless sample.
[b] Samples with necrotic ringspot symptoms on areca palm leaves.
[c] Samples with necrotic spindle-spots on areca palm leaves.
[d] Among the 7 positive samples detected by ANRSV RT-LAMP, 3 samples were negative by ANRSV RT-PCR.

reactions could be easily visualized by color changes and did not require the use of potentially harmful UV light. The RT-LAMP assays developed for the detection of ANRSV and ANSSV in this study were rapid and efficient, and could be useful tools for monitoring both viruses in the main areca palm growing regions.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Guangyuan Zhao: Conceptualization, Methodology, Software. **Wentao Shen:** Writing - original draft. **Decai Tuo:** Visualization, Investigation. **Hongguang Cui:** Methodology, Resources. **Pu Yan:** Methodology, Software. **Qinghua Tang:** Resources. **Guopeng Zhu:** Project administration. **Xiaoying Li:** Supervision. **Peng Zhou:** Funding acquisition. **Yindong Zhang:** Conceptualization.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113795.

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