Front. Biol. China 2009, 4(3): 314–320
DOI 10.1007/s11515-009-0026-5

RESEARCH ARTICLE

Quantitative detection of Cymbidium mosaic virus by real time PCR

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Abstract The technique of SYBR Green-based quantitative real-time reverse transcription polymerase chain reaction (real-time RT-PCR) was applied to quantitative detect a 764 bp nucleotide sequence containing total *coat protein (cp)* gene of Cymbidium mosaic virus (CyMV). The plasmid containing the target sequence was constructed to prepare the standard curve and detect the sensitivity. The standard curve was drawn based on the linear relationship between the logarithm (base 10) of the quantity of target sequence and cycle threshold [C(T)]. While the concentration of plasmid DNA falling within the range of 2.6×10^7 to 2.6×10^2 copies per tube established a regression equation, $y = -0.3583x + 10.32$, and related coefficient: $r^2 = 0.995$. The real-time RT-PCR assay for CyMV had a minimum detectable quantity of two copies per tube. The naturally infected samples of *Phalaenopsis* sp. and the artificially inoculated samples of *Arachnis* sp. with trace CyMV were quantitatively detected using this method. CyMV in the positive samples of *Phalaenopsis* sp. and *Arachnis* sp. was confirmed by DNA sequencing and *cp* gene homeology blast. The results showed that CyMV extracted from the leaves of orchid in Hangzhou, Zhejiang Province, China, could be derived from Kunming city (KM), Yunnan Province, China. This method characterized by high sensitivity, specificity, and precision is suitable for early diagnosis and quantitative detection of CyMV.

Keywords Cymbidium mosaic virus (CyMV), coat protein gene, quantitative detection, real-time reverse transcription polymerase chain reaction (real-time RT-PCR), SYBR Green

Received September 11, 2008; accepted November 24, 2008

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1 Introduction

Orchids have been reported to be infected with at least 25 types of viruses. Of these, Cymbidium mosaic virus (CyMV) has been reported to be one of the most prevalent viruses (Zettler et al., 1990; Hu et al., 1993; Wong et al., 1994; Khentry et al., 2006). Orchid cultivars infected with CyMV often show reduced flower quality, necrosis, and flower disfigurement, as well as foliage symptoms, resulting in great economic loss. As such, some diagnostic techniques have been developed over the years for detecting CyMV in infected orchids.

A host-plant bioassay and electron microscopy has been employed for the detection of CyMV (Vejaratpimol et al., 1999), but it is laborious and expensive for large-scale tests.

Recently, enzyme-linked immunosorbent assay (ELISA) (Hsu et al., 1992; vejaratpimol et al., 1998) and reverse transcription polymerase chain reaction (RT-PCR) (Lim et al., 1993; Seoh et al., 1998; Ryu and Park, 1995; Srifah et al., 1996) have been the most common methods because of their reliability, rapidity, and relatively low cost.

Although traditional RT-PCR techniques described above could detect a low titer for RNA of CyMV, they could not be used for quantitative analysis. Real-time PCR allows rapid detection and accurate quantification when used with a standard curve (Ashton and Headrick, 2007).

Quantitative (real-time) PCR relies on the ability to measure the accumulation of PCR products during each amplification cycle. This is achieved by measuring the increase in a fluorescent signal, which is proportional to the amount of double-stranded DNA present. The current study focuses on the application of one-step quantitative (SYBR Green-based real time) RT-PCR (qRT-PCR) for the measurement of CyMV, which was extracted from the leaves of orchids (although appropriate for other tissues also).

2 Materials and methods

2.1 Materials and instruments

Plants of orchid *Phalaenopsis* sp., suspected of being infected with CyMV due to the presence of a variety of CyMV-infection symptoms, were collected from the Hangzhou Qian-jiang Flower-bird Market, Zhejiang province, China.

Healthy control plants of orchid *Arachnis* sp. were collected from the orchid garden of the Horticulture Institute, Hangzhou Academy of Agricultural Sciences, Hangzhou, China.

The UNIQ-10 histogram total RNA extraction kit (Sangon Corporation, Shanghai, China), MMLV one-step RT-PCR amplification kit (Sangon Corporation, Shanghai, China), and one-step SYBR PrimeScript^{TM} RT-PCR kit (Takara Corporation, Dalian, China) were used.

The instruments included the Opticon2 real-time PCR instrument (Bio-Rad, USA), Multiskan Mk3 microplate reader (Thermo Lab systems, Shanghai, China) for ELISA, TC-96/H/G PCR amplification (Ferrotec Corporation, Hangzhou, China), and Cary 50 UV-spectrophotometer (Varian, USA).

2.2 Early ELISA screening, biological separation, and inoculation

Fresh leaves of *Phalaenopsis* sp. were early screened by ELISA based on anti-Cymbidium mosaic virus. The positive infected samples (sample 1#) (0.5 g) were frozen in liquid nitrogen.

Similarly, fresh leaves of *Arachnis* sp. were early screened by ELISA. The negative control plants were grown in a greenhouse for three weeks. They were regarded as healthy plants on the condition that their leaves were tested to be noninfected by ELISA. These negative samples (sample 3#) (0.5 g) were frozen in liquid nitrogen.

Donated sap was extracted from the infected leaves of *Phalaenopsis* sp. and then from the leaves of healthy control *Arachnis* sp. The plants were cut with V-shape wounds and inoculated with the donated sap. Plants were grown at room temperature and watered when needed with special fertilizer for orchids. The leaves of *Arachnis* sp. that had no wound (sample 4#) (0.5 g) were collected and frozen in liquid nitrogen seven days later.

2.3 Primer design and testing

RT-PCR primers for viral *coat protein (cp)* gene of CyMV with an expected amplified size of 764 bp were designed (Zhou et al., 2004). Forward primer PCymv1 (5′-CCTGGCGAGGGTTAAGTTAC-3′) was constructed to amplify the *cp* gene from nt 5441 to 5460, and reverse primer PCymv2 (5′-GCCAGTAGTGGAAACAAACT-3′) was constructed to amplify the *cp* gene from nt 6204 to 6185. The amplified PCR fragment contained total *cp* gene of CyMV corresponding to nt 5481 to 6152 (672 bp) (NC-001812) and some upstream and downstream sequences. The RT-PCR primers were tested in Primers-BLAST at the National Center for Biotechnology Information (NCBI), USA. Subsequently, the primers for viral *cp* gene of CyMV were synthesized by Sangon Corporation, Shanghai.

2.4 Total RNA isolation and quantification

The orchid leaves were collected and immediately frozen in liquid nitrogen. The material was then ground to a fine powder with a precooled pestle and mortar under liquid nitrogen.

DNA samples were extracted from the naturally infected leaves of *Phalaenopsis* sp. and the artificially inoculated leaves of *Arachnis* sp., as well as from noninfected leaves of *Arachnis* sp. Each specimen was homogenized and extracted using the UNIQ-10 histogram total RNA extraction kit.

After extraction, to determine the total RNA concentration and purity, the absorbance of the isolate was measured at wavelengths of 230, 260, and 280 nm using the Cary 50 UV-spectrophotometer. The result indicated that the total RNA yield ranged from 480 to 720 μg per gram of tissue with an average purity measured as OD 260/230 of more than 2.00 and OD 260/280 more than 1.90. The purified total RNA was stored at -20°C. Healthy orchid materials were used as negative controls.

2.5 Preparation of plasmid standards

Isolated total RNA of sample 1# was used for amplification by the conventional RT-PCR. The amplified PCR product that was purified CyMV *cp* gene was cloned into the pUCm-T vector using the T-Vector PCR products cloning kit (Sangon Corporation, Shanghai, China), and then, the plasmid was extracted. The purified plasmid was quantified to 100 ng/μL (accounting for 2.6×10^{10} DNA recombinant plasmids/μL or 5.2×10^{10} copies/μL). This purified plasmid was used as a standard to determine the sensitivity of real-time RT-PCR. The 10-fold serial dilutions (10^7 to 10^0 copies per μL) of purified plasmid were ready to use.

2.6 Quantitative detection

The plasmid standards were diluted serially from 10^7 to 10^0 copies per μL at one log unit intervals with nuclease free water. Sample 1# was diluted to 10^3 and 10^5 times, sample 3# one time and, and sample 4# 1, 2 times for use.

The SYBR Green I was used as the fluorescent dye, and RT-PCR reactions were carried out using the one-step SYBR PrimeScript^{TM} RT-PCR kit. The reaction took place in a final volume of 25 μL, containing 0.5 μL template

(RNA or plasmid standards), 12.5 μL of one step SYBR RT-PCR buffer III, 0.5 μL of TaKaRa Ex Tap® HS (5 U/L), 0.5 μL of PrimerScript™ RT Enzyme Mix II, 10 μL of RNase free dH_2O, 0.5 μL of forward primer, and 0.5 μL of reverse primer. Nuclease free water was used as negative control, and then, it was sealed and centrifuged (1000 r/min for 1 min at 4°C). The Bio-Rad Opticon2 real-time PCR instrument was used for thermal cycling and to record changes in fluorescence intensity. The thermal cycling profile for real time RT-PCR was as follows: 1 cycle of 45°C for 25 min (reverse transcription) and 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and 5 s, followed by reading the plate and a final extension of 72°C for 10 min.

To test the specificity of the PCR, control DNA samples from sample 3# were subjected to the same amplification procedure.

2.7 Melting curve and sequencing

The experiments were replicated twice with parallel double samples. The amplicons achieved from the first experiment were used for melting-curve analysis, and the amplicons achieved from the second experiment were used for sequencing.

Following the PCR, the melting-curve analysis of the amplicons was conducted with the Bio-Rad Opticon2 real-time PCR instrument by increasing the temperature from 55°C to 95°C at every 0.5°C reading and holding 00:00:05.

The amplicons achieved from sample 4# were cloned and sequenced by Sangon Corporation in Shanghai, China.

3 Results

3.1 Melting curve analysis

In order to determine the specificity of the amplification based on real-time RT-PCR technique, PCR-coupled melting analysis of the amplicons achieved from the plasmid standard, sample 1#, and sample 4# were performed. The results showed that they had the same melting temperature (shown in Fig. 1 and Table 1). Therefore, the specificities of amplicons which contained the target gene were confirmed.

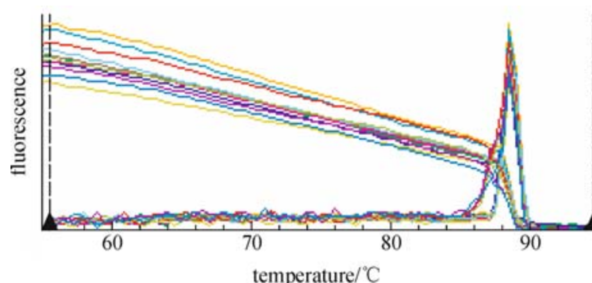

Fig. 1 Melting curve graph

3.2 Standard curve

Purified plasmid standards (concentrations of 2.6×10^7 to 2.6×10^0 plasmids/μL, each 0.5 μL) were added to tubes, and the target gene sequence in the constructed plasmid was encoded in double-stranded DNA, thus, corresponding to containing 2.6×10^7 to 2.6×10^0 copies per tube, respectively.

The fluorescent intensity of the negative control solution was lower than 0.015, and the cycle threshold [C(T)] was set at 0.053 (3.5 times more than that of the negative control). The standard curve was drawn based on the linear relationship between the logarithm (base 10) of the quantity of the target sequence and C(T). The data of six reactions with the concentration of 2.6×10^7 to 2.6×10^2 copies per tube of plasmid DNA as template were used to plot the standard curve related to C(T) value and copy number. The equation was obtained as $y=-0.3583x+$

Table 1 Melting temperature, Tm

well	dye	content	description	Tm
C3	SBG1	standard	—	88.5
C4	SBG1	standard	—	88.5
C5	SBG1	standard	—	88.5
C6	SBG1	standard	—	88.5
C7	SBG1	standard	—	88.5
C8	SBG1	standard	—	88.5
C9	SBG1	standard	—	88.5
C10	SBG1	standard	—	88.5
E4	SBG1	sample	1#	88.5
E5	SBG1	sample	1#	88.5
E4	SBG1	sample	4#	88.5
E5	SBG1	sample	4#	88.5

10.32, and R^2= 0.995 (where x stands for log quantity and y for cycle number). The amplification result and standard curve are shown in Figs. 2 and 3.

While the concentrations of 2.6×10^1 and 2.6×10^0 copies per tube as template were used, related C(T) values were 21.65 and 22.04, respectively, and these two points diverged from the standard line (shown in Fig. 3). For this reason, the copy numbers of the target gene were lower than 2.6×10^2. Although it could be qualitatively detected, it could not be used for quantitative analysis.

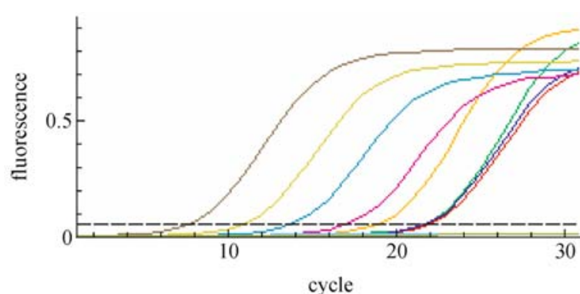

Fig. 2 Amplification profile of detection for the *cp* gene of CyMV. From left to right, the amplification contained 2.6×10^7, 2.6×10^6, 2.6×10^5, 2.6×10^4, 2.6×10^3, 2.6×10^2, 2.6×10^1, and 2.6×10^0 copies of plasmid DNA, respectively.

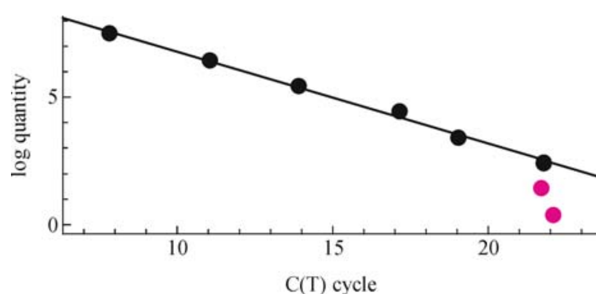

Fig. 3 Standard curve created by the analysis of known copies of target nucleic acid. Reactions with copy numbers of the target gene from 2.6×10^7 to 2.6×10^2 were used for creating the standard curve. The standard curve was plotted by the log concentration of copy numbers against cycle threshold [C(T)] values. Control graph $y=-0.3583x+10.32$; r^2= 0.995. While the concentrations of 2.6×10^1 and 2.6×10^0 copies per tube (shown in red dots) as template were used, related C (T) values were 21.65 and 22.04, respectively, and these two points diverged from the standard line.

3.3 Results of samples quantitative detection

The cycle threshold of each sample was then compared to a standard curve made by diluting genomic DNA (10-fold serial dilution).

The amplification profile curves of sample 1# are shown in Fig. 4, and the results of quantitative detection for RNA in orchids samples are shown in Table 2. The relation standard of deviation was 4.2% (sample 1#, $10^3\times$dilution, tested in parallel, $n=2$). Sample 1# ($10^5\times$dilution) was quantified to 351 and 453 copies per tube (average 402 copies per tube, tested in parallel, $n=2$), which is close to the optimal theoretical value (average 370 copies per tube).

The concentration of the target RNA could be accurately quantified within the scope of the standard curve.

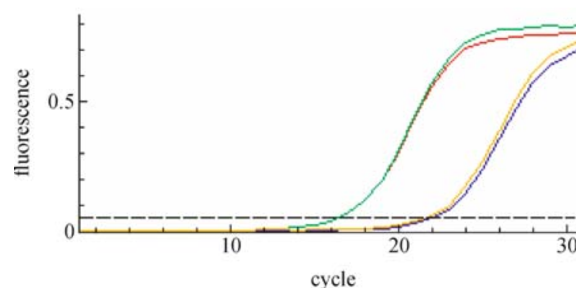

Fig. 4 Amplification profile of detection for target RNA in sample 1# (*Phalaenopsis* sp.). From left to right, the amplification contained (the left side two curves were of 10^3 times dilution; the right side two curves were of 10^5 times dilution) purified total RNA, respectively.

3.4 Results of trace quantitative detection

While the concentration of one time dilution of sample 4# as template was used, the related mean C(T) value ($n=2$) was 22.37, and the related C(T) value of plasmid standards (2.6 copies per tube) was 22.04. As such, the amount of genomic template from sample 4# was estimated at less than 3 copies per tube. The templates from two times dilution of samples 4# (equaling to 1–2 copies per tube) could be amplified and qualitatively detected, and its amplification fluorescence curves are shown in Fig. 5 and samples 3# (one time dilution, negative control, $n=2$) were not detected (shown in Fig. 5). Therefore, the smallest amount of genomic template from which the target RNA could be amplified and qualitatively detected by real-time RT-PCR was estimated at 1–2 copies per tube.

3.5 BLAST analysis

The amplicons contained total *cp* gene of CyMV corresponding to nt 5481 to 6152 (672 bp) and some upstream and downstream sequences.

The sequence analysis showed that amplicons of CyMV strain shared between 86% to 98% sequence identity with those of other CyMV strains in the NCBI GenBank. Although the nucleotide sequences of DNA fragments amplified from different orchid species or different farms with CyMV primer showed slight diversity, they had high homology to *cp* gene of CyMV isolates around the world. Therefore, the virus infecting orchids in Hangzhou was identified as CyMV.

BLAST analysis revealed that our isolate was most

Table 2 Results of quantitative detection of RNA in Orchids samples

sample types	serial number	diluted times	C(T)	RNA template copy number
Phalaenopsis sp. (positive)	1#	10^3	16.18	3.34×10^4
	1#	10^3	16.16	3.40×10^4
Phalaenopsis sp. (positive)	1#/100	10^5	21.70	351
	1#/100	10^5	21.39	453
Arachnis sp. (negative)	3#	1	N/A	not detected
	3#	1	N/A	not detected
Arachnis sp. (trace virus)	4#	1	22.28	–
	4#	1	22.46	–
	4#/2	2	22.47	–
	4#/2	2	22.52	–

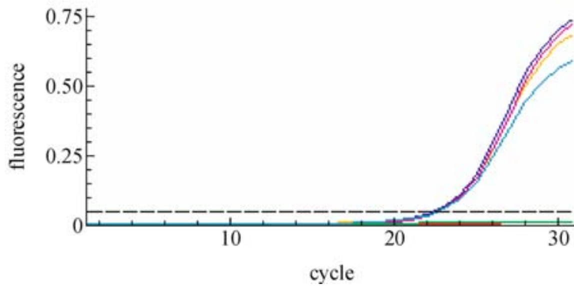

Fig. 5 Amplification profile of detection for target RNA in the samples (*Arachnis* sp.). Sample 4#: S-shape amplification profile curves ($n = 4$), Sample 3# (negative controls): nearly parallel to x-coordinate amplification lines ($n = 2$).

closely related to domestic CyMV strain AM055640.2 (data from NCBI) that was isolated from Kunming city (KM), Yunnan Province. There were only 10 different base-pair types, and 98% sequence of them was homologous (Fig. 6).

4 Discussion

4.1 Quantitative (real-time) PCR (qPCR) represents a highly sensitive, sequence-specific, and reproducible technique for quantitation of nucleic acids and detection method for trace amount of the virus

This real-time PCR offers a sensitive, high-throughput, and rapid method for CyMV detection. In our experiments, the standard curve was drawn based on the linear relationship between the logarithm (base 10) of the quantity of target sequence and cycle threshold. While the concentration of plasmid DNA falling within the range from 2.6×10^7 to 2.6×10^2 copies per tube established a regression equation, $y = -0.3583x + 10.32$, and a related coefficient, $r^2 = 0.995$. The amounts of starting templates in infected orchid leaves were estimated from the standard curves. As little as 1 to 2 copies per tube of CyMV could be detected with the *cp* gene as the target.

Real-time RT-PCR is currently the most sensitive

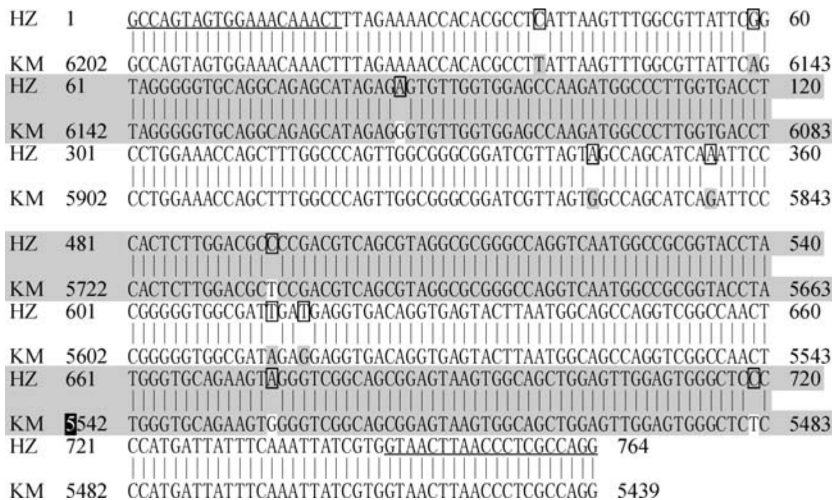

Fig. 6 Comparison of amplicons of CyMV strain isolated from Hangzhou (HZ), Zhejiang Province, with those isolated from Kunming city (KM), Yunnan Province

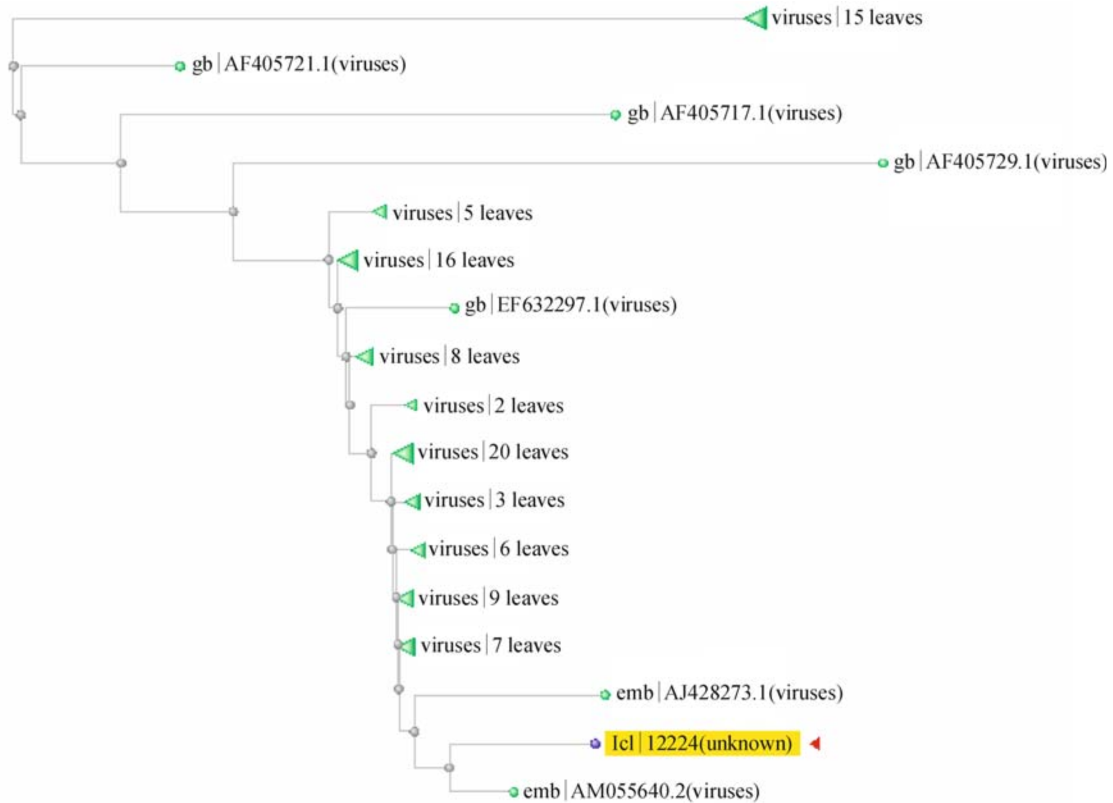

Fig. 7 Distance tree of the *cp* genes from different CyMV strains. 12224(unknown): sample 4# isolates; AM05640.2 (viruses): Kunming (KM) isolates.

method for detecting low viral concentrations, and it can be applied for quantitation of early infected trace CyMV before being used for mass production by tissue culture.

4.2 Comparison of sensitivity between ELISA, conventional RT-PCR, and real-time RT-PCR

Although traditional RT-PCR techniques can detect a low titer for RNA of CyMV, they are not quantitative. qPCR represents a highly sensitive, sequence-specific, and reproducible technique for the gel-free detection and quantitation of nucleic acids.

To evaluate the difference in sensitivity between ELISA and real-time RT-PCR, two samples of *Arachnis* sp. with trace CyMV by artificial infection were used in our experiments. The results showed that CyMV could be tested by real-time RT-PCR in Sap-inoculation seven days later, whereas it will take 10–14 days later by ELISA. Therefore, real-time RT-PCR was more sensitive and less time consuming than ELISA or traditional RT-PCR. The real-time RT-PCR assay developed is a valuable new tool for detection and titer quantitation of CyMV.

Although the detection of CyMV by PCR has been reported to be highly sensitive, ELISA tests are still frequently used for routine viral screening by orchid growers and the industry because of their specificity, the scope provided for standardization, and inexpensive assay facilities.

4.3 Distance tree analysis

The accession numbers for these complete *cp* gene nucleotide sequences of CyMV were obtained from the GenBank nucleotide database at NCBI. These nucleotide sequences were aligned and analyzed by nucleotide blast in NCBI, and the results show that our isolate was most closely related to domestic CyMV strain AM55640.2 that was isolated from Kunming (KM), Yunnan Province (shown in Fig. 7). Because Kunming city, Yunnan Province, is a major cultivation area of orchids in China, we speculate that the CyMV of orchids in Hangzhou, Zhejiang Province, may be derived from those of Kunming city, Yunnan Province, China.

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