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Project Title:

Development of Polymerase Chain Reaction for Rapid Detection of Grapevine Leafroll
Associated Viruses 1 through 5

Principal Investigator:

Adib Rowhani, Plant Pathology Specialist, Department of Plant Pathology/Foundation Plant
Materials Service, University of California, One Shields Avenue, Davis, CA 95616
Phone: (530) 752-5401
Fax: (530) 752-2132
E-mail: akrowhani@ucdavis.edu

Cooperator:

Deborah A. Golino, Associate Cooperative Extension Specialist, Department of Plant
Pathology/Foundation Plant Materials Service, University of California, One Shields
Avenue, Davis, CA 95616
Phone: (530) 754-8102
Fax: (530) 752-2132
E-mail: dagolino@ucdavis.edu

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Concise General Summary of Current Years Results

In the funding cycle 2002-2003 we mostly focused on grapevine leafroll associated viruses 1 and 2 (GLRaV). In our investigation we found that 3 isolates (among 17) of GLRaV 1 which initially were tested positive by ELISA were negative when used in reverse transcriptase-polymerase chain reaction (RT-PCR) using primers adopted for the detection of this virus. In addition, the PCR primers designed to amplify the coat protein (CP) gene used for sequencing and sequence comparison among different isolates failed to amplify the CP of these 3 isolates. Also in the same population we found 3 other isolates which were tested positive by ELISA and detection primers but not by the CP-specific primers. When we investigated the primers binding sites for these isolates, we found that at least two nucleotide mis-match (nucleotides in the viral genome used for designing the primers which were not complementary with the specific PCR primers sequences) were existed between PCR primers and viral genome.

For GLRaV-2 also the same problem existed for 2 and 2 different isolates which were tested positive by ELISA only or by ELISA and RT-PCR using detection primers, respectively. In this case only one isolate from each group showed that having sequence mis-match at the PCR primers' sites (as described for GLRaV 1). For two other isolates, we found that both were infected with GLRaV 1. The reason that we could get positive reaction in ELISA using GLRaV 2-ELISA system, because the antibody produced for GLRaV-2 also has the antibody component for GLRaV-1.

Based on the new sequence information we obtained for GLRaV 1 and 2 (see above), new sets of PCR-detection primers were designed and evaluated to detect all possible isolates of each in a single reaction and reduce the chance of getting false negative data.

All PCR-detection primers designed for GLRaVs 1 to 5 were further evaluated. The simplified sample preparation and one step RT-PCR protocols were also further evaluated using different types of tissue in different time of the year, especially for the sample preparation using the blotting methodology.

Objectives and Experiments Conducted to Accomplish Objectives:

The objective of this project is to develop fast, sensitive and reliable reverse transcriptase-polymerase chain reaction (RT-PCR) methods for the detection of grapevine leafroll associated viruses (GLRaV) 1 through 5 in grapevine. The specific objectives will include:

1. Develop RT-PCR methodologies for the detection of GLRaVs 1 through 5.
 - Sequence fragments of viral genome for viruses that do not have such sequences already available. The sequence information is necessary for designing PCR primers.
 - Design and evaluate virus-specific and universal primers for detection of single and multiple leafroll associated viruses, respectively.
2. Develop a simple and reliable sample preparation method for grapevine tissue for testing large number of samples at a time.
3. Investigate strain diversity among leafroll associated virus types. This will aid in design of primers that can detect all strains.
4. Investigate and develop protocols for colorimetric RT-PCR assay.

Experimental procedures:

Objective 1: To develop RT-PCR methodology for the detection of GLRaVs 1 through 5: Complementary DNAs (cDNA) were synthesized from viral genomes derived from total RNA extractions or double-stranded RNAs (dsRNA) and cloned. Cloned cDNAs were sequenced using the facilities available at UC Davis. Oligonucleotide primers (20-30 bases) were made from the sequences for RT-PCR use. For some of these viruses (e.g., GLRaV 2 and 3), the complete genome sequences published by other investigators were available from the GenBank. In these cases we used the available information for designing PCR primers. In addition, for GLRaVs-1 to -3, sequences obtained from the same genome segment of up to 10 different isolates of each (obtained either from the Genbank or cloned and sequenced in our laboratory) were piled up and specific primers were designed to identify all isolates of the same virus (objective 3). The specificity of each primer pair in detecting the virus was evaluated by testing them against number of different virus isolates collected from different regions. The primer pairs which specifically amplified each viral genome and its different isolates and strains were selected for virus detection application and field survey work. We also investigated the use of nested RT-PCR in detection of these viruses. This method is reported to be more sensitive than regular PCR. In this method, our general PCR primers were used to amplify a target sequence. Then part of the amplified product was diluted and an aliquot was added to a different PCR reagent containing new set of primers which were designed to internally amplify a segment from the original PCR product.

Objective 2: Develop a simple and reliable sample preparation method for grapevine tissue for testing large number of samples at a time: Grapevine tissues contain large amounts of polysaccharides, phenolic compounds and other substances that have been proven to make both nucleic acid extraction and virus detection by RT-PCR difficult and unreliable. Therefore, preparing samples from grapevine tissues for RT-PCR usually is a time consuming process and may take hours to prepare. In this objective, different buffers, chemical additives and treatments will be tested and an optimum sample preparation method will be developed.

Objective 3: Investigate strain diversity among leafroll associated virus types. This will aid in design of primers that can detect all strains: We have gathered ELISA data from large number of different isolates on GLRaV 1 through 5. These ELISA data indicated that the OD405 reading values (reading was done by an ELISA plate reader) for different plants varied from very low and boarder line readings to very high readings. These data suggested the possibility of the occurrence of more than one strain of the virus in different plants. Therefore, we designed specific PCR primers to amplify the coat protein gene of these viruses and study their sequences. For each virus we will select at least 10 different isolates (or less for those viruses which are not reported to be widely spread and we do not have access to many), preferably from different locations or regions, and compare and study their sequences. To compare the sequences we will use a computer Pileup program. Finally, specific PCR primers will be designed from the region exhibiting perfect nucleotide match among all these isolates for each virus. These primers will be evaluated and adopted for RT-PCR detection of the above mentioned viruses.

Objective 4: Investigate and develop protocols for colorimetric RT-PCR assay: We have developed a much simplified colorimetric assay for the detection of some of the viruses attacking fruit and nut trees (7). In this protocol the RT-PCR products were analyzed in an ELISA plate and the resulting color development (for virus-positive samples) were evaluated and quantified by an ELISA reader. In colorimetric PCR, the microtiter plates were coated with streptavidine. After 1 hour incubation at 37C, the plates were washed and then incubated with virus-specific biotin-labeled detection probes (an oligonucleotide complementary to an internal site of PCR product for a given virus) and incubated for 30 min at 37C. After washing, the denatured PCR product labeled with Digoxigenin (PCR product was labeled by adding Digoxigenin-labeled dUTP in the PCR reaction) was added and incubated for 1.5 hr at 46C. Plates were washed and incubated with alkaline phosphatase-conjugated antibody prepared to Digoxigenin and incubated for 1 hr at 37C. Finally, the plates were washed and incubated at room temperature with colorimetric substrate for 1-2 hours. Color development was then analyzed by measuring absorbance at 405 nm with an ELISA plate reader.

Summary of Research by Objectives:

Objective 1: We have designed and evaluated specific detection PCR primers for GLRaVs-1 to 5 (Fig. 1). These primers were designed based on the information we have gathered on sequence pileup experiments (objective 3) to ascertain that the primers sequences have almost 100% match with the sequences of all the isolates for each virus used in this investigation. Nested PCR primers were also designed for all these five different GLRaVs and are being used for their detection in

samples which regular PCR test results are inconsistent or weak (due to low virus concentration or uneven distribution in the plant) and they worked quite well (Fig. 2). For example, the detection limit using regular PCR primer for LR-1 was at 1:1 dilution of the sample while with nested PCR this dilution increased to 1:100 (Fig. 2A). The same was true for GLRaV-3 in which its limit of detection with the regular PCR primer was at 1:10 while when using nested PCR a strong band (representing the PCR product) was visible at 1:100 dilution (Fig. 2B).

Objective 2: A simple sample preparation method for RT-PCR detection of grapevine viruses in grape tissue was developed. This simplified method is not any more complex or labor intensive compared to the method used in ELISA. In this method grape tissues were homogenized in ELISA extraction buffer (a carbonate-based buffer containing PVP40, BSA, and Tween 20) at a ratio of 1:20 to 1:40 of sample:buffer, and two microliters from this homogenate were added to 25 microliters of GES buffer (glycine buffer containing EDTA, 2-mercaptoethanol, and NaCl) and boiled at 95C for 5-10 min. Finally, 2 microliters from this preparation was added to PCR cocktail and amplified. We also have developed a sample blotting method for collecting samples in the field. In this method, a cross section was made on leaf petioles or young shoots and the cut area blotted on special type of nitrocellulose or nylon membrane and air dried. Later in the laboratory each blot (representative of a sample) was cut and processed in GES buffer, then an aliquot from this processed sample was added to PCR reaction for amplification and detection. This is a very simple method for sample collection, it does not require any technical training to collect and blot the samples on the membrane. The blotted membranes could simply be shipped in an envelop to the laboratory for processing and testing. If necessary samples prepared by this method could be stored for a long time at room temperature before processing for PCR detection.

RT-PCR is a two step reaction procedure. In the first step, reaction cocktails are prepared to synthesize the first cDNA strand from the viral RNA genome (most plant viruses including all known grapevine viruses contain RNA as their genome) to be used by an enzyme called Taq DNA polymerase (which make copies only from DNA templates) and in the second step, a different cocktail containing Taq DNA polymerase enzyme is added to use the first cDNA strand as a template and amplify the desired segment from the viral genome. We have modified this two step RT-PCR and developed a one step reaction in which all the reagents necessary for the first cDNA synthesis and for amplification were mixed together including the dye and other reagent needed for gel electrophoresis and proceeded with the reactions in a thermocycler. This one-tube (or one step) method requires less sample manipulation, therefore, significantly reduces chance of cross contamination. Also it reduces the time required for set up of the PCR reaction and preparation of the product for gel electrophoresis and analysis.

Objective 3: In order to develop a reliable PCR detection methodology, it is very important to identify variations among different isolates (or strains) of each one of these GLRaVs and accordingly design PCR primers that could detect all diverse isolates. We maintain a collection of GLRaVs here in Davis and have evaluated the reactivity of number of these isolates selected from different types of GLRaVs by ELISA. In this study we have selected 17, 17, 10, and 5 different isolates respectively, for GLRaVs-1, -2, -3, and -5. For GLRaV-4 only one isolate was available.

These selected isolates were all tested positive by ELISA with different degrees of reaction. The coat protein gene from these isolates was amplified by RT-PCR (using specific primers designed for amplification of the coat protein gene) and sequenced. For GLRaV-1, 680 nt sequences of the coat protein from 17 different isolates were compared with each other and with the one published in the GeneBank (GB). An example of sequence pile up and nucleotide comparison is represented in Fig. 3. The complete sequence matches in this figure are designated by asterisks. These isolates also included 3 which were tested positive by ELISA and PCR (using the primers used for the detection of the virus) but did not amplify the coat protein gene and 3 other isolates which were positive by ELISA but negative by detection and coat protein primers. Data showed that these 6 isolates had at least 2 nucleotide mis-match at the primers site, therefore, the PCR primers could not attach to the target sequences and failed the amplification. Overall, we found that all these isolate had 91-94% homology with the sequence of the isolate published in the GB and 88-99% sequence homology among themselves at the coat protein gene segment compared. For GLRaV-2 also about 654 nt sequences covering the coat protein gene of 17 different isolates were compared with each other and with the one published in the GB. From this sequence, one pair of primers, LR2CP1 and LR2CP2, which flank the coat protein region, was designed. At the beginning we had problem amplifying the coat protein gene by this pair of primers. Repeated efforts using different types of tissue or use of dsRNA as template did not produce any product. Finally, we developed a nested RT-PCR method (which showed to be 10-100 times more sensitive than regular RT-PCR) and designed another pair of primers, LR2CP3 and LR2CP4, and used them in combination with previously designed primers LR2CP1 and LR2CP2. Initial RT-PCR was carried out with primers LR2CP3 and LR2CP4. This reaction resulted in a PCR product of 715 nt in size (because of low amount it was non detectable on gel). This PCR product was then diluted 15 times and used in a second round of nested PCR using primers LR2CP3 and LR2CP2 resulted in a product of 654 nt in size. Fifteen isolates of this virus were eventually analyzed by using nested-RT-PCR. The PCR products were cloned, sequenced, and the sequences were analyzed. Thirteen of these isolates were very similar in their coat protein sequences, ranging from 99% to 100% in nucleotide sequence homology. These 13 isolates had 89% homology to the published GLRaV 2 virus sequence. four of these isolates were more divergent and they had 89% and 92% sequence homology to the rest of the isolates. Among all the isolates included in this investigation, 2 isolates were tested positive by ELISA and negative by detection and coat protein primers, also another 2 which were positive by ELISA and detection primers but negative by the coat protein primers. By modifying the sample preparation methodology and using the total RNA extract we could amplify the coat protein gene of one of the isolates from each of the first and second groups and study their sequences. We were not able to amplify any product from the other 2 isolates in these groups even from the one in the second group which was tested positive once by detection primers indicating that we had a false positive or contamination in our first experiment. The same two isolates were ELISA positive indicating that the antibody we use for the detection of GLRaV-2 also contains antibody for other leafroll viruses, hence can detect viruses other than GLRaV-2 (the source of antibody used in this experiment also contains antibody to GLRaV-1). For GLRaV-3 10 isolates were compared and found that 9 of them had about 98-99% sequence homology (they were almost identical) among each other. Only one of these isolates was showing some differences (about 92% homology with other 9 isolates). . For GLRaV-4, the coat protein sequence is not available yet. We attempted several times using different strategies to clone and sequence part or the genome of this virus including the coat protein

gene but all our efforts failed. We used isolate LR106 in our investigation. The vine infected with this virus seemed to be singly infected with GLRaV-4. However, we tried to extract virus-specific dsRNA and use in our cloning and sequencing, but we could not obtain enough dsRNA for our experiment due to the low titer of virus in the host. We increased the amount of tissue used for this purpose, but again the efforts failed. Some preliminary data indicated that this virus is closely related to GLRaV-5, therefore, we tried to use the coat protein sequence information available for GLRaV-5 and design degenerate PCR primers and use them for amplification of the coat protein of GLRaV-4. In this effort we obtained a PCR product from GLRaV-4 infected vine with expected size revealed in gel electrophoresis, but when we sequenced the product and compared it with other GLRaVs, it did not show any homology with any of these viruses.

For GLRaV-5 we had access to only 5 isolates. We designed a pair of PCR primer that could amplify the coat protein gene of this virus. We used this pair of primer, amplified the coat protein gene and sequenced. This sequence consisted of 786 nt. Sequence comparison among these 5 isolates showed 95-99% homology indicating that these tested viruses were quite homogeneous.

Objective 4: For this objective, we designed specific probes for each of the viruses used in this investigation for trapping the PCR products in ELISA plates. Our many attempts failed to detect these viruses by the colorimetric PCR. Finally we concluded that one of the necessary components used in this reaction, Dig-11-dUTP labeled reagent, was not stable enough to maintain through the PCR reaction. In the control reactions for each virus, when dTTP (substitute for dUTP in regular PCR) was used and the products were analyzed by gel electrophoresis, the related PCR products were observed on the gel, while the results with Dig-11-dUTP on the gel were not consistent and usually a smeared bands (instead of sharp intense bands) were observed. Our experiments indicated that the problem is related to the quality of the product. Boehringer Mannheim Chemical Co. had the monopoly on the product and their labeling product was working very well in our PCR system. About 2 years ago the company was merged with Roche Chemical Co. and since then their product has not been working well. We also repeatedly contacted the Roche Co. and consulted with their technical personnel about the product but unable to fix the problem

Reliability and sensitivity of RT-PCR for the detection of GLRaVs-1 to -5 was compared with ELISA and with symptom expression of leafroll disease on its biological indicator. From 137 vines tested in this experiment, 26, 16, and 29, respectively tested positive on biological indicator, ELISA, and RT-PCR. The results indicate that the RT-PCR is more sensitive than either indexing on biological indicator host or ELISA. For GLRaV-5 in this experiment only PCR test was compared, because we did not have access to a reliable ELISA reagent for this virus. We have established confidence on our PCR procedure for GLRaVs and have started using the procedure routinely for testing all quarantine and newly introduced grape selections (brought to FPMS for disease testing and elimination). Also we have provided the RT-PCR detection services to grapevine nurseries and growers for testing their valuable and propagative source plants for different viruses.

Publications or Reports:

1. Golino, D.A., Sim, S., Gill, R., and Rowhani, A. 2002. California mealybugs can spread grapevine leafroll disease. *Cal. Agri.* 56:196-201.
2. Golino, D.A., Sim, S., and Rowhani, A. 2000. Identification of the latent viruses associated with young vine decline in California. Abstract and presentation in the "13th Meeting of the International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine". 12-17 March 2000. Adelaide, South Australia.
3. Routh, G., Zhang, Y.P., Saldarelli, P., and Rowhani, A. 1998. Use of degenerate primers for partial sequencing and RT-PCR-based assay of grapevine leafroll-associated viruses 4 and 5. *Phytopathology* 88: 1238-1243.
4. Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y.P., Chin, J., and Green, M. 2000. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. Abstract and presentation in the "13th Meeting of the International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine". 12-17 March 2000. Adelaide, South Australia.
5. Rowhani, A., Chin, J., Zhang, Y.P., Biardi, L., and Golino, D.A. 1999. Simplified extraction method for sample preparation for PCR and development of a colorimetric detection technique for analysis of PCR products of virus-infected grapevine. *Am. J. Enol. Vitic.* 50: 374.
6. Weber, E., Golino, D.A., and Rowhani, A. 2002. Laboratory testing for grapevine diseases. *PWV*, January/February:13-26.
7. Zhang, Y.P., and Rowhani, A. 2000. A strategy for rapid cDNA cloning from double-stranded RNA templates isolated from plants infected with RNA viruses by using Taq DNA polymerase. *J. Virol. Methods* 80: 59-63.

Research Success Statements:

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a very sensitive and reliable molecular methodology which have been developed and used for the detection of many plant RNA viruses (viruses which contain RNA as their genome constituent, which include all known viruses in grapevine). In this project we developed RT-PCR detection methodology for the detection of grapevine leafroll associated viruses 1 to 5 (GLRaV) and a working protocol was developed.

In order to develop a reliable RT-PCR detection method, it is very important to identify variations among different isolates (or strains) of each one of these GLRaVs and accordingly design PCR primers that could detect all diverse isolates. In this experiment, we cloned and sequenced the coat protein gene from 5-17 different isolates of each one of these isolates, sequences were compared among isolates selected for each virus, and PCR primers were designed in order to detect all variants.

In general, sample preparation for PCR test is a tedious and time consuming process, especially when grapevine tissues are involved. These tissues contain large amounts of polysaccharides and phenolic compounds that are detrimental to different enzymes used in PCR procedure, therefore, they should be eliminated. A simple sample preparation methodology was developed and refined to use for testing grapevine plants for grapevine leafroll associated viruses 1 to 5 by RT-PCR. In addition, a simple blotting methodology for sample collection in the field was developed. In this method, leaf petioles or young shoots are cut and blotted on specific nylon or nitrocellulose

membrane and then these membranes are brought to the laboratory for processing and virus detection. The advantages of this method are: no technical training is required, and if necessary, the samples can be stored for a long time before they are processed for testing.

Reliability and sensitivity of RT-PCR for the detection of GLRaVs-1 to -5 was compared with ELISA and with symptom expression of leafroll disease on its biological indicator, Cabernet Franc. The results indicated that RT-PCR was more sensitive than either indexing on biological indicator host or ELISA.

The methodologies developed in this investigation were shared with other private laboratories in the State to improve their sampling and testing methods, and with other scientists in the nation and worldwide.

FIGURE 1

Gel Analysis of RT-PCR Products. Petiole samples from infected and healthy grapevines were prepared and used in PCR detection of GLRaVs 1-5.

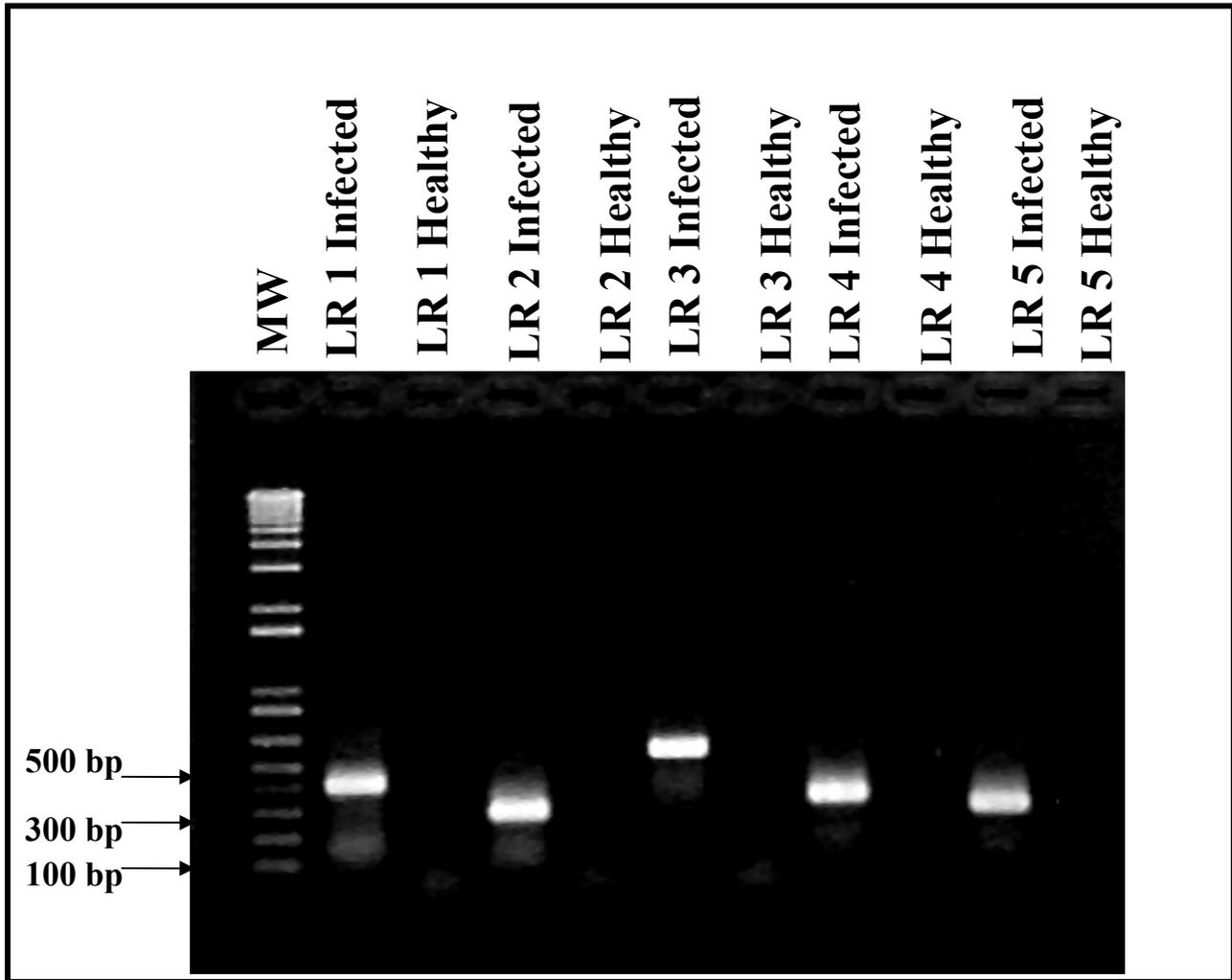


Fig. 2. Nested RT-PCR method used for amplifying GLRaV 1 (panel A) and 3 (panel B) in comparison to regular RT-PCR. Samples were prepared and serially diluted from 1:1 to 1:10,000 and tested by regular RT-PCR (Lanes a, c, e, g, and i). Then an aliquot from regular RT-PCR product was added to nested PCR reagent and amplified again using nested primers (Lanes b, d, f, h, and j). Regular, represents the regular RT-PCR product and Nested, represents nested RT-PCR product.

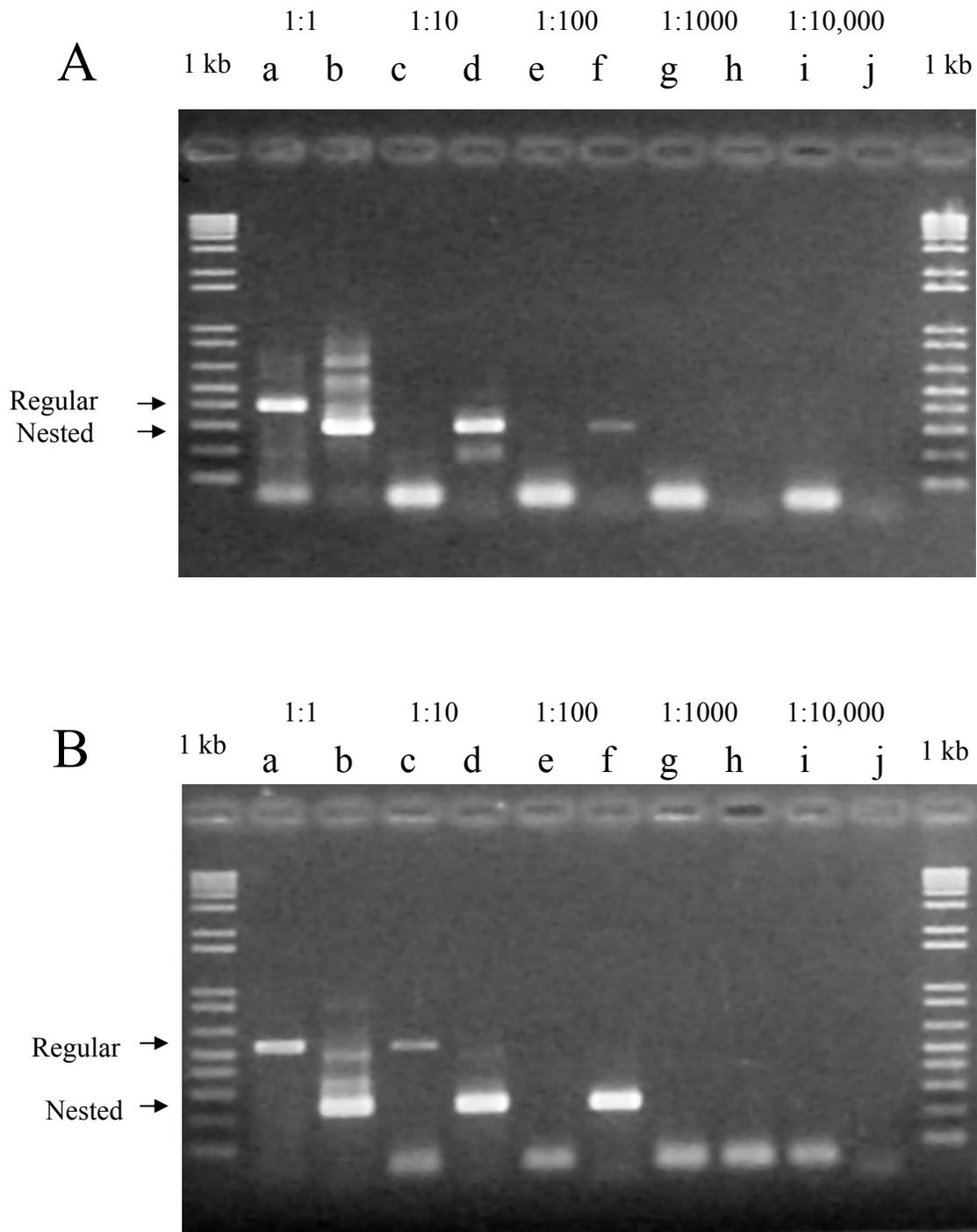


Fig. 3. Sequence alignment among 17 different isolate of GLRaV-1 and the sequence published in the GeneBank (LR1-GB-CP). Asterisks indicate complete match among the sequences. Total of 680 nucleotide (nt) from each isolate has been compared.

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A2V49-CP      CTCTCCGTCCTTCTCGACCATCTACGGGTCAGCGTTCGGGGGATTCTGAGGTGGCCAGAC 60
A1V49-CP      CTCTCCGTCCTTCTCGACCCTCTACGGGTCAGCGTTCGGGGGATTCTGAGGTGGCCAGAC 60
A2V35-CP      CACTTCGCCAATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
A1V1-CP       CACTTCGCCAATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
A1V79-CP      CACTTCGCCAATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
A2V43-CP      CACTTCGCCAATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
LR1-9-CP      CACAACGCCTATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAAAC 60
LR1-13-CP     CACAACGCCTATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
LR1-02-CP     CACAACGCCTATCTCGACCCTCAACGGGTCAGCGTTTAGGGGATTCTGAGGTAGCCAGAC 60
LR1-16-CP     CACAACGCCTATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
LR1-20-CP     CACAACGCCTATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCTAGAC 60
LR1-4-CP      CACAACGCCTATCTCGACCCTCTACGGGCCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
LR1-7-CP      CACAACGCCTATCTCGACCCTCTACGGGTCAGCGTTCAGGGGATTCTGAAGTGGCCAGAC 60
LR1-22-CP     CACAACGCCTATCTCGACCCTCAACGGGTCAGCGTTCAGGGGATTCTGAGGTGGCCAGAC 60
LR1-23-CP     CACTCCGCCCATCTCGACCCTCGACGGGTCAGCGTTCGGGGGATTCTGAGGTGGCTAGAC 60
A3V87-CP     CACTCCGCTTGTCTCGACCCTCGACGGGTCAGCGTTCGGGGGATTCTGAGGTGGCCAGAC 60
LR1-18-CP     CACAACGCCTATCTCGACCCTCGACGGGTCAGCGTCGCGGGGATTCTGAGGTGACCAGAC 60
LR1-GB-CP     CACAACGCCTATCTCGACCCTCTACAGGTCAGCGATTTGGGGATTCTGAGGTGGTCAGAC 60
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A2V49-CP      GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
A1V49-CP      GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
A2V35-CP      GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
A1V1-CP       GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
A1V79-CP      GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
A2V43-CP      GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
LR1-9-CP      GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
LR1-13-CP     GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTTACTAATCCCG 120
LR1-02-CP     GTGAAATGGGTGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATTTTCACTAATCCCG 120
LR1-16-CP     GTGAAATGGGTGGCAAGCTTAAAAGAACTTTCAATATCGCAGAAATCTTTTCTAATCCCG 120
LR1-20-CP     GTGTAATAGGTGACAAGCTAAAAGAACTTTTAATATAGCGGAAATCTTTACTAATCCCG 120
LR1-4-CP      GTGAAATGGGCGACAAACTTAAAAGAACTTTTAATATCGCAGAAATCTTTACTAATCCCG 120
LR1-7-CP      GTGAAATGGGTGACAAGCTTAAAGAGAACTTTTAATATCGCAGAGATCTTTACTAATCCCG 120
LR1-22-CP     GTGATATGGGCGACAAGCTTAAAAGAACTTTTAATATCGCAGAAATCTTCACTAATCCCG 120
LR1-23-CP     GTGAAATGGGCGATAAGCTTAAACGAACTTTTTAATATTGCCGAAATCTTTACCAACCCCG 120
A3V87-CP     GTGAAATGGGGGACAAGCTTAAACGTACTTTTTAATATTGCCGAAATCTTCACTAATCCTG 120
LR1-18-CP     GTGAAATGGGTGACAAGCTTAAACGAACTTTTTAATATTGCAGAAATCTTTACAAACCCCG 120
LR1-GB-CP     GTGAAATGGGCGACAAGCTTAAAAGAACTTTTAATATAGCAGAAATCTTTACCAACCCCG 120
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A2V49-CP      AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180
A1V49-CP      AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180
A2V35-CP      AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180
A1V1-CP       AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180
A1V79-CP      AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180
A2V43-CP      AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180
LR1-9-CP      AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180
LR1-13-CP     AGATGAATATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCTGTAGTTGTACCAACCG 180

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LR1-02-CP AGATGAATATTATTTTTGAACCACCAAAGGACATGGAGGTTTCGGTAGTAGTACCAACTG 180
 LR1-16-CP AGATGAACATCATCTTTGAACCACCGAAGGAAATGGAGGTTTCGGTGGTAGTACCAACAG 180
 LR1-20-CP AGATGAACATTATCTTTGAACCACCAAAGGAAATGGAGGTTTCGGTAGTGGTACCAACCG 180
 LR1-4-CP AGATGAACATCATCTTTGAACCACCAAAGGACATGGAGGTTTCGGTAGCAATACCAACCG 180
 LR1-7-CP AGATGAATATCATCTTTGAACCACCAAAGGACATGGAGGTTTCGGTAATAGTACCAACCG 180
 LR1-22-CP AGATGAATATCATCTTTGAACCACCAAAGGATATGGAGGTTTCGGTAATAGTACCAACCG 180
 LR1-23-CP AGATGAATGTTATCTTTGAACCACCAAAGGACATGGAGGTTTCGGTAGTAGTACCAACCG 180
 A3V87-CP AGATGAATATTATCTTTGAACCACCAAAGGACATGGAGGTTTCGGTAGTAGTACCAACCG 180
 LR1-18-CP AGATGAATATCATTTTTGAACCACCAAAGGATATGGAGGTTTCGGTAATAGTGCCAACCG 180
 LR1-GB-CP AGATGAATATCATCTTTGAACCACCAAAGGATATGGAGGTTTCGGTAGTATTACCAACCG 180
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A2V49-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACGGAATTAATAAATTTGT 240
 A1V49-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACGGAATTAATAAATTTGT 240
 A2V35-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACGGAATTAATAAATTTGT 240
 A1V1-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACGGAATTAATAAATTTGT 240
 A1V79-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACGGAATTAATAAATTTGT 240
 A2V43-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACAGAATTAATAAATTTGT 240
 LR1-9-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACAGAATTAATAAATTTGT 240
 LR1-13-CP GGCCCGGCTTGGTTACGCCAGCGGTGGCAACAGCTATTTCAACAGAATTAATAAATTTGT 240
 LR1-02-CP GACCCGGCTTGGTTACACCAGCGGTGGCGACAGCTATTTCAACAGAATTAATAAATTTAT 240
 LR1-16-CP GGCCCGGCTTAGTTACACCAGCGGTGGCGACAGCAATATCAACAGAATTAATAAATTTAT 240
 LR1-20-CP GACCCGGCTTGGTTACACCAGCGGTGGCAACGGCTATATCAACAGAATTAATAAATTTAT 240
 LR1-4-CP GACCCGGCTTGGTTACACCAGTGGTGGCAACAGCTATTTTCGTCAGAATTAATAAATTTAT 240
 LR1-7-CP GGCCTGGCTTGGTTACACCAGCGGTGGCGACCGCTATTTTCGACAGAATTAATAAATTTAT 240
 LR1-22-CP GACCCGGCTTGGTTACGCCAGCGGTGGCGACGGCCATTTCTACAGAATTAATAAATTTAT 240
 LR1-23-CP GACCCGGCTTGGTCACGCCGGCGGTGGCAACTGCAATTTCCACAGAATTAATAAATTTAT 240
 A3V87-CP GACCCGGCTTGGTCACACCAGCGGTGGCAACTGCAATTTCCACAGAATTAATAAATTTAT 240
 LR1-18-CP GACCCGGCTTGGTGACACCGCGGTAGCAACTGCGATTTCTACAGAATTAATAAATTTAT 240
 LR1-GB-CP GACCCGGCTTGGTGACGCCGGCGGTGGCAACTGCAATTTCCACAGAATTAAGAATTTAT 240
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A2V49-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 A1V49-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 A2V35-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 A1V1-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 A1V79-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 A2V43-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 LR1-9-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 LR1-13-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACTGACTTCTTCTTGGCAATGT 300
 LR1-02-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTTACGGACTTCTTCTTGGCGATGC 300
 LR1-16-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTTACAGACTTCTTCTTGGCAATGT 300
 LR1-20-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTTACGGACTTCTTCTTAGCAATGT 300
 LR1-4-CP GTGCTGAAGTGATGGGTAACACGGATCAGAAAAGTCTTACAGACTTCTTCTTGGCAATGT 300
 LR1-7-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAGTCTCACAGACTTCTTCTTGGCGATGT 300
 LR1-22-CP GTGCTGAAGTGATGGGTAATACGGATCAGAAAAGTCTCACAGACTTCTTCTTGGCGATGT 300
 LR1-23-CP GTGCTGAAGTGATGGGTAATACAGATCAGAAAAGTCTTACGGACTTCTTCTTGGCGATGT 300
 A3V87-CP GTGCTGAAGTGATGGGTAATACAGATCAGAAAAGTCTTACGGACTTCTTCTTGGCGATGT 300
 LR1-18-CP GTGCTGAAGTAATGGGTAATACAGATCAGAAAAGTCTTACGGACTTCTTCTTGGCGATGT 300
 LR1-GB-CP GTGCTGAAGTGATGGGTAATACGGATCAAAAAGTCTTACAGACTTCTTCTTGGCAATGT 300
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A2V49-CP TGCAACTGATGTTGACATTTAGCACACCACCAGACGCAGAGAGCAAAGAAGAGTATTTTG 360
 A1V49-CP TGCAACTGATGTTGACATTTAGCACATCACCAGACACAGAGAGCAAAGAAGAGTATTTTG 360

LR1-20-CP TCTCTCAATTTAGCAACGATGCCATTAGAGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660
 LR1-4-CP ACTCTCAATTTAGCAACGATGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660
 LR1-7-CP ACTCTCAATTTAGCAACGATGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660
 LR1-22-CP ACTCTCAATTTAGCAACGATGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660
 LR1-23-CP ACTCTCAGTTTGTAGCAACGACGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660
 A3V87-CP ACTCTCAGTTTGTAGCAACGATGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660
 LR1-18-CP ACTCTCAGTTTGTAGCAACGATGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660
 LR1-GB-CP ACTCTCAATTTAGCAACGATGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG 660

A2V49-CP GGCGGAAAAGTAATGTGA-CA 680
 A1V49-CP GGCGGAAAAGTAATGTGAACA 681
 A2V35-CP GGCGGAAAAGTAATGGGA-CA 680
 A1V1-CP GGCGGAAAAGTAATGTGA-CA 680
 A1V79-CP GGCGGAAAAGTAATGTGA-CA 680
 A2V43-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-9-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-13-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-02-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-16-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-20-CP GGCGGAAAAGTAATGTGA-AA 680
 LR1-4-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-7-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-22-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-23-CP GGCGGAAAAGTAATGTGA-CA 680
 A3V87-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-18-CP GGCGGAAAAGTAATGTGA-CA 680
 LR1-GB-CP GGCGGAAAAGTAATGTGA-CA 680
