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REOVIRUS-LIKE SEQUENCES ISOLATED FROM ADULT ASIAN CITRUS PSYLLID, (HEMIPTERA: PSYLLIDAE: *DIAPHORINA CITRI*)

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ABSTRACT

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, (Insecta: Hemiptera: Psyllidae), has been identified as a damaging pest and an efficient vector of the plant infecting bacterium (*Candidatus* Liberibacter *asiaticus*) which is strongly associated with the disease Huanglongbing (HLB), known as 'Citrus greening disease'. Huanglongbing has caused extensive economic losses in the citrus industries worldwide. Traditional control measures of the psyllid have proven to be ineffective and costly. Biological control measures have been shown to provide environmentally friendly management tools for insect pests. In this study, an expression library was prepared from adult psyllids in search of new pathogens that can be use as biological control agents. We identified 2 viral sequences: one 616 base pairs and a second, 792 base pairs. Both had significant similarity to viruses within the insect Reovirus group. Phylogenetic and homology comparisons indicated that the viral sequences were most closely related to the viruses in the Family *Reoviridae*, Genus *Fijivirus*, specifically *Nilaparvata lugens reovirus,* NLRV.

Key Words: Psyllidae, psyllids, citrus, HLB, Huanglongbing, Reoviridae, pathogen, virus

RESUMEN

El sílido asiático de los cítricos, *Diaphorina citri* Kuwayama, (Insecta: Hemiptera: Psyllidae), ha sido identificado como una plaga dañina y un vector eficiente de la bacteria que infecta las plantas (*Candidatus* Liberibacter *asiaticus*) que esta fuertemente asociado con la enfermedad Huanglongbing (HLB), conocida como 'La enfermedad de enverdecímento de cítricos'. Huanglongbing ha causado pérdidas económicas extensivas en la industria de cítricos por todo el mundo. Las medidas tradicionales de control del sílido han sido infructuosas y costosas. Las medidas de control biológico han mostrado que proveen herramientas de manejo favorable al medio ambiente para plagas de insectos. En este estudio, una "biblioteca de expresiones" fue preparada de los adultos sílidos en búsqueda de nuevos patógenos que puedan ser usados como agente de control biológico. Identificamos 2 secuencias de virus, uno de 616 pares de bases y una segunda de 792 pares de bases, que tuvieron una similitud significativa de los virus entre el grupo Reovirus de insectos. Compariciones filogenéticas y de homologia indicaron que las secuencias de los virus fueron mas relacionadas cercanamente con los virus en la familia Reoviridae, Genero *Fijivirus*, específicamente el *reovirus Nilaparvata lugens,* NLRV.

The Asian Citrus Psyllid, *Diaphorina citri* (Hemiptera: Psyllidae) is the most serious pest of citrus, due to the ability to transmit *Candidatus* Liberibacter *asiaticus*, CLa, a bacterium found in association with a severe disease in citrus trees, Huanglongbing, HLB (also known as Citrus Greening disease), and threatens the viability and production of citrus worldwide (Halbert & Manjunath 2004). To limit the spread of HLB, suppression of *D. citri* populations will require multi-tactic integrated pest management approaches (Hoy 1998). Currently chemical control is the easiest method of control. However, widespread use of chemical insecticides will result in the eventual development of resistant psyllids (Hoy 1998). Therefore, alternative approaches are

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needed. Like most animals, insects are susceptible to diseases caused by viruses, and many viruses have been applied as insecticides (Tweeten et al. 1981; Moscardi 1999). In the United States, Nuclear polyhedrosis viruses (NPVs) are used as insecticides (Thorne et al. 2007). The NPVs suppress the corn earworm moth (*Heliothis zea*), the Asian gypsy moth (*Lymantria dispar*), and the Douglas fir tussock moth (*Orgria pseudotsugata*) (Shieh & Bohmfalk 1980). These viruses have been shown to provide environmentally friendly management tools for insect pests. In this study, annotation of sequences extracted from whole *D. citri* resulted in the discovery and validation of viral sequence, which had significant homology to viruses within the family *Reoviridae*, genus, *Reovirus*. This is the first report of a virus in *D. citri*.

Materials and Methods

Library Construction

A cDNA library was constructed from wild adult *D. citri* from citrus trees in Picos Farm in Fort Pierce, FL in 2005 to investigate the biology and pathology of adult psyllids. About 5,000 insects were ground in liquid nitrogen and total RNA was extracted with guanidinium salt-phenol-chloroform procedure as previously described by Strommer et al. (1993). Poly (A) + RNA was purified with the Poly (A) Pure™ kit according to the manufacturer's instructions (Ambion, Austin, TX). A directional cDNA library was constructed in Lambda Uni-ZAP® XR vector with Stratagene's ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA). The resulting DNA was packaged into Lambda particles with Gigapack® III Gold Packaging Extract (Stratagene). An amplified library was generated with a titer of 1.0×10^9 plaque-forming units per mL. Mass excision of the amplified library was carried out by Ex-Assist® helper phage (Stratagene). An aliquot of the excised, amplified library was used for infecting XL1-Blue MRF cells and subsequently plated on LB agar containing 100 µg/mL ampicillin. We recovered 5,760 bacterial clones containing excised pBluescript SK (+) phagemids by random colony selection.

Sequencing of Clones

We grew pBluescript SK (+) phagemids overnight at 37°C and 240 rpm in 96-well culture plates containing 1.7 mL of LB broth, supplemented with 100 µg/mL ampicillin. Archived stocks were prepared from the cell cultures with 75 µL of a LB-amp (100 µg/mL) -25% glycerol mixture and 75 µL of cells. Plasmid DNA was extracted by the Qiagen 9600 liquid handling robot and the QIAprep® 96 Turbo miniprep kit according to the recommended protocol (Qiagen, Valencia, CA). Sequencing reactions were performed with the ABI PRISM® BigDye™ Terminators V 3.0 (Applied Biosystems, Foster city, CA) along with a universal T3 primer. Reactions were prepared in 96-well format with the Biomek 2000™ liquid handling robot (Beckman Coulter, Fullerton, CA). Sequencing reaction products were precipitated with 70% isopropanol, resuspended in 15 µL sterile water and loaded onto an ABI 3700 DNA Analyzer (Applied Biosystems).

Sequence Verification and Analysis

When individual clonal DNA fragments were sequenced, they were verified and processed at 5× coverage. Base confidence scores were assigned with TraceTuner® (Paracel, Pasadena, CA). Low-quality bases (confidence score< 20) were trimmed from both ends of sequences. Quality trimming, vector trimming and sequence fragment alignments were executed with Sequencher® software (Gene Codes, Ann Arbor, MI). Sequences less than 100 nucleotides in length after both vector and quality trimming were excluded from the analysis. Additional ESTs that corresponded to vector sequences were removed from the dataset. Vector and low-quality sequence were trimmed and the sequences filtered for a minimum length (200 bp), producing 4972 high-quality ESTs. Putative function of cDNA clones were determined based on BLAST homology searches with the National Center for Biotechnology Information BLAST server (BLASTX, TBLASTX, BLASTN, http://www.ncbi.nlm.nih.gov). To estimate the number of genes represented in the library and the redundancy of specific genes, ESTs were assembled into "contigs" by Sequencher® based on the parameters of minimum overlap of 50 bases and 95% identity match. Two clones were identified for further analysis. To ensure sequence accuracy clones were bidirectionally sequenced 3 times. The resulting sequences were assembled into a contig with Sequencher® based on the same parameters used above.

Protein Analysis

The amino acid sequences were predicted with the 'Translate' program on the ExPASy server (http://au.expasy.org). The resulting sequences were then analyzed with BLASTP. The top 5 returns from BLAST analyses were used for phylogenetic comparison, as these included viruses related to the *Reoviridae*. Multiple sequence alignments of predicted psyllid-*Reovirus* amino acid sequences were performed with CLUSTAL W (DNA database of Japan; http://www.ddbj.nig.ac.jp/search/clustalw-j.html) with the neighbor-joining (NJ) method (Saitou & Nei 1987) based on genetic distances computed with

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Kimura's two-parameter model (Kimura 1980). TreeView was used to draw the NJ phylogenetic tree. NJ bootstrap analyses of 2,000 replicates were performed on each data set base based on a heuristic search to identify the most optimal unrooted tree. *Infectious bursal disease virus*, a dsRNA virus in *Birnaviridae* family, was used as an outgroup. Sequences used in the phylogenetic analysis included the 136.6 KD protein of *Nilaparvata lugens reovirus* (NP_619777), 'B' spike structural protein of *Fiji disease virus* (YP_249761), hypothetical protein of *Mal de Rio Cuarto virus* (YP_956845), P4 protein of *Rice black streaked dwarf virus* (NP_620461), RNA polymerase of *Nilaparvata lugens reovirus* (NP_619776), *Mal de Rio Cuarto virus* (YP_956848), *Fiji disease virus* (YP_249762) and hypothetical protein (P1) from *Rice black streaked dwarf virus* (NP_620452), p3 of *Heliothis armigera cypovirus* 5 (YP_001883321), an unnamed protein product of *Diadromus pulchellus idnoreovirus* (CAA56651), VP1 RNA-dependent RNA polymerase of *Infectious bursal disease virus* (NP_690839), and a putative surface protein of *Infectious bursal disease virus* (CAI43281).

Reovirus Population Evaluation

In May 2008, 100 Asian citrus psyllids were sampled from the U. S. Horticultural Research Lab, Picos Research Farm, Fort Pierce, FL, area to evaluate the incidence of the Reovirus in psyllid populations. For population evaluation, individual psyllids were homogenized in 20 µL of water and centrifuged at 8,000 × *g* for 1 min. Then 1.5 µL of dimethyl sulfoxide, DMSO (Sigma, St. Louis, MO) was added to 8.5 µL of supernatant and heated at 100°C for 5 min to denature the dsRNA. Two µL were used as a template for RT-PCR with the SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, Carlsbad, CA). RT-PCR was conducted with Dc-reo2F (5´-GGGCGATTGATGCTATCGTA-3´) and Dc-reo2R (5´-TGAGCGTATCGAATTTGACG-3´) with cycling condition of 50°C for 30 min, then 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s followed by 72°C for 10 min.

Results and Discussion

Two *Reovirus*-like sequences were isolated from psyllid cDNA library: a 616 bp of Dc-Reo1 (accession number; AB45810) and 712 bp of Dc-Reo 2 (accession number; AB455528). RT-PCR was performed to determine whether these sequences were of psyllid or virus origin. To eliminate psyllid RNA, RNAs were treated with S1 nuclease (Invitrogen). This enzyme degrades single strand RNA. Also as a negative control, RT-PCR was conducted without Reverse transcriptase. Result shows when dsRNA was template, virus gene were amplified with S1 nuclease treatment (Fig. 1). This shows that PCR was derived from double strand RNA not single strand RNA, i.e., from virus RNA not psyllid RNA. The deduced Dc-Reos amino acid sequences had the highest homology to *Nilaparvata lugens reovirus* (NLRV), a *Fiji disease virus* (FDV) (McQualter et al. 2003), and next highest homology to a *Mal de Rio Cuarto virus* (MRCV) (Distéfano et al. 2003) (Fig. 2). Multiple sequence alignments of predicted psyllid-Reo1 amino acid sequences resulted in 48% shared identity to RNA polymerase of NLRV, 39% identity to RNA polymerase of the MRCV, 38% identity to RNA polymerase of FDV, and 22% identities to p3 of *Heliothis armigera cypovirus* 5 (Ha-CPV5) (Fig. 2A). Multiple sequence alignments of predicted Dc-Reo2 amino acid sequences resulted in 30% shared identity to segment S2 of the NLRV, 25% identity to a 'B' spike structural protein from segment 3 of FDV, 24% identity to segment S2 of MRCV, 25% identity toP4 protein of *Rice black streaked dwarf virus* (RBSDV) segment 4 and 20% identity to an unnamed protein product of *Diadromus pulchellus idnoreovirus* 1 (DpRV) (Fig. 2B). The NLRV segment S2 is pro-

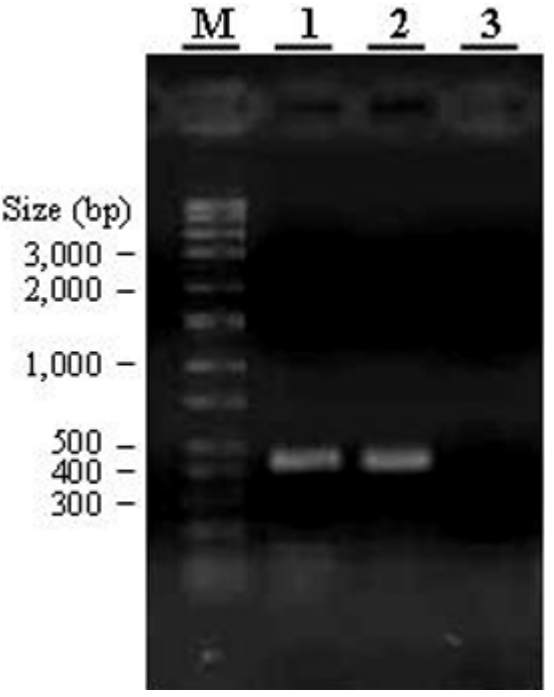
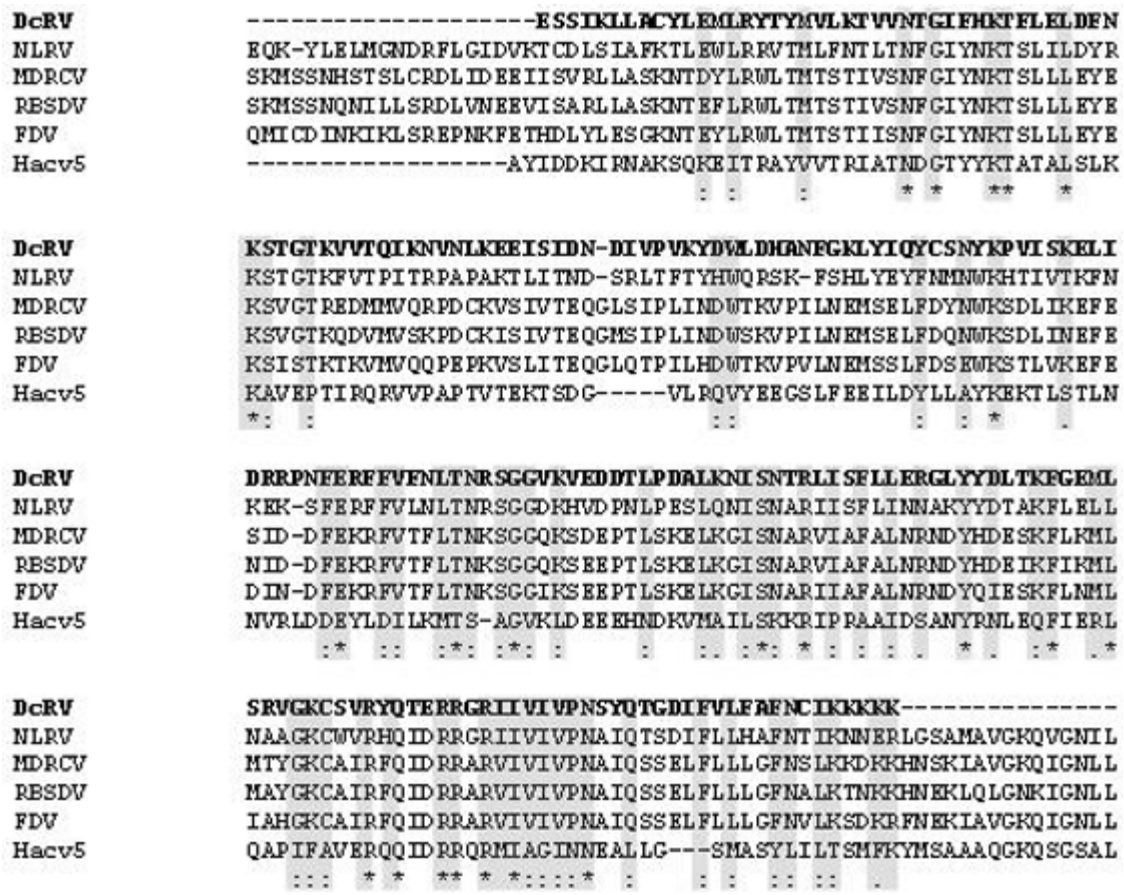


Fig. 1. Detection of DcRV from *Diaphorina citri* with Dc-reo2 primers. M; Direct Load™ Wide Range DNA Marker (Sigma, Saint Louis, Missouri). Lane 1; RT-PCR product from total RNA from psyllids as a template. Lane 2; RT-PCR product from S1 nuclease treated total RNA from psyllids as a template. Lane 3; PCR product from RNA from psyllids as a template.

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Figs. 2A and 2B. (2A) Multiple alignment of amino acid sequences from *Diaphorina citri* of Dc-Reo1; (2B) Alignment with the homologous proteins of *Nilaparvata lugens reovirus* (NLRV), *Fiji disease virus* (FDV), *Mal de Rio Cuarto virus* (MRCV) *Rice black streaked dwarf virus* (RBSDV), *Heliothis armigera cypovirus* 5 (HaCV) and *Diadromus pulchellus idnoreovirus* (DpRV). Similar or identical amino acids are highlighted.

posed to be the B-spike protein located on the surface of the inner core of the virus coat protein (Nakashima et al. 1996).

Reoviruses have wide host ranges and are classified into 11 genera: *Orthorevirus, Obrivirus, Rotavirus, Coltivirus, Seadornavirus, Aquareovirus, Cypovirus, Idnoreovirus, Fijivirus, Phytoreovirus,* and *Oryzavirus* in the family *Reoviridae* by the International Committee for the Taxonomy of Viruses (2000). The genus *Fijivirus* are further classified into five groups based on vectors, plant hosts, and serological and nucleotide sequence similarities. *Fiji disease virus* (FDV) is the sole member of group 1, while group 2 contains *rice black streaked dwarf virus* (RBSDV), *maize rough dwarf virus* (MRDV), *Mal de Rio Cuarto virus* (MRCV), and *Pangola stunt virus* (PaSV). *Oat sterile disease virus* (OSDV) is the sole member of group 3, while group 4 and 5 contain *Garlic dwarf virus* (GDV) and *Nilaparvata lugens virus* (NLRV), respectively. Members of the genus usually have 10 dsRNA genome segments and most of them replicate in their plant hosts, in which they induce growth abnormalities. *Heliothis armigera cypovirus* 5 (Ha-CPV5) belongs to the genus *Cypovirus*, which resides in the family *Reoviridae* (Tan et al. 2008). Ha-CPV5 is a pathogen of *Helicoverpa armigera*, the most dangerous cotton pest in P.R. China. The *Diadromus pulchellus idnoreovirus* (DpRV) is a member of *Reoviridae* family, genera *Idnoreovirus* (Rabouille et al. 1994). The virus is found in the gut of *Diadromus pulchellus* (Hymenoptera) and is thought to be nonpathogenic toward *D. pulchellus* (Rabouille et al. 1994).

To analyze *D. citri Reovirus* (DcRV) relationships with other reoviruses, a phylogenetic tree was constructed by the neighbor-joining (NJ) methodology (Fig. 3). The topology of the tree showed that DcRV is most closely related to NLRV. Viruses within the *Fijivirus* genera are

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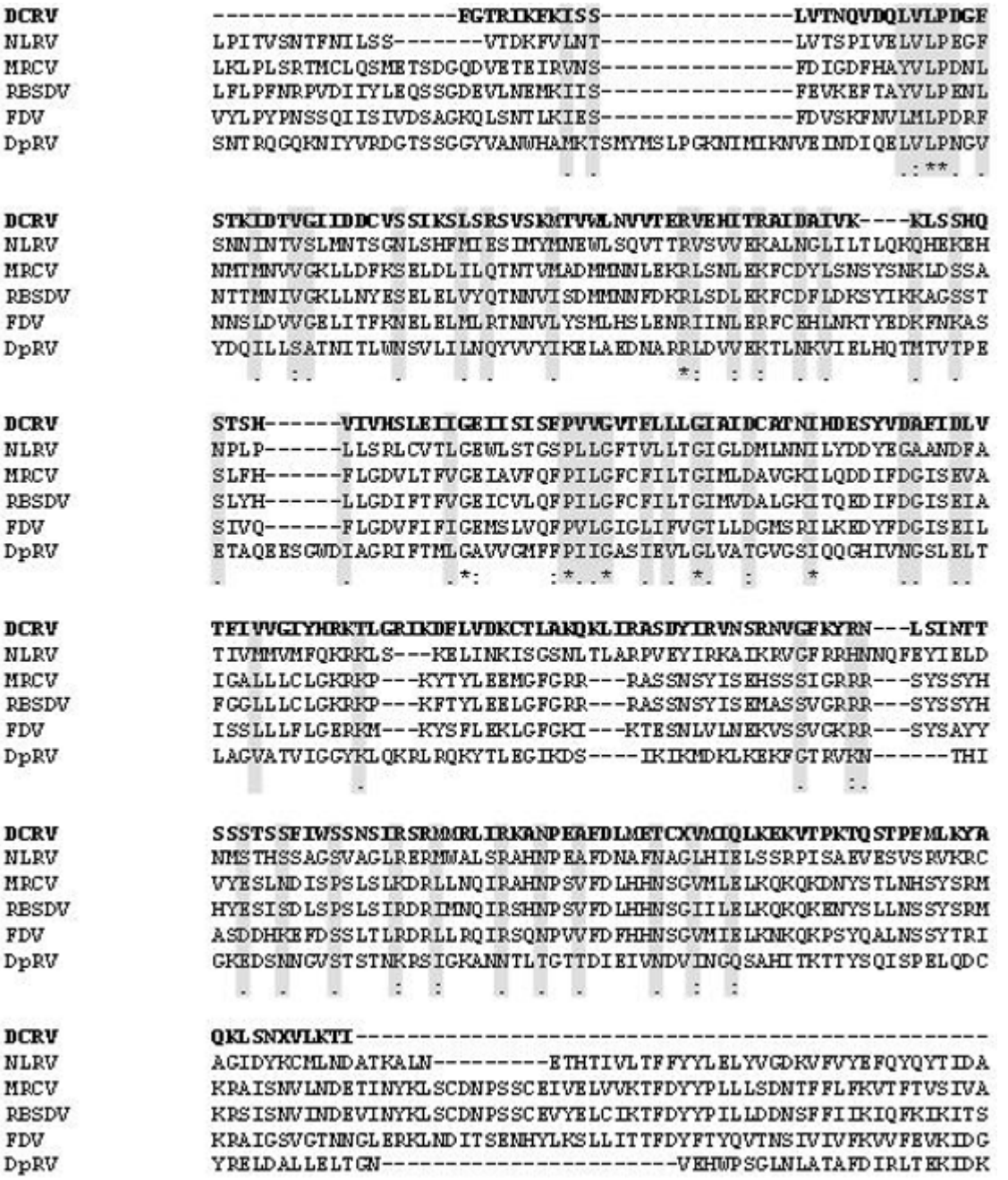


Fig. 2B.

grouped into 5 groups as mentioned before. Our phylogenetic tree also grouped 5 of the 7 sequences into their respective *Fijivirus* groups: FDV in group 1, MRCV and RBSDV in group 2 and the DcRV and NPLV in group 5. Ha-CPV5 and DpRV are different genus *Reovirus* members therefore they did not fit into a *Fijivirus* group. *Infectious bursal disease virus*, ds RNA virus in *Birnaviridae* family, was used as an out-group.

To confirm the incidence of psyllids infected by this *reovirus*, psyllids were collected from the Picos Research Farm, Fort Pierce, FL and assayed for the virus by RT-PCR with Dc-reo2 specific primers. Psyllids which were collected from the

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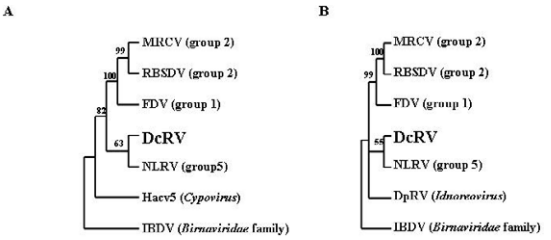


Fig. 3. (A) Phylogenetic tree conducted with *Diaphorina citri Reovirus* amino acid, Dc-Reo1; (B) amino acid sequences and related sequences by the NJ method. Numbers on the inside of nodes represent bootstrap values (%). Virus abbreviations: *Nilaparvata lugens reovirus* (NLRV), *Fiji disease virus* (FDV), *Mal de Rio Cuarto virus* (MRCV), *Rice black streaked dwarf virus* (RBSDV), *Heliothis armigera cypovirus* 5 (HaCV), *Diadromus pulchellus idnoreovirus* (DpRV) and *Infectious bursal disease virus* (IBDV) (as outgroup).

field (May 2008) resulted in ~55% virus positive. Fig. 4 shows RT-PCR results to detect Dc-Reo2 from 10 individual psyllids. Six of 10 were amplified with 442bp, which were considered positive. No immediate pathogenic effects were observed in psyllids so far. Viruses may case latent infection where we are unable to see obvious deleterious effects. To understand reovirus ecology, more research with multiple dates and locations will be needed. The report of NPLV as replicating in the brown planthopper, as a non-pathogenic infection (Nakashima & Noda 1995), suggests that DcRV may also be non-pathogenic to D. *citri*. Pathogenicity tests of D. *citri-Reovirus* to psyllids will be evaluated at a later time. In this case, the spread of a virus through the psyllid population provides an excellent opportunity to use them as delivery mechanisms/tools for the RNAi strategy to reduce psyllids. Virus acquisition and transmission may be occurring due to a combination of the *D. citri* feeding behavior and wide host range, which overlaps with *reovirus* host plants. This is the first report of a *reovirus* in *D. citri*. Knowledge of host range, mode of transmission and genome organization of the *D. citri Reovirus* is important information which will help us understand the virus-vector interactions and illuminate possible roles this virus may have in the development of new management strategies against *D. citri* to reduce the impact of HLB in citrus trees.

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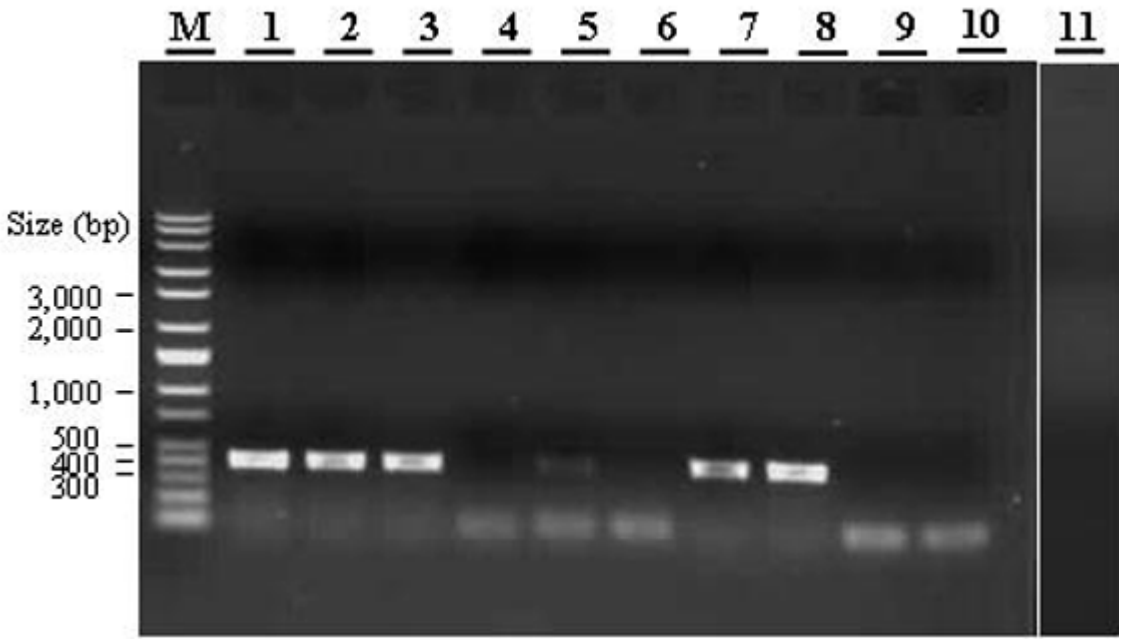


Fig. 4. Detection of DcRV from field collected *Diaphorina citri* with Dc-reo2 primers. Lanes 1-3,5,7,8, were positive. M; Direct Load™ Wide Range DNA Marker (Sigma, Saint Louis, Missouri, USA). Lane 1-10; RT-PCR product from individual psyllid as a template. Lane 11; Negative control with water as a template.

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