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BRIEF REPORT

Characterization of taro reovirus and its status in taro (*Colocasia esculenta*) germplasm from the Pacific

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Abstract

Taro reovirus (TaRV) has been reported infecting taro (*Colocasia esculenta*) in the South Pacific, but information on the virus is limited. Here, we report the genome sequence of a reovirus infecting taro in Papua New Guinea that had 10 genomic segments ranging from 1.1 to 3.9 kilobase pairs (kbp) in length with a total genome length of 26.3 kbp. TaRV was most closely related to rice ragged stunt virus (RRSV) but did not cross-react with RRSV polyclonal antisera. TaRV was not detected in 82 germplasm accessions of taro in Hawaii, or samples collected in American Samoa, Fiji, Guam, Palau, or Vanuatu.

Taro (*Colocasia esculenta*) is an important starch staple in the tropics and has considerable economic and cultural importance for Pacific Island countries. As a vegetatively propagated crop, taro is prone to several viral diseases, including dasheen mosaic, taro vein chlorosis, and the alomae-bobone complex [1]. A virus infecting taro tentatively identified as an oryzavirus (family *Reoviridae*) has been reported previously in Papua New Guinea (PNG), the Solomon Islands, and Vanuatu [2], although little information on this virus, designated taro reovirus (TaRV), is currently available. The genus *Oryzavirus* currently includes two species, the type species *Rice ragged stunt virus* and *Echinochloa ragged stunt virus*. Oryzavirus genomes are composed of 10 linear segments of double-stranded RNA (dsRNA) ranging from 1.1 to 3.8 kilobase pairs (kbp) with a total genome length of approximately 26 kbp. The purpose of this work was to characterize TaRV and determine its status in taro germplasm maintained in Hawaii as well as in various taro samples collected from Pacific Island countries.

Between 2017 and 2019, taro samples collected from American Samoa, Fiji, Guam, Palau, PNG, and Vanuatu were shipped in RNAlater (Invitrogen) storage solution to the University of Hawaii under USDA Permits P526P-17-03915 and P526P-19-04245 and stored at -20°C upon arrival. The storage solution provided sample preservation and protection from RNA degradation during extended periods of exposure to elevated temperatures and obviated the need for a state import permit. Additional samples from 82 accessions belonging to the University of Hawaii's taro germplasm collection maintained at the University of Hawaii's Waimanalo Research Station were collected in 2020.

dsRNA was isolated from a ~5 g composite of the PNG samples using cellulose chromatography [3], and an aliquot was resolved by agarose gel electrophoresis. A second aliquot was used for library construction [4] and was prepared for high-throughput sequencing (HTS) using a Nextera DNA Library Prep Kit (Illumina). HTS (300 cycles) was performed on a MiSeq platform (Illumina) at the University of Hawaii's Advanced Studies in Genomics, Proteomics, and Bioinformatics laboratory. Approximately 7.3 M reads were trimmed and filtered for quality (>Q20) using Trimmomatic [5] and assembled using Trinity v.2.9.1 [6]. Of the 2,168 contiguous sequences (contigs) generated, BLASTx analysis [7] indicated that 39 of the contigs shared low to moderate sequence identity to rice ragged stunt virus (RRSV) sequences in the GenBank database. An iterative mapping procedure in Geneious R10 (Biomatters) generated 10 contigs with homology to the 10 segments of RRSV with mean coverage values ranging from 10,177 to 59,941 (Table 1). To determine terminal sequences of each segment, 5' and 3' rapid amplification of cDNA ends (RACE) was performed using dsRNA polyadenylated with *E. coli* poly(A) polymerase (New England Biolabs). Primers used in the

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Table 1 Properties of taro reovirus genomic segments 1 through 10, including mapped reads and coverage from high-throughput sequencing data. RRSV, rice ragged stunt virus

Segment	Genbank accession	Size (bp)	Coding region	Protein	Protein function	Protein predicted Weight (kDa)	Protein identity to RRSV homolog	Mapped reads	Coverage		
									Min	Mean	Max
1	MW148346	3,878	211-3,765	P1	Virus core associated (B Spike)	131.2	37.9%	340,981	2	11,840	66,434
2	MW148347	3,803	126-3,740	P2	Inner Core Capsid	135.8	43.1%	287,533	1	10,177	54,733
3	MW148348	3,763	132-3,286	P3	Major Core Capsid	133.3	52.2%	595,490	1	19,018	251,280
4	MW148349	3,813	13-3,768	P4a	RNA-depent RNA polymerase	142.7	54.6%	955,850	3	46,457	425,267
			513-1,367	P4b	Unknown	32.1	34.8%				
5	MW148350	2,665	51-2,429	P5	Capping enzyme	89.8	36.1%	874,978	4	59,941	287,699
6	MW148351	2,242	42-2,144	P6	Unknown	78.5	16.4%	417,985	1	24,022	77,968
7	MW148352	1,933	20-1,846	P7a	Non-structural	68.3	39.5%	726,746	2	47,503	138,927
			1,200-901	P7b	Unknown	10.9	45.3%				
8	MW148353	1,905	23-1,795	P8	Precursor protease (Major Capsid)	67.8	31.6%	270,524	1	19,786	84,555
9	MW148354	1,132	14-1,030	P9	Spike protein	39	48.2%	109,681	3	10,392	25,379
10	MW148355	1,168	90-1,061	P10	Non-structural	35.7	38.6%	200,696	1	20,529	76,937

RACE experiments are listed in Supplementary Table S1. All amplification products were cloned into pGEM-T Easy Vector (Promega), and three to five clones for each terminus were used for Sanger-based sequencing to determine a consensus terminal sequence.

The genome of the TaRV isolate from PNG possessed 10 segments ranging from 1,132 to 3,878 bp, with a total genome size of 26,302 bp (Fig. 1A). This was consistent with the dsRNA banding pattern revealed by gel electrophoresis (Fig. 1B). The segments were assigned numbers based on homology to corresponding RRSV segments. Open reading frames (ORFs) were predicted using the National Center for Biotechnology's ORFfinder tool. The specific characteristics of each segment and the predicted protein products encoded are summarized in Table 1. Overall, the genome organization of TaRV is similar to that of RRSV, and the corresponding protein products of the two viruses share between 16.4 to 54.6% identity. Segment 7 is predicted to encode two proteins: P7a, a non-structural protein 68.3 kDa in size with homology to P7 of RRSV, and P7b, a small (10.9 kDa) protein of unknown function encoded by a small ORF in the negative orientation. RRSV S7 also possesses an ORF in this position, and the protein it encodes shares 45.3% amino acid sequence identity with TaRV P7b. If expressed, this would represent the first ORF in the negative sense identified for oryzaviruses. Additional experimentation is necessary to determine if P7b is expressed. The terminal sequences of each TaRV segment were found to be conserved, initiating and terminating (in the positive sense) as follows: 5'-AAUAAAAA(U/A/C)…(U/C/A)GAUGAU GC-3' (Fig. 1A).

The phylogenetic relationship between TaRV and selected members and putative members of the family *Reoviridae* was determined by aligning RdRp protein sequences using Clustal [8] and analyzing this alignment using the maximum-likelihood (ML) algorithm (model LG+G) in MEGA7 [9]. Branch support was provided by 1,000 bootstrap replications, and rice dwarf virus (genus *Phytoreovirus*) was used as an outgroup. This analysis indicated that TaRV is a member of the oryzavirus clade and is most closely related to RRSV (Fig. 2). The unclassified reoviruses raspberry latent virus [10] and grapevine Cabernet Sauvignon reovirus [11] formed a sister clade to TaRV and RRSV and could also be potential members of the genus (Fig. 2).

Using the complete genomic sequence of TaRV, multiple primer sets were designed to validate each of the 10 TaRV segments using RT-PCR (Supplementary Table S1). Additionally, an RT-PCR assay using primers Taro-Reo-RdRp-F/R targeting the TaRV RdRp gene was developed for virus detection assays. Total nucleic acid and total RNA were extracted from ~200 mg of taro leaf tissue using a CTAB-based protocol [12] and a NucleoSpin® RNA Plus Kit (Macherey-Nagel), respectively, and cDNA synthesis was

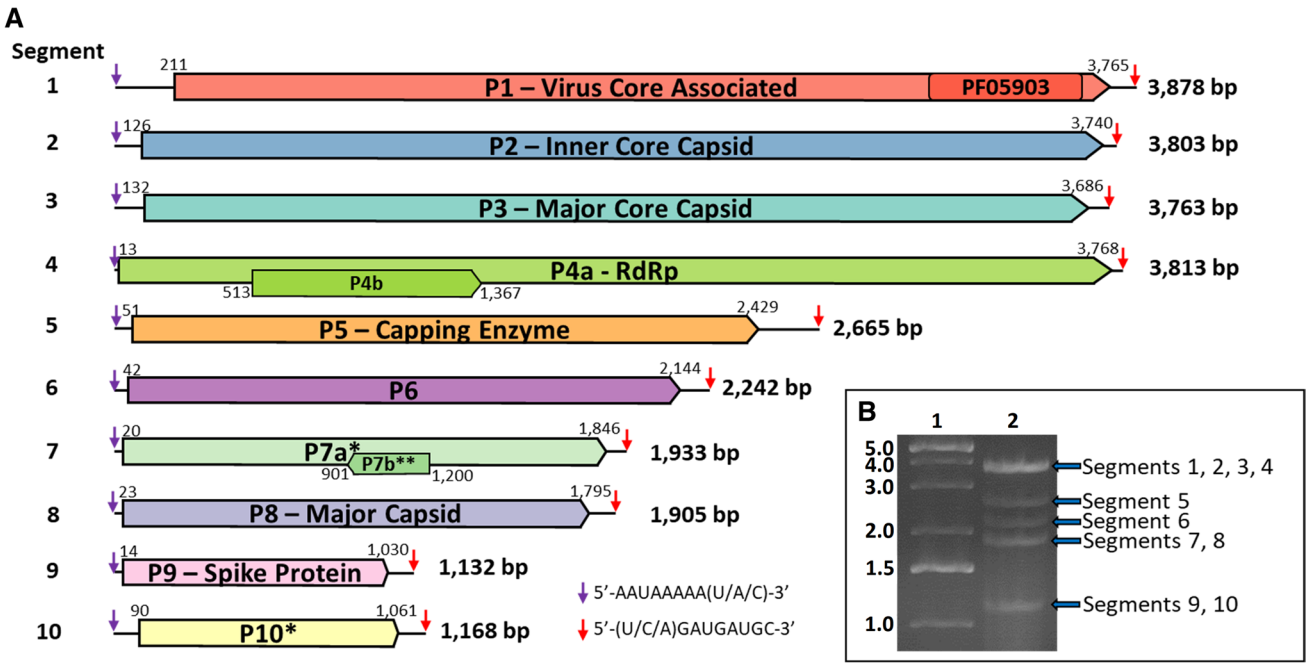

Fig. 1 **A.** Genome organization of taro reovirus (TaRV). Segments are identified at the left with lengths in base pairs (bp) indicated at the right. The 3'- and 5'-terminal sequences, indicated by vertical blue and red arrows, respectively, are provided at the bottom center. Colored boxes represent open reading frames (ORFs), with the tapered end indicating the coding direction. The start and stop position of each ORF is identified immediately above the box. ORFs are labeled by protein name (P1 through P10), and the putative function and protein family (PF number) is provided if known. RdRp, RNA-dependent RNA polymerase. *, non-structural proteins. **, predicted ORF of unknown function. **B.** Agarose gel electrophoresis of TaRV double-stranded RNA segments (lane 2) and partial 1 kb Plus Ladder (Thermo Fisher Scientific) with sizes in kilobases at the left (lane 1)

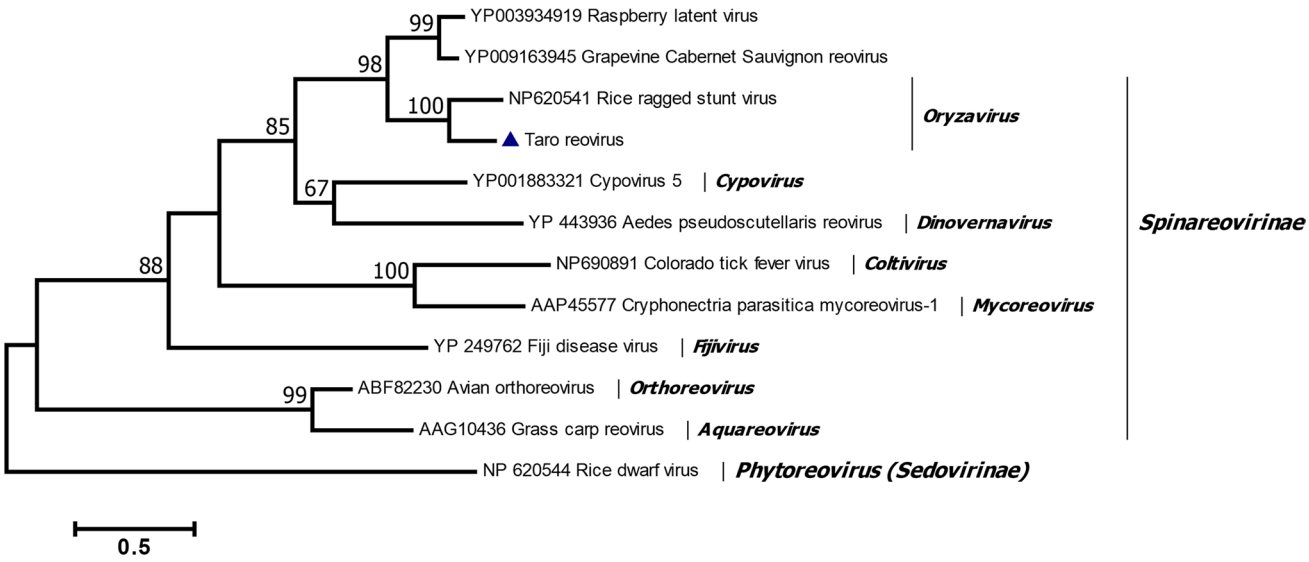

Fig. 2 Phylogenetic placement of taro reovirus (blue triangle) based on the RNA-dependent RNA polymerase amino acid sequence, using the maximum-likelihood algorithm. Branch numbers represent support based on 1,000 bootstrap replications, and the scale represents the number of substitutions for the given distance. Raspberry latent virus and grapevine Cabernet Sauvignon reovirus are unclassified viruses that putatively belong the subfamily *Spinareovirinae*. Accession numbers are provided before each virus name

performed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Promega). PCR conditions for the TaRV detection assay were an initial denaturation at 94 °C for 3 min followed by 40 cycles of 94 °C (30 s), 54 °C (30 s), and 72 °C (30 s), with a final extension step of 72 °C for 5 min. Using this TaRV detection assay, 82 taro germplasm accessions maintained by the University of Hawaii were evaluated for the presence of the virus. Additionally, taro samples from American Samoa (11 samples), Fiji (6 samples), Guam (3 samples), Palau (4 samples), PNG (5 samples), and Vanuatu (4 samples) were assayed for the presence of TaRV. Of these, only two samples from PNG produced an amplification product of the expected size (389 bp), indicating that the samples were positive for the virus. The amplification products for each of the positive samples were sequenced directly. The amplicons from the two PNG samples were 100% identical to each other and to the TaRV sequence generated using HTS data.

With TaRV having the closest genetic and phylogenetic relationship to RRSV, the serological relationship between the two viruses was evaluated using a commercial RRSV-specific double-antibody sandwich (DAS)-ELISA kit (Nano Diagnostics) following the manufacturer's protocol. TaRV-positive taro samples from PNG were assayed in duplicate wells, alongside positive and negative tissue and buffer controls. Color development at 405 nm was recorded at 1 h and 24 h after substrate addition using an Epoch plate reader (BioTek), and these readings were analyzed using Gen5 Data Analysis software (BioTek). Neither the TaRV-positive taro samples nor negative control tissues were found to be positive using the RRSV DAS-ELISA, with buffer-corrected absorbance readings of <0.01 and <0.05 after 1 h and 24 h, respectively. Conversely, RRSV-positive tissue had average corrected absorbance readings of 1.7 and 3.0 after 1 h and 24 h, respectively.

Based on a partial genomic sequence, TaRV was predicted to be a reovirus of the genus *Oryzavirus* [Devitt et al., as reported in reference 2]. The complete genome sequence and phylogenetic placement of TaRV reported here support classification of TaRV as an oryzavirus. A phylogenetic analysis indicated that TaRV was most closely related to RRSV, but the low sequence similarity between TaRV and RRSV in the taxonomically informative RdRp and capsid proteins indicates that TaRV represents a distinct species in the genus. This was further supported by a lack of serological relationship between TaRV and RRSV, as indicated by DAS-ELISA. The species demarcation criteria for the family *Reoviridae* include low levels of sequence similarity among cognate genome segments (but typically above 33% amino acid sequence identity for RdRp), a lack of serological relationship, and in the case of the genus *Oryzavirus*, a distinct host range [13]. Based on the amino acid sequence analysis, serological comparison, and host range, TaRV meets the species demarcation criteria for members of the family *Reoviridae*, and we propose that TaRV be included as a new member of the genus *Oryzavirus*.

An earlier, comprehensive survey of taro virus incidence in South Pacific Island countries revealed that TaRV was present in PNG, the Solomon Islands, and Vanuatu [2]. Details of the RT-PCR assay used for TaRV detection, however, were not described. In this study, we developed an RT-PCR assay for TaRV detection that targets the RdRp gene. A primary goal of this study was to determine whether TaRV was present in taro germplasm accessions in Hawaii, as propagules from these collections are commonly distributed to farmers. We further examined a limited number of taro samples collected from elsewhere in the Pacific. Of these, only two samples from PNG (from which the isolate of TaRV was sequenced for this study), tested positive for TaRV. The RT-PCR assay developed in this study will allow for more comprehensive screening for this virus in countries where taro is grown.

RRSV is an important pathogen of rice, particularly in southeast Asia, where it causes stunting, leaves that are twisted and deformed and appear ragged, and galling [13, 14]. Rice plants can be coinfected with RRSV and other viruses, often leading to synergistic effects or masking the impact of single infections [14, 15]. In the case of taro, symptoms caused by TaRV are unclear, as it has been reported previously only in plants hosting multiple viruses [2]. In this study also, TaRV was found only in mixed infections with other viruses (data not shown). Interestingly, both TaRV-infected plants from PNG examined in this study displayed symptoms of the alomae-bobone disease complex, including severe stunting, leaf deformation, and petiole galling. Further studies are necessary to determine the impact of TaRV on taro in a single infection and in coinfections with other viruses.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05108-9.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethics statement This work does not contain any studies performed by the authors that included human participants or animals.

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