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Partial characterisation of a novel *Tobamovirus* infecting *Actinidia chinensis* and *A. deliciosa* (Actinidiaceae) from China

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Abstract *Actinidia chinensis* and *A. deliciosa* plants from China, showing a range of symptoms, including vein clearing, interveinal mottling, mosaics and chlorotic ring spots, were found to contain ~300 nm rod-shaped virus particles. The virus was mechanically transmitted to several herbaceous indicators causing systemic infections in *Nicotiana benthamiana*, *N. clevelandii*, and *N. occidentalis*, and local lesions in *Chenopodium quinoa*. Systemically-infected leaves reacted with a *Tobacco mosaic virus* polyclonal antibody in indirect ELISA. PCR using generic and specific *Tobamovirus* primers produced a 1,526 bp sequence spanning the coat protein (CP), movement protein (MP), and partial RNA replicase genes which showed a maximum nucleotide identity (88%) with *Turnip vein clearing virus* and *Penstemon ringspot virus*. However, when the CP sequence alone was considered the highest CP sequence identity (96% nt and 98% aa) was to *Ribgrass mosaic virus* strain Kons 1105. The morphological, transmission, serological and molecular properties indicate that the virus is a member of subgroup 3 of the genus *Tobamovirus*.

Keywords *Actinidia* · Kiwifruit · *Tobamovirus* · *Ribgrass mosaic virus*

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Introduction

The genus *Actinidia* consists of 66 species widely distributed in eastern Asia, with 62 species in China alone, indicating it is the centre of genetic diversity for the genus (Ferguson 1990; Huang et al. 2000; Li et al. 2002). *Actinidia chinensis* and *A. deliciosa* are extensively cultivated in New Zealand and other parts of the world for their nutritious berries, commonly referred to as kiwifruit (Ferguson and Bollard 1990). Nearly a million tonnes of kiwifruit are produced every year in New Zealand, exports being worth NZ $659 million in 2004 (Kerr et al. 2004). The constant endeavour to improve the quality of kiwifruit to meet the demands of industry and consumers led to the importation of new *Actinidia* germplasm into New Zealand. Both bacterial and fungal diseases are known to sometimes cause considerable crop loss, but there are no documented cases of viral diseases being associated with significant losses of kiwifruit (Clover et al. 2003). Although viral symptoms have been recorded in kiwifruit in Fujian province, China, the centre of origin of genetic stocks (Lin and Gao

1995), and during grafting experiments in Japan (Nitta and Ogasawara 1997), the causal agents were not identified. The first definitive identification of a virus in kiwifruit was *Apple stem grooving virus* (genus *Capillovirus*) in *A. chinensis* from China, held in post-entry quarantine in New Zealand (Clover et al. 2003). This paper reports the partial characterisation of a *Tobamovirus* which is one of several additional viral-like agents detected in the same consignment of *Actinidia* plants (Pearson et al. 2007).

Materials and methods

Source plants

Male and female *Actinidia* accessions from Shaanxi province, China, were imported into post-entry quarantine in New Zealand as woody cuttings in February 2001 and whip-grafted (Hartmann et al. 2001) onto healthy rootstock of *A. chinensis* cv. Hort 16A. Dormant plants were *chilled* at 4°C for a period of 3 weeks to hasten bud break. The spring and summer growth was observed for viral symptoms for 4 years and symptomatic plants were photographed.

Sap transmission experiments

Herbaceous indexing was carried out during both the spring and summer months of 2003–2005. Leaf sap extracts from both young and mature leaves of *A. chinensis* and *A. deliciosa* were used for sap transmission tests. Leaf tissue (1–2 g) was homogenised in 4 ml 0.1 M phosphate buffer, pH 7.5 (Sweet 1975) containing 5% polyvinylpyrrolidone and 0.12% sodium sulphite, using a pestle and mortar. The homogenate was mixed with 400 mesh carborundum powder and mechanically inoculated to the indicators *Chenopodium amaranticolor, C. quinoa*, *Nicotiana benthamiana, N. clevelandii, N. glutinosa, N. occidentalis* 37B, and *Phaseolus vulgaris* cv. The Prince. Buffer-inoculated plants were used as negative controls and all inoculated plants were maintained in the greenhouse at 20–22°C for up to 6 weeks and observed for viral symptoms. Grafting of the *Tobamovirus* infected scions onto healthy *Actinidia* stocks and transmission of the virus from indicators onto healthy *Actinidia* were not performed, as this was not permitted by the Ministry of Agriculture and Forestry, Biosecurity New Zealand.

Transmission electron microscopy(TEM)

TEM grids were prepared from leaves of symptomatic *Actinidia, N. clevelandii*, and *N. occidentalis* 37B. Approximately 0.25 g of infected leaf sample was squashed in 0.01 M Sørensen's phosphate buffer, pH 7. A drop (~20 μl) of the extract was placed on carbon-stabilised, formvar-coated, 400 mesh copper EM grid (ProSciTech, Qld, Australia) for 1 min, the excess removed using filter paper, and the grid stained with 2% (*w/v*) aqueous potassium phosphotungstate, pH 7 (Hill 1984). The grids were observed using a Phillips Tecnai 12 electron microscope (FEI, Eindhoven, Netherlands) and the particle lengths determined using the microscope's internal calibration and by comparison to *Tobacco mosaic virus* photographed at the same magnification.

Enzyme-linked immunosorbent assay (ELISA)

Leaf samples from symptomatic *A. chinensis*, *A. deliciosa,* and herbaceous indicators were tested by indirect ELISA, using a rabbit polyclonal antiserum raised against purified TMV at The University of Auckland. The microtitre plates (Falcon #. 353279) were coated with leaf extracts prepared at 1:10 dilution in sodium carbonate sample extraction buffer, pH 9.6 (Hill 1984) plus 2% polyvinyl pyrrolidone (PVP) (MW 40,000). *Actinidia chinensis* and various *Nicotiana* spp. were used as negative controls and TMV-infected *N. tabacum* was used as a positive control. A 50:50 mixture of sap from TMV-infected *N. tabacum* and healthy *Actinidia* was included to determine whether kiwifruit leaf sap had any inhibitory effect on the serological reaction. The microtitre plates were incubated at room temperature for 2 h then washed three times with phosphate buffered saline, pH 7.4, plus 0.05% Tween 20 (PBST) (Hill 1984). TMV antiserum diluted 1:1,000 in ECI Buffer (PBST, 0.2% bovine serum albumin, 2% PVP) was added, the plates incubated for 2 h at room temperature and washed three times with PBST. Goat anti-rabbit IgG alkaline phosphatase conjugate, (BIO-RAD # 170-6518) diluted 1:2,000 in ECI buffer was added, the plates incubated at room temperature for 2 h and washed three times with PBST. The substrate,

p-nitrophenyl phosphate (PNP) (1 mg ml^{-1}) in PNP buffer (Hill 1984) was added, the plates incubated in the dark at room temperature for 60 min and the A_{405} measured using BIO-RAD Microplate plate reader (model 550). Samples with A_{405} values greater than the mean plus three standard deviations of the negative controls were considered positive.

Samples from infected indicator plants were also tested using the following RMV-specific antisera AS-0029, AS-0030, AS-0213 prepared against the DSMZ RMV isolates PV-0147, PV-0147 and PV-0436 respectively (DSMZ, Braunschweig, Germany) and V051 (AC Diagnostics, Fayetteville, AR 72701, USA).

PCR and sequencing

RNA was extracted from 100 mg leaf samples of symptomatic *Actinidia* and indicators using Qiagen RNeasy® Plant mini kit (Cat # 74903, Gmbh, Germany) according to the manufacturer's protocol, with the following modification. The RLC lysis buffer containing guanidine hydrochloride, was modified by the addition of 2 M sodium acetate to a final concentration of 0.2 M and polyvinyl pyrrolidone (MW 40,000) to a final concentration of 2.5% (*w/v*), and the pH adjusted to 5.0 (MacKenzie et al. 1997). RNA was eluted in molecular grade water or 1 mM sodium citrate RNA storage solution at pH 6.4 (Ambion, UK). Reverse transcription was performed at 37°C for 1 h using a 25 µl reaction volume containing 3 µl of total RNA, 5 µl of 5x first strand reverse transcription buffer, 1 µl 100 mM of DTT, 2.5 µl of 10 mM dNTPs, 1.25 µl of RNaseOUT (Invitrogen, CA Cat # 10000840), 10.25 µl molecular grade water, pH 7, and either (a) 1 µl of reverse primer Tob Uni 1 (Letschert et al.2002) and 100 units of *Moloney murine leukaemia virus* (M-MLV) reverse transcriptase (Invitrogen, CA Cat # 28025-013), or (b) 1 µl of the *Ribgrass mosaic virus* primer, 8R (Zhu et al. 2001) and SuperScript III (Invitrogen CA Cat # 1808044), according to the manufacturer's protocol.

The polymerase chain reaction was performed in a 50 µl reaction volume containing 5 µl of 10 x PCR buffer (Applied Biosystems, California, USA), 5 µl of 25 mM $MgCl_2$, 2.5 µl of 10 mM dNTPs, 1 µl each of forward and reverse primers, 0.2 µl (1 unit) of Ampli*Taq*® DNA Polymerase (Applied Biosystems, California), 3 µl of cDNA and 32.3 µl of molecular grade water, pH 7. DNA amplification was achieved using either Tob Uni 1 and Tob Uni 2 primers (Letschert et al. 2002) or Tob Uni 1 plus a TMV-specific forward primer (5'-CGG TCA GTG CCG AAC AAG AA-3') which binds at nts 5589–5609. The programme consisted of 94°C for 5 min followed by 25 cycles of 94°C for 60 s, 60°C for 45 s, and 72° C for 60 s, followed by 72°C for 5 min. Alternatively primers 8R and 5F (Zhu et al. 2001), which amplify a 1.2 kb product encompassing the coat protein and movement protein genes and part of the RNA replicase gene, were used with the following programme: 94°C for 5 min, followed by 35 cycles of 94°C for 60 s, 45°C for 45 s, 72°C for 60 s, followed by 72°C for 5 min. All the primers were used at a concentration of 20 pico moles μl^{-1}.

PCR products were analysed by agarose gel electrophoresis, stained with ethidium bromide and visualised using a BIO-RAD transilluminator. For cloning, DNA was purified from excised bands using 'Perfectprep Gel Cleanup' purification kit (Eppendorf, Hamburg, Germany), ligated into pGEMT easy vector (Promega, Madison, WI, USA) and cloned in *E. coli* DH5α competent cells (Invitrogen Corp., Carlsbad, USA), and selected on LB medium supplemented with XGal, IPTG and Ampicillin (pGEM®-T Easy Vector Systems, Technical manual No 042, Promega, Madison, WI, USA). Plasmids were extracted using FastPlasmid®Mini kit (Eppendorf, Hamburg, Germany) and the inserts sequenced with an ABI PRISM automated DNA sequencer (University of Auckland, New Zealand).

Phylogenetic analysis

Consensus nucleotide sequences for the putative movement and coat protein genes, and the partial RNA replicase read-through component gene were created from the forward and reverse sequences using Sequencher 4.5 (Gene Codes Corporation, Michigan 48108, USA) and translated into amino acid sequence using the BioEdit biological sequence alignment editor (Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008). Sequences were compared with sequences from GenBank using BLAST (Altschul et al. 1997; Schäffer et al. 2001) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al. 2004). The neighbour-joining method was used to construct phylogenetic

trees with Poisson corrected amino acid distances and pairwise gap deletion options. The node significance was evaluated with 50,000 bootstrap random addition replicates to create a consensus tree. Both uniform and unequal rates of evolutions were tested to evaluate the movement and coat protein gene phylogenies.

Recombination analysis

The *Actinidia Tobamovirus* sequence, comprising partial replicase gene, complete movement and coat protein genes, and 3'UTR, was analysed for recombination events with nine different closely related (sub-group III) *Tobamoviruses* (TVCV [U03387.1], PenRSV [DQ658743.1], TMV-Crucifer isolates [Z29370.1, AB017503.1, AB017504.1, AY318866.1], YoMV [U30944.1], RMV isolates Shanghai [AF254924] and Impatiens [DQ223770.1]) using the analysis package RDP3 Beta 24-Recombination (Martin et al. 2005a), which incorporates Automated RDP (Martin et al. 2005b), Bootscan/Recscan (Martin et al. 2005b), MaxChi (Maynard Smith 1992), Chimera (Posada & Crandall 2001) and SiScan (Gibbs et al. 2000).

Results

Symptoms on *Actinidia* and mechanically-inoculated indicators

Symptoms on *Tobamovirus*-infected *A. chinensis* accessions included chlorosis of the leaf veins and adjacent tissue at the tip and middle regions of shoots during spring (Fig. 1a), followed by chorotic mottles, mosaics, and ring spots during summer. Some leaves with vein chlorosis also displayed mild hypertrophy of the mesophyll tissue (Fig. 1b). Symptoms on *A. deliciosa* plants included chlorotic mottling or mosaic during spring (Fig. 1d) and ring spots during summer months (Fig. 1c).

Symptoms on indicators were as follows: *C. amaranticolor* and *C. quinoa* - local chlorotic lesions within 1 week followed by fine systemic chlorotic lesions; *N. benthamiana* - systemic mosaic and distortion 2–3 weeks post-inoculation; *N. clevelandii* – systemic necrotic ring spots in older leaves (Fig. 1e) and chlorotic vein banding and dark green blistering and distortion (Fig. 1f) and/or chlorotic spotting; *N. glutinosa* – necrotic lesions developed on the inoculated leaves within 1 week post-inoculation followed by a systemic mottle; *N. occidentalis* – localised necrotic ring spots after *c.* 2 weeks (Fig. 1g) followed by systemic distortion and mottling and/or vein chlorosis 2–3 weeks post-inoculation (Fig. 1h); *P. vulgaris* – mild systemic mottle.

Electron microscopy

Rigid rod-shaped particles with a modal length ~300 nm and an obvious central axial canal were observed in leaf sap extracts from *A. chinensis* and *A. deliciosa* and from mechanically-inoculated *N. benthamiana*, *N. clevelandii* and *N. occidentalis*. Particles in partially purified preparations from *N. clevelandii* had a mean length of 307 nm (SD=19, n=107) and width of 17 nm (SD=1.6).

ELISA

Leaf extracts from ten different cultivars (three of *A. deliciosa*, seven of *A. chinensis*) were inoculated onto herbaceous indicators, and both the original *Actinidia* plants and symptomatic indicators were tested using TMV antiserum. Indicators inoculated from five of the cultivars (two of *A. deliciosa* and three of *A. chinensis*) tested positive, but extracts from the infected *A. chinensis* and *A. deliciosa* plants tested negative. Samples containing equal proportions of leaf sap from healthy *Actinidia* and TMV-infected *N. tabacum* also tested negative. Using specific RMV antisera in a DAS-ELISA format, no reaction was obtained with antisera AS-0029 or AS-0030. A weak reaction was obtained with extracts of indicator plants and antiserum AS-0213, but not against extracts from kiwifruit. Antiserum V051 reacted weakly with extracts from frozen samples of infected *N occidentalis* and *A. chinensis.* This result needs to be confirmed when fresh tissue samples are available.

PCR and sequencing

PCR and sequencing were carried out on *Actinidia Tobamovirus* isolates maintained in *N. clevelandii* or *N. occidentalis*. The generic *Tobamovirus* primers, Tob Uni1 and Tob Uni 2 (Letschert et al. 2002) generated a ~700 bp product (650 bp excluding

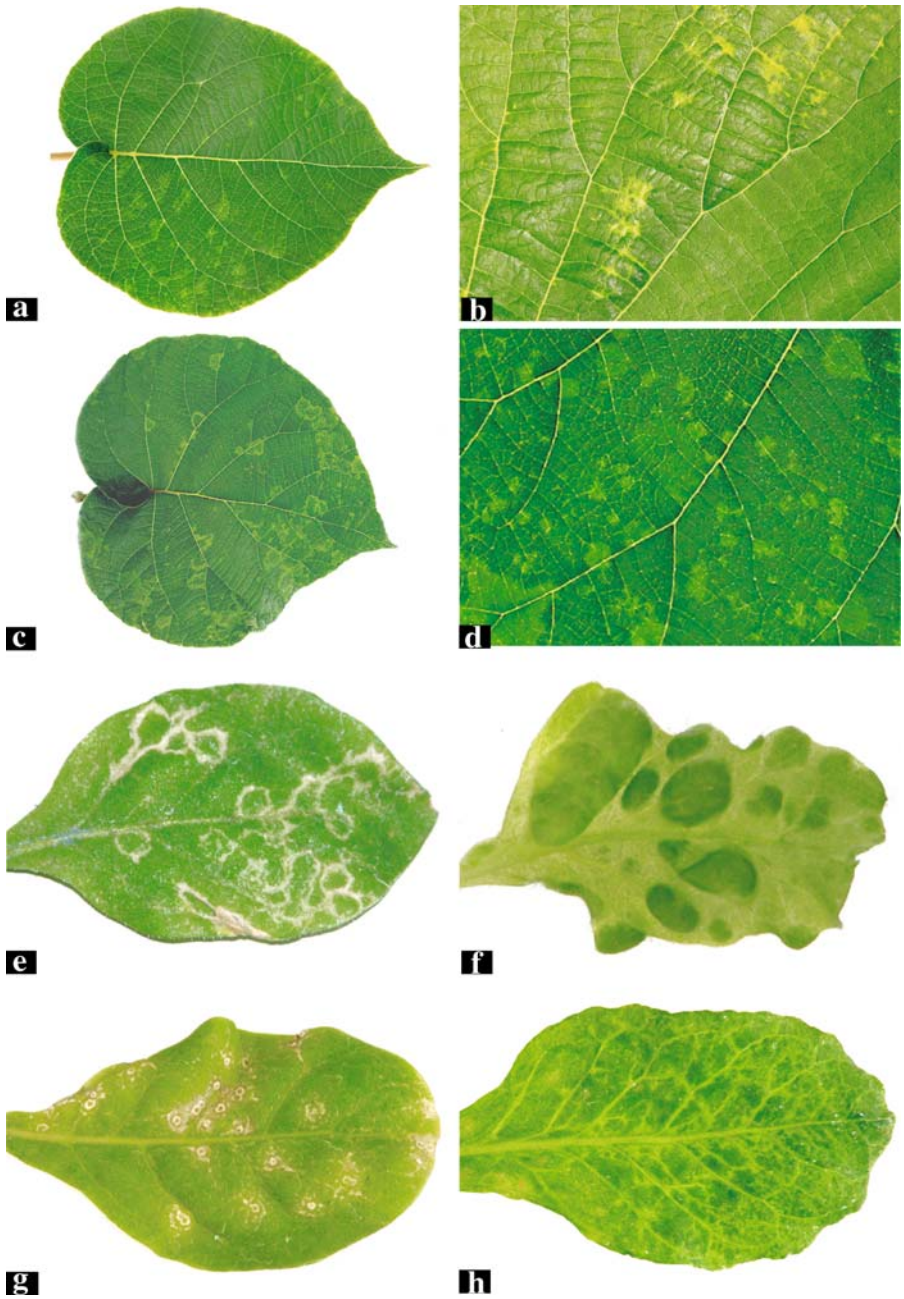

Fig. 1 Symptoms associated with infection by *Actinidia Tobamovirus* isolate. **a** *A. chinensis* - mild mottle, **b** *A. chinensis* – vein chlorosis and hypertrophy of tissues, **c** *A. deliciosa* – ring spots and mild mottles, **d** *A. deliciosa* – mottle, **e** *N. clevelandii* (inoculated from *A. deliciosa*) – ringspots, **f** *N. clevelandii* (inoculated from *A. deliciosa*) – leaf distortion, chlorosis and dark green islands, **g** *N. occidentalis* (inoculated from *A. chinensis*) – chlorotic lesions and ring spots, **h** *N. occidentalis* (inoculated from *A. deliciosa*) – vein clearing and leaf distortion

primer sequences) covering the coat protein gene and part of the 3' UTR of the *Actinidia Tobamovirus,* and a ~800 bp product for the TMV positive control. Seven distinct *Actinidia* sequences, representing three *A. chinensis* cultivars (four sequences) and one *A. deliciosa* cultivar (three sequences) showed complete identity. Primer set 8R and 5F (Zhu et al. 2001) amplified a 1.2 kb product covering the coat protein, movement protein and partial RNA replicase read-through component genes, which overlapped the ~700 bp product by 296 nucleotides giving a combined sequence length of 1,526 nucleotides. Two independent, identical 1,526 sequences were generated from *A. chinensis* and *A. deliciosa* isolates (GenBank accessions EF409996 and EF409997 respectively).

Table 1 Pairwise alignments of putative movement and coat protein nt and aa sequences of *Tobamovirus* isolated from *Actinidia chinensis and A. deliciosa* (accession EF409996 and EF409997 respectively) with different related *Tobamoviruses*

	EF409996 EF409997 *Actnidia* isolate	DQ658743 *Penstemon ringspot virus*	NC_001873 *Turnip vein clearing virus*	Z29370 TMV *Crucifer*	AB017504 *Crucifer tobamovirus*	AY318866 *Crucifer tobamovirus*	AF254924 *Ribgrass mosaic virus* (Shanghai)	DQ223770 *Ribgrass mosaic virus* (Impatiens)	NC_004422 *Youcai mosaic virus*	AM040967 *Ribgrass mosaic virus* Kons 1105, R14
Putative movement protein										
Actnidia isolate (EF409996, EF409997)	–	87%	86%	85%	84%	81%	81%	81%	80%	*
Penstemon ringspot virus (DQ658743)	**87%**	–	99%	94%	82%	81%	82%	81%	81%	*
Turnip vein clearing virus (NC_001873)	**87%**	**100%**	–	95%	82%	81%	81%	81%	81%	*
TMV [crucifer] (Z29370)	**86%**	**97%**	**97%**	–	82%	81%	81%	83%	81%	*
Crucifer tobamovirus (Tochigi) (AB017504)	**82%**	**83%**	**83%**	**82%**	–	83%	83%	83%	85%	*
Crucifer tobamovirus (AY318866)	**82%**	**81%**	**81%**	**83%**	**87%**	–	99%	97%	97%	*
Ribgrass mosaic virus (Shanghai) (AF254924)	**82%**	**83%**	**83%**	**82%**	**87%**	**98%**	–	96%	96%	*
Ribgrass mosaic virus (Impatiens) (DQ223770)	**82%**	**81%**	**81%**	**82%**	**87%**	**98%**	**97%**	–	96%	*
Youcai mosaic virus (NC_004422)	**82%**	**81%**	**81%**	**82%**	**88%**	**98%**	**97%**	**98%**	–	*
Ribgrass mosaic virus (AM040967)	*	*	*	*	*	*	*	*	*	*
Putative coat protein										
Actnidia isolate (EF409996, EF409997)	–	88%	88%	87%	85%	84%	83%	83%	83%	96%
Penstemon ringspot virus (DQ658743)	**90%**	–	99%	94%	87%	86%	85%	85%	85%	90%
Turnip vein clearing virus (NC_001873)	**90%**	**99%**	–	94%	87%	86%	86%	85%	85%	90%
TMV [crucifer] (Z29370)	**88%**	**95%**	**94%**	–	86%	84%	84%	84%	84%	88%
Crucifer tobamovirus (Tochigi) (AB017504)	**87%**	**91%**	**91%**	**89%**	–	91%	91%	91%	91%	85%

Crucifer tobamovirus (AY318866)	**88%**	**88%**	**88%**	**85%**	**93%**	–	99%	99%	98%	85%
Ribgrass mosaic virus (Shanghai) (AF254924)	**88%**	**88%**	**88%**	**85%**	**93%**	**100%**	–	99%	98%	85%
Ribgrass mosaic virus (Impatiens) (DQ223770)	**88%**	**88%**	**88%**	**85%**	**93%**	**100%**	**100%**	–	98%	85%
Youcai mosaic virus (NC_004422)	**87%**	**87%**	**87%**	**84%**	**92%**	**99%**	**99%**	**99%**	–	85%
Ribgrass mosaic virus (AM040967) Kons 1105, R14	**98%**	**91%**	**91%**	**88%**	**88%**	**89%**	**89%**	**89%**	**89%**	–

Normal and bold numerals represent percentage homologies of nucleotide and amino acid sequences respectively. * = Movement protein data not available

Phylogenetic analysis

Pairwise BLAST (NCBI), using the combined 1,526 nucleotide sequence for coat protein, movement protein, and partial RNA replicase read-through component genes of the *Actinidia Tobamovirus*, showed a maximum nucleotide identity of 88% with *Penstemon ringspot virus* (PenRSV) DQ658743 and 87% with *Turnip vein clearing virus* TVCV (NC-001873) which share 99% identity across this region and are both considered strains of TVCV (http://www.dpvweb.net/notes/showgenusmembers.php?genus=Tobamovirus). Separate comparisons of the *Actinidia* virus MP and CP genes with the eight sequences showing the greatest similarity across the entire *c*. 1,526 sequence showed the highest identity with *Penstemon ringspot virus* DQ658743, of 87% and 88% nucleotide identity, and 87% and 90% amino acid identity, for the MP and CP genes respectively (Table 1). However, when the individual gene sequences were BLASTed against all *Tobamovirus* CP sequences in GenBank the highest CP sequence identity (96% nt and 98% aa) was to *Ribgrass mosaic virus* strain Kons 1105, AM040967 (Heinze et al. 2006). The highest sequence identity for MP was to *Penstemon ringspot virus* DQ658743 (88% nt), but there is no MP sequence for Kons 1105 on Genbank.

Neighbour-joining trees (Figs. 2 and 3) based on the deduced amino acid sequences of the *Actinidia Tobamovirus* MP and CP genes compared with the same isolates used for the *Tobamovirus* trees presented in the Eighth ICTV report (Lewandowski 2005) place the *Actinidia* isolate in sub-group 3 as defined by Heinze et al. (2006), together with the PenRSV, TVCV, *Ribgrass mosaic virus* isolate 'Shanghai' (RMV), and *Wasabi mottle virus* (WMoV), *Youcai mosaic virus* (YoMV) clusters. A neighbour-joining tree based on 30 CP amino acid sequences for sub group 3 *Tobamovirus* clusters the *Actinidia Tobamovirus* with *Ribgrass mosaic virus* strains infecting members of Brassicaceae, Caryophyllaceae, Lilliaceae, Plantaginaceae and Scrophulariaceae (Fig. 4). Similar analysis for the MP was not possible due to lack of the corresponding sequence data in Genbank.

Recombination analysis

None of the programmes detected recombination in the *Actinidia Tobamovirus*.

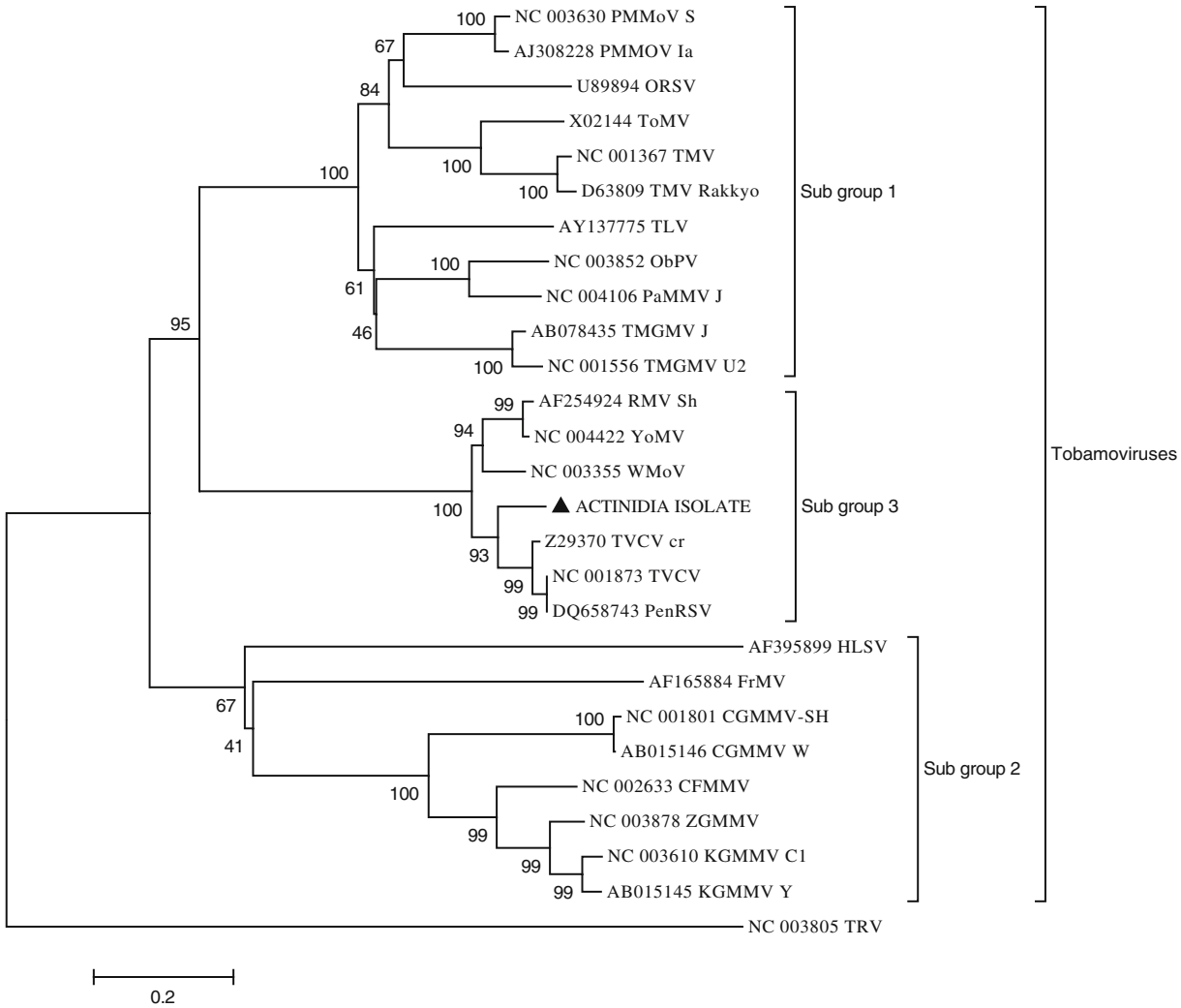

Fig. 2 Phylogenetic relationship of aminoacid sequence of putative movement protein gene of *Actinidia Tobamovirus* with selected strains of tobamovirus species. The tree topology was derived using neighbour joining method, with Poisson corrected aa distances, pairwise gap deletion and uniform rate of evolution options. The nodal significance evaluated by 50,000 bootstrap random replicates. NC_002633 (CFMMV– *Cucumber fruit mottle mosaic virus*), NC_001801 (CGMMV-SH, *Cucumber green mottle mosaic virus*-SH), AB015146 (CGMMV W– *Cucumber green mottle mosaic virus*-W), AF165884 (FrMV, *Frangipani mosaic virus*), AF395899 (HLSV, *Hibiscus latent Singapore virus*), NC_003610 (KGMMV-C1, *Kyuri green mottle mosaic virus*-C1), AB015145 (KGMMV Y, *Kyuri green mottle mosaic virus*-Yodo), NC_003852(ObPV, *Obuda pepper virus*), U89894 (ORSV, *Odontoglossum ringspot virus*), NC_004106 (PaMMV J, *Paprika mild mottle virus*-J), DQ 658743 (Pen RSV, *Penstemon ringspot virus*), AJ308228 (PMMOV-Ia, *Pepper mild mosaic virus*-Ia), NC_003630 (PMMOV-S, *Pepper mild mosaic virus*-S), AF254924 (RMV-Sh, *Ribgrass mosaic virus* isolate 'Shanghai'), AY137775 (TLV, *Tobacco latent virus*), AB078435 (TMGMV J, *Tobacco mild green mosaic virus*-Japan), NC_001556 (TMGMV U2, *Tobacco mild green mosaic virus*-U2), NC_001367 (TMV, *Tobacco mosaic virus*), D63809 (TMV Rakkyo, *Tobacco mosaic virus*- Rakkyo), X02144 (ToMV, *Tomato mosaic virus*), NC_001873 (TVCV, *Turnip vein clearing virus*), Z29370 (TVCV-cr, *Turnip vein clearing virus*-cr), NC_003355 (WMoV, *Wasabi mottle virus*), NC_004422 (YoMV, *Youcai mosaic virus*), NC_003878 (ZGMMV, *Zucchini green mottle mosaic virus*). The corresponding aminoacid sequence of putative movement protein gene from an isolate of *Tobacco rattle virus, TRV* (NC_003805), the type species of *Tobravirus* genus was used as the out group

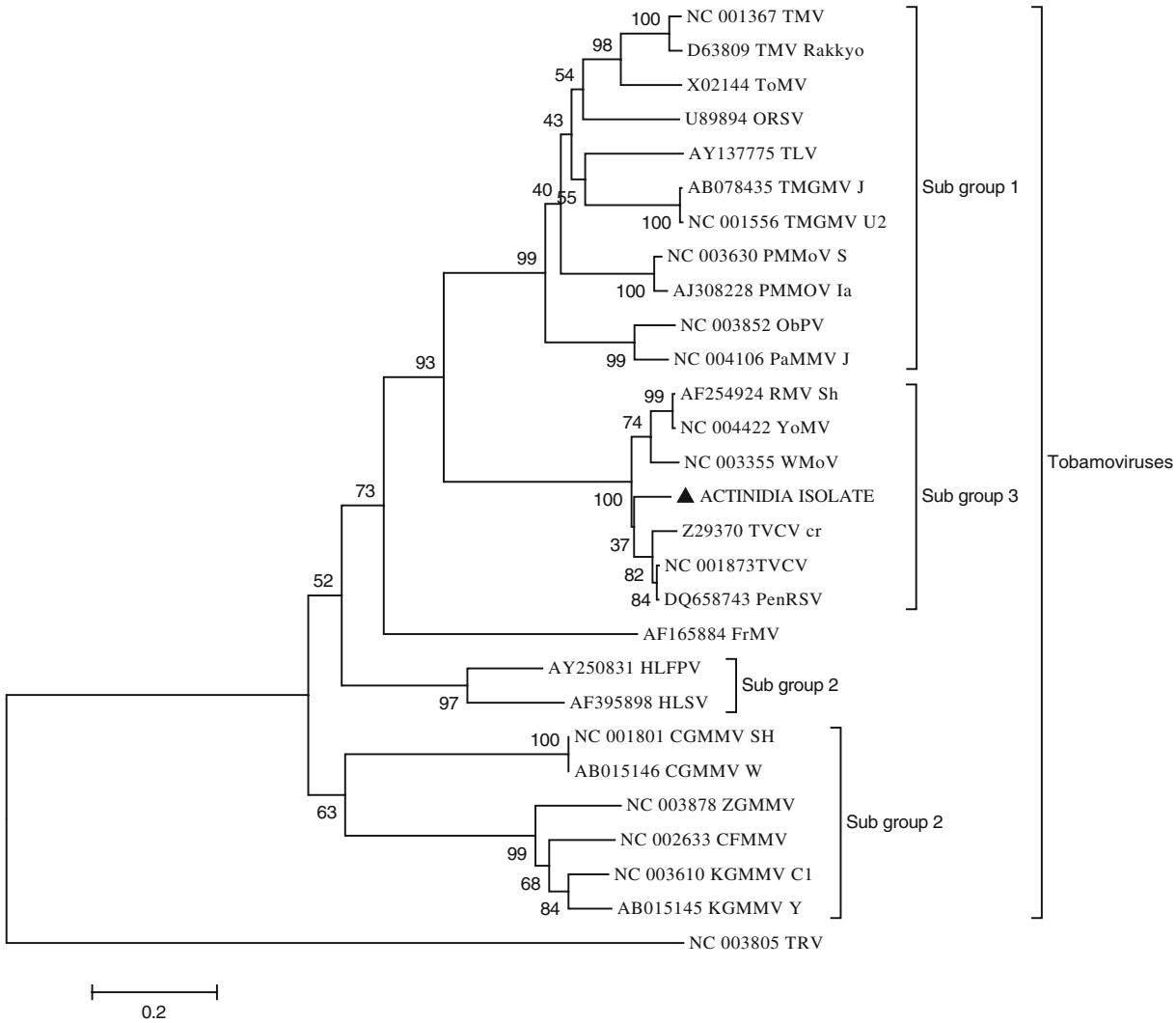

Fig. 3 Phylogenetic relationship of aminoacid sequence of putative coat protein gene of Actinidia tobamovirus with selected strains of tobamovirus species. The tree topology was derived using neighbour joining method, with Poisson corrected aa distances, pairwise gap deletion and uniform rate of evolution options. The nodal significance evaluated by 50,000 bootstrap random replicates. NC_002633 (CFMMV- *Cucumber fruit mottle mosaic virus*), NC_001801 (CGMMV-SH, *Cucumber green mottle mosaic virus*-SH), AB015146 (CGMMV W-*Cucumber green mottle mosaic virus*-W), AF165884 (FrMV, *Frangipani mosaic virus*), AY250831 (HLFPV, *Hibiscus latent Fort Pierce virus*), AF395898 (HLSV, *Hibiscus latent Singapore virus*), NC_003610 (KGMMV-C1, *Kyuri green mottle mosaic virus*-C1), AB015145 (KGMMV Y, *Kyuri green mottle mosaic virus*-Yodo), NC_003852(ObPV, *Obuda pepper virus*), U89894 (ORSV, *Odontoglossum ringspot virus*), NC_004106 (PaMMV J, *Paprika mild mottle virus*-J), DQ 658743 (Pen RSV, *Penstemon ringspot virus*), AJ308228 (PMMOV-Ia, *Pepper mild mosaic virus*-Ia), NC_003630 (PMMOV-S, *Pepper mild mosaic virus*-S), AF254924 (RMV-Sh, *Ribgrass mosaic virus* isolate 'Shanghai'), AY137775 (TLV, *Tobacco latent virus*), AB078435 (TMGMV J, *Tobacco mild green mosaic virus*-Japan), NC_001556 (TMGMV U2, *Tobacco mild green mosaic virus*-U2), NC_001367 (TMV, *Tobacco mosaic virus*), D63809 (TMV Rakkyo, *Tobacco mosaic virus*- Rakkyo), X02144 (ToMV, *Tomato mosaic virus*), NC_001873 (TVCV, *Turnip vein clearing virus*), Z29370 (TVCV-cr, *Turnip vein clearing virus*-cr), NC_003355 (WMoV, *Wasabi mottle virus*), NC_004422 (YoMV, *Youcai mosaic virus*), NC_003878 (ZGMMV, *Zucchini green mottle mosaic virus*). The corresponding aminoacid sequence of putative coat protein gene from an isolate of *Tobacco rattle virus, TRV* (NC_003805), the type species of *Tobravirus* genus was used as the out group

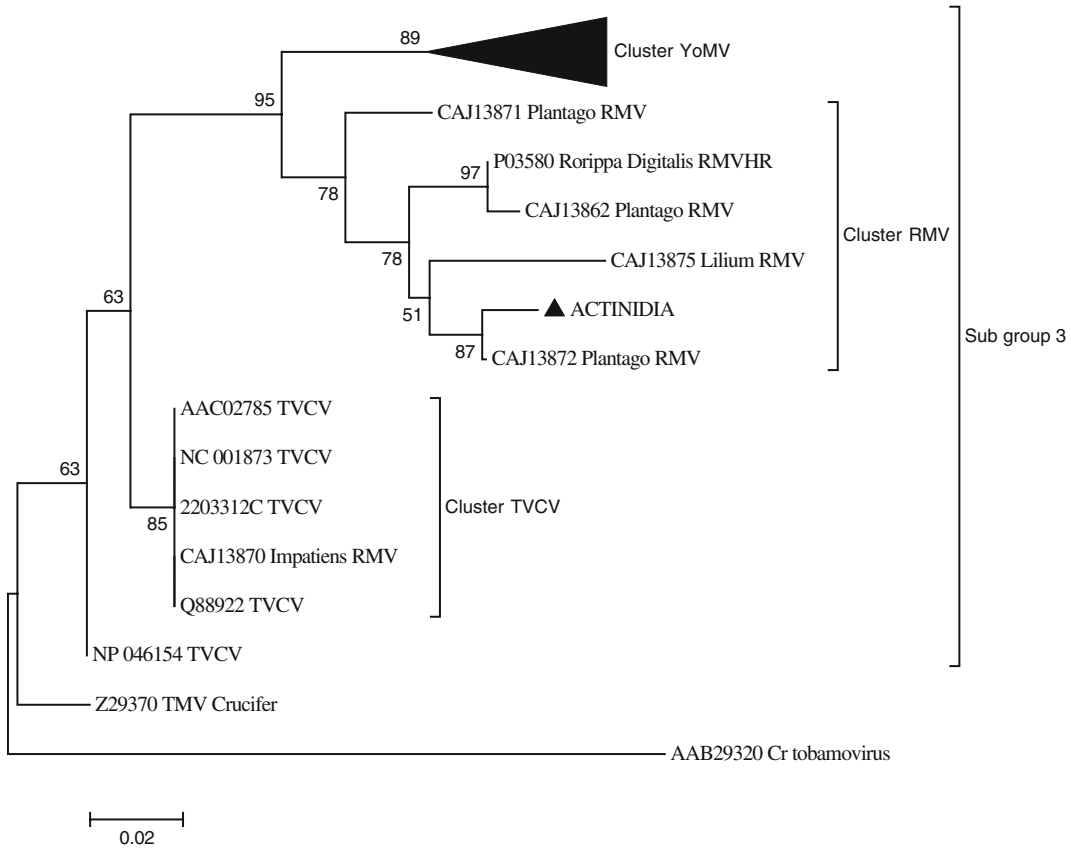

Fig. 4 Phylogenetic relationship of aminoacid sequence of putative coat protein gene of Actinidia tobamovirus with available CP aa sequences of sub group tobamoviruses. The tree topology was derived using neighbour joining method, with Poisson corrected aa distances, pairwise gap deletion and uniform rate of evolution options. The nodal significance evaluated by 50,000 bootstrap random replicates. Cr tobamovirus – Crucifer-infecting tobamovirus, RMV–*Rib grass mosaic virus* (name of the specific host is indicated as prefix), RMVHR– Holmes *Rib grass mosaic virus,* TVCV–*Turnip vein clearing virus,* YoMV– *Youcai mosaic virus*. The corresponding aminoacid sequence of putative coat protein gene from an isolate of Crucifer-infecting tobamovirus (AAB29320) was used as the out group. The *Youcai mosaic virus* cluster is condensed for simplification

Discussion

Although virus-like symptoms have been reported in *Actinidia* from China (Lin and Gao 1995) and Japan (Nitta and Ogasawara 1997) the causal agents were not identified. The only previously characterised virus from *Actinidia* was *Apple stem grooving virus* (Clover *et al.* 2003). The results presented in this paper provide definitive evidence of a *Tobamovirus* naturally infecting *Actinidia*. The virus has typical *Tobamovirus* morphology, and the systemic infection of various *Nicotiana* species and local lesions on *C. amaranticolor* and *C. quinoa* are common features of *Tobamoviruses*. Sequence analysis clearly places it in sub-group 3 of the *Tobamoviruses*, as defined by Heinze et al. (2006). The genus *Tobamovirus* comprises 22 accepted and one tentative species (Lewandowski 2005). The viruses have a wide geographical distribution, and infect a wide range of host plants, including members of the families Brassicaceae, Cucurbitaceae, Fabaceae, Solanaceae, Plantaginaceae and Orchidaceae (Lartey et al. 1996; Gibbs 1999). However, no *Tobamovirus* has previously been found to infect members of Actinidiaceae (order Ericales).

Some of the symptoms observed in the *Tobamovirus*-infected *Actinidia* (chlorotic mottling, mosaics and chlorotic ring spots) resemble the virus-like symptoms previously described in *Actinidia* from China (Lin and Gao, 1995), and also ASGV-infected *Actinidia* (Clover et al. 2003). During the current study variable

symptoms were observed in different cultivars infected by the *Tobamovirus*, although some of the plants were co-infected with other viruses. At this stage it is not possible to associate symptoms in kiwifruit with specific viruses.

The positive reaction of the *Actinidia Tobamovirus* with antiserum raised against TMV (in ELISA) is not surprising as antisera raised against intact virions of TMV have previously been reported to cross-react with other *Tobamoviruses* (Altschuch et al. 1985; Van Regenmortel et al. 1993). The failure to detect the virus directly from infected *Actinidia* is presumably due to the properties of the plant extract (e.g. masking or inactivation of the epitopes by polysaccharides and polyphenolic compounds) since the assay also failed to detect TMV when mixed with *Actinidia* leaf extract. The virus was readily detected from herbaceous indicators by PCR using *Tobamovirus* generic primers, but not using TMV-specific primers (Letschert et al. 2002). This is consistent with the low (46%) nucleotide identity between the *Actinidia Tobamovirus* (sub-group III) and TMV (sub-group I) CP genes.

The 100% identity shown by seven *c.* 650 bp sequences indicates that all of the cultivars of *A. chinensis* and *A. deliciosa* tested were infected by the same strain of *Tobamovirus*. Analysis of the sequence data for the separate CP and MP genes produces somewhat equivocal results since there was no commonality between the nine closest MP sequences and the 13 closest CP sequences. When the CP alone is considered, the *Actinidia* isolate clusters with the RMV brassica isolates, the closest match being RMV strain Kons 1105, AM040967 (Heinze et al. 2006) which was isolated from *Plantago* sp. In contrast, the MP shows the highest identity with TVCV (NC-001873) and PenRSV (DQ658743). This could signify differential rates of evolution for the two genes within the viral genome, as reported for other *Tobamoviruses* (Lartey et al. 1996; Gibbs 1999), or may simply reflect a greater number and range of sequences available for the CP gene. Based on the combined 1,526 nucleotide sequence covering partial RNA replicase read-through component, the movement protein, coat protein, genes, and 3' UTR, the closest matches are 88% (nt) with PenRSV (DQ658743) and 87% (nt) with TVCV (NC-001873). If this 1,526 nt sequence is representative of the whole genome, then based on the ICTV guideline that different species share <90% sequence similarity (Lewandowski 2005) the *Actinidia Tobamovirus* could be considered a distinct species of virus from all previously described *Tobamovirus* species for which there is an equivalent or greater amount of sequence.

The evolution of *Tobamoviruses* has been comprehensively analysed by several authors (Gibbs 1999; Aguilar et al. 1996; Lartey et al. 1996; Wang et al. 1997; Heinze et al. 2006). Based on sequence data three clusters of *Tobamoviruses* are recognised: subgroups I, II, and III, infecting solanaceous species, cucurbits and legumes, and cruciferous plants, respectively (Lartey et al. 1996; Gibbs 1999). From the phylogenetic analysis it is clear that the *Actinidia* isolate clusters with PenRSV, TVCV, YoMV, and crucifer isolates of RMV, which are placed in subgroup III based on their common host range, serological-cross reactivity and the aa composition of their coat protein (Lartey et al. 1996; Gibbs 1999, Heinze et al. 2006). If, as suggested by Lartey et al. (1996), the brassica-infecting *Tobamoviruses* represent a distinct sub lineage of *Tobamoviruses* the *Actinidia* virus may have originated from a cruciferous host. Heinze et al. (2006) recognised TVCV, RMV and YoMV clades within sub group 3 *Tobamoviruses* based on CP analysis. Our analysis (Fig. 4) places the *Actinidia* isolate in the RMV clade with viruses that infect members of Brassicaceae, Caryophyllaceae, Liliaceae, Plantaginaceae and Scrophulariaceae. However, *Actinidia Tobamovirus* did not react strongly with any of the specific RMV antisera tested. Although RMV has an extensive host range, infecting at least 67 different species belonging to 15 diverse dicot and monocot families, it has not been recorded from the order Ericales, to which *Actinidia* belongs (http://image.fs.uidaho.edu/vide/descr683.htm). Among the diagnostically susceptible host species, RMV causes localised brown necrotic local lesions or necrotic concentric rings on *N. glutinosa and* small necrotic or chlorotic local lesions or occasional systemic necrotic spotting on *Chenopodium amaranticolor*. (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB). The *Actinidia Tobamovirus* produced similar symptoms caused on *N. glutinosa, Chenopodium amaranticolor* and *C. quinoa.* However, the symptoms caused on *N. clevelandii* are similar to those caused by TVCV (Lartey et al. 1993); it caused mild mottles in *Phaseolus vulgaris* which is listed as

non-susceptible to RMV (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB). On the basis of the currently available information we conclude that the *Tobamovirus* from *Actinidia* is most closely related to the Kons1105 strain of RMV.

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