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Identification, characterization and full-length sequence analysis of a novel dsRNA virus isolated from the arboreal ant *Camponotus yamaokai*

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A novel dsRNA virus was identified from the arboreal ant *Camponotus yamaokai*. The complete nucleotide sequence analysis of the virus revealed that the virus consisted of 5704 bp with two ORFs. ORF1 (3084 nt) encoded a putative capsid protein. ORF2 (1977 nt) encoded a viral RNA-dependent RNA polymerase (RdRp). ORF2 could be translated as a fusion with the ORF1 product by a −1 frameshift in the overlapping ORF1. Phylogenetic analyses based on the RdRp revealed that the virus from *C. yamaokai* was most likely a novel totivirus, but it was not closely related to the previously known totiviruses in arthropods. Transmission electron microscopy revealed isometric virus particles of ~30 nm diameter in the cytoplasm, which was consistent with the characteristics of the family *Totiviridae*. The virus was detected by reverse transcription-PCR in all caste members and developmental stages of ants, including eggs, larvae, pupae, adult workers, alates (male and female) and queens. To our knowledge, this is the first report of a member of the family *Totiviridae* in a hymenopteran; the virus was designated *Camponotus yamaokai* virus.

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INTRODUCTION

The family *Totiviridae* contains a broad range of viruses characterized as small, non-enveloped, isometric particles with dsRNA 4.6–7.0 kbp in length (Wickner *et al.*, 2012). The viral genome contains two large ORFs, encoding major capsid proteins (CPs) and an RNA-dependent RNA polymerase (RdRp), respectively. Five genera are recognized in the family *Totiviridae*: *Totivirus*, *Giardiavirus*, *Leishmaniavirus*, *Trichomonasvirus* and *Victorivirus*. Viruses in the genera *Totivirus* and *Victorivirus* infect yeast, smut fungi and filamentous fungi; those in the genera *Trichomonasvirus*, *Giardiavirus* and *Leishmaniavirus* infect parasitic protozoa.

Penaeid shrimp infectious myonecrosis virus was isolated from the Pacific white shrimp *Litopenaeus vannamei* at aquaculture farms in Brazil and Indonesia. IMNV possesses molecular characteristics of a totivirus (Nibert, 2007). IMNV was the first totivirus found to infect a host other than a fungus or a protozoan. A virus genetically related to IMNV was found to infect a cultured *Drosophila* cell and was designated *Drosophila* totivirus (DTV) (Wu *et al.*, 2010). Totiviruses have also been isolated from mosquitoes caught in China (Zhai *et al.*, 2010) and Japan (Isawa *et al.*, 2011). These viruses isolated from mosquitoes are most genetically similar to IMNV and DTV (Isawa *et al.*, 2011). A totivirus that is most closely related to giardiaviruses is also found in salmon (Haugland *et al.*, 2011).

The GenBank/EMBL/DDBJ accession number for the sequence of the novel dsRNA virus is LC026053.

Three supplementary tables are available with the online Supplementary Material.

Arboreal ants (*Camponotus yamaokai*) have been reported to possess endogenous dsRNA elements (Miyazaki *et al.*, 1996). In this study, the complete nucleotide sequence of the dsRNA element in *C. yamaokai* was determined. Genetic characterization and phylogenetic analysis revealed that the dsRNA in *C. yamaokai* is the genome of a member of the family *Totiviridae*, which led us to designate the virus as *Camponotus yamaokai* virus (CYV). To our knowledge, CYV is the first totivirus isolated from the order Hymenoptera.

RESULTS

Characterization of dsRNA from *C. yamaokai*

To determine the character of the dsRNA element in *C. yamaokai*, total nucleic acids were extracted from *C. yamaokai* and *Camponotus nawai*, and treated with nucleases (Fig. 1). The nucleic acids extracted from *C. yamaokai* produced a band with a size of ~5.7 kbp in 1 % agarose gel, whilst those from *C. nawai* did not. The band was resistant to DNase I treatment, S1 nuclease treatment and RNase A treatment under high salt conditions, whilst it was degraded by RNase A treatment under low salt conditions, indicating that the band extracted from *C. yamaokai* was composed of dsRNA.

Genetic characterization of the dsRNA and identification of a totivirus-like genome

The complete sequence of the dsRNA appeared to be 5704 nt in length (GenBank accession number LC026053), which was roughly consistent with the size estimated by agarose gel electrophoresis. The sequence was predicted to contain two large ORFs in different frames (Fig. 2). ORF1 (204–3287 nt) was found to encode a 1028 aa protein (116 kDa). ORF2 (3584–5560 nt) was found to encode a 659 aa protein (76 kDa). These two ORFs were separated by a 296 nt intergenic region. The protein sequence of ORF1 had significant similarity to proteins of *Drosophila yakuba* (GenBank accession number XP 002090380.1, $E=2\times10^{-40}$), *C. floridanus* (GenBank accession number EFN67052.1, $E=1\times10^{-6}$) and *Trichuris suis* (GenBank accession number KFD67269.1, $E=0.001$). The protein sequence of ORF2 had significant similarity to the protein of *D. yakuba* (GenBank accession number XP 002090381.1, $E=2\times10^{-91}$) and to the RNA-dependent RNA polymerase (RdRp) of members or putative members of the family *Totiviridae*, such as *Piscine myocarditis virus* (YP_004581250.1, $E=9\times10^{-12}$), *Giardia lamblia virus* (GenBank accession number AAB01579.1, $E=6\times10^{-8}$), *Leishmania RNA virus 2-1* (GenBank accession number NP_043465.1, $E=2\times10^{-4}$), *Omono river virus* (OMRV; GenBank accession number BAJ21513.1, $E=8\times10^{-4}$), *Trichomonas vaginalis virus 1* (GenBank accession number AAA62868.1, $E=3\times10^{-4}$), and IMNV (GenBank accession number AIC34742.1, $E=0.001$). ORF2 exhibited eight RdRP motifs similar to other totiviruses (Table S1, available in the online Supplementary Material) (Isawa *et al.*, 2011). The presence of virus genome-like dsRNA and the protein sequence homology of ORF2 to the RdRp sequences of family *Totiviridae* suggested the possibility that the dsRNA was the genome of a novel virus species.

Although the first start codon (AUG at nt 3584–3586) in ORF2 was found to begin 294 nt downstream of the ORF1 stop codon (UAA at nt 3288–3290), no in-frame stop codons existed upstream of the ORF2 start codon until UAA at position 2843–2845 nt. Thus, the ORF2 region, when defined as the regions devoid of stop codons, began 442 nt upstream of the end of ORF1, i.e. ORF1 and ORF2

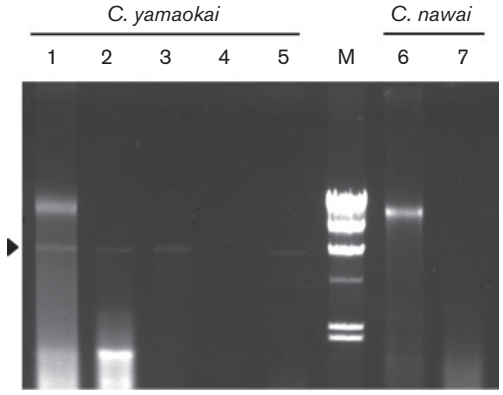

Fig. 1. Agarose gel electrophoresis of nucleic acids extracted from *C. yamaokai* and *C. nawai* after nuclease treatments. Lane M, molecular mass marker (λ-*Hin*dIII digest); lanes 1–5, nucleic acid of *C. yamaokai*; lanes 6 and 7, nucleic acid of *C. nawai*. Lanes 1 and 6 received no treatment; lanes 2–5 and 7 were treated with DNase I; lane 3 was treated with S1 nuclease; lane 4 was treated with RNase A under low salt conditions; lane 5 was treated with RNase A under high salt conditions. Arrowhead indicates viral genomic dsRNA.

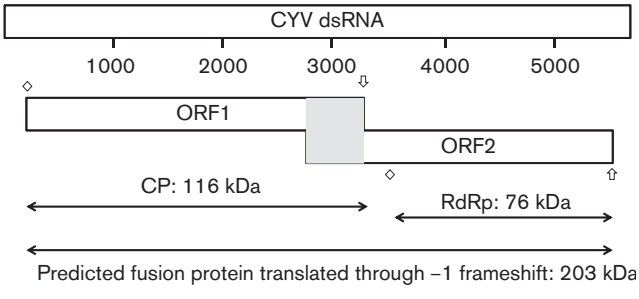

Fig. 2. Genome organization of CYV. The sizes of products are shown. The shaded area represents the overlap region between ORF1 and ORF2 (442 nt), where the −1 frameshift is predicted to occur. Diamonds and arrows indicate the start and the end, respectively, of the conventional ORFs.

were overlapping. This suggested that ORF2 was translated as a fusion with ORF1 through a −1 ribosomal frameshift within the region of overlap. Similar genomic arrangements have been described for several totiviruses (Wickner *et al.*, 2012). The ribosomal −1 frameshift site typically consists of a 'shift' (or 'slippery') heptamer fitting the consensus motif XXXYYYZ, where X represents any nucleotide, Y represents A or U and Z represents A, C or U, preceded by a GC dinucleotide that favours a frameshift (Jacks *et al.*, 1988; Bekaert *et al.*, 2003). The heptamer is followed by a short 'spacer' region and then a stable RNA secondary structure, such as a pseudoknot or hairpin. Sequence analysis of CYV revealed the existence of a potential slippery heptamer AAAAAAC at nt 2903–2908 (Fig. 3). A class 4 pseudoknot of 25 nt (nt 3096–3120) was identified with the HPknotter program.

Phylogenetic analysis

The pair-wise amino acid identities of the CYV RdRp and other totiviruses were low, ranging from 5 to 17 % (Tables 1 and 2). CP identities ranged from 4 to 8 %. Similarities between amino acid sequences of viruses were 43 to 57 % for both RdRp and CP.

To study the genetic relationships of CYV with other viruses, including 22 viruses in the family *Totiviridae*, and two viruses and four arthropod viruses tentatively assigned to the family *Totiviridae*, a phylogenetic tree by the maximum-likelihood method using amino acid sequences of RdRp was reconstructed (Fig. 4). CYV was distantly related to the other viruses in arthropods, i.e. *Armigeres subalbatus* virus (AsTV), DTV, IMNV and OMRV. The phylogenetic trees reconstructed using neighbour-joining and Bayesian

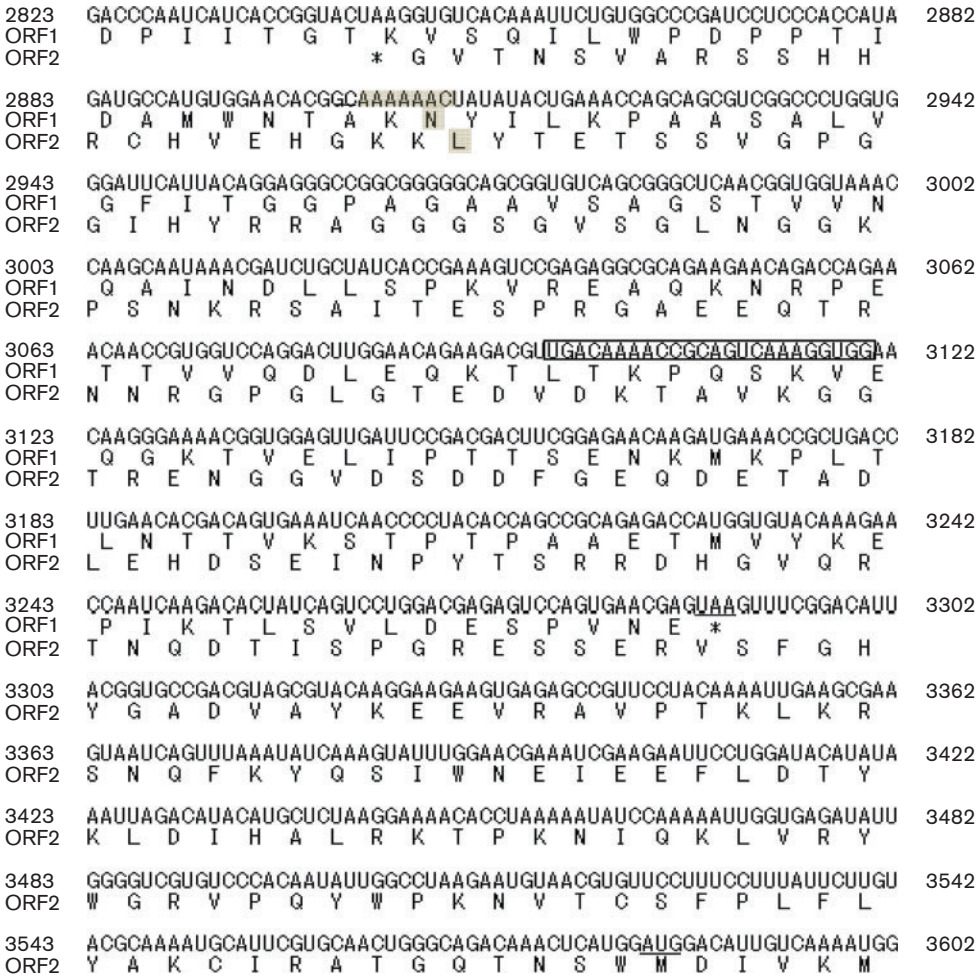

Fig. 3. Overlap between ORF1 and ORF2 of CYV. The stop codon of ORF1 is located at nt 3288–3290. ORF2 starts at position 2846. The start codon of ORF2 is located at nt 3584–3586. This means that the end of ORF1 overlaps with the start of ORF2 by 442 nt. Within the overlap region, a heptamer (AAAAAAC) complies with the definition of slippery heptamers that favour −1 frameshifts, preceded by a GC dinucleotide (underlined). A predicted pseudoknot was located at nt 3096–3120, downstream of the slippery heptamer.

Table 1. Members of the family *Totiviridae* and related viruses used in this study

Virus	Abbreviation	Genus	GenBank accession no.
Saccharomyces cerevisiae virus L-A	ScV-L-A	*Totivirus*	J04692
Saccharomyces cerevisiae virus L-BC	ScV-L-BC	*Totivirus*	U01060
Ustilago maydis virus H1	UmV-H1	*Totivirus*	U01059
Giardia lamblia virus	GLV	*Giardiavirus*	L13218
Leishmania RNA virus 1-1	LRV1-1	*Leishmaniavirus*	M92355
Leishmania RNA virus 1-4	LRV1-4	*Leishmaniavirus*	U01899
Leishmania RNA virus 2-1	LRV2-1	*Leishmaniavirus*	U32108
Botryotinia fuckeliana totivirus	BfTV1	*Victorivirus*	AM491608
Chalara elegans RNA virus 1	CeRV1	*Victorivirus*	AY561500
Coniothyrium minitans RNA virus	CmRV	*Victorivirus*	AF527633
Epichloe festucae virus 1	EfV1	*Victorivirus*	AM261427
Gremmeniella abientina RNA virus L1	GaRV-L1	*Victorivirus*	AF337175
Gremmeniella abientina RNA virus L2	GaRV-L2	*Victorivirus*	AY615210
Helicobasidium mompa totivirus 1-17	HmTV1-17	*Victorivirus*	AB085814
Helminthosporium victoriae virus 190S	Hv190SV	*Victorivirus*	U41345
Magnaporthe oryzae virus 1	MoV1	*Victorivirus*	AB176964
Magnaporthe oryzae virus 2	MoV2	*Victorivirus*	AB300379
Sphaeropsis sapinea RNA virus 1	SsRV1	*Victorivirus*	AF038665
Sphaeropsis sapinea RNA virus 2	SsRV2	*Victorivirus*	AF039080
Trichomonas vaginalis virus	TVV1	*Trichomonasvirus*	U08999
Trichomonas vaginalis virus II	TVV2	*Trichomonasvirus*	AF127178
Trichomonas vaginalis virus III	TVV3	*Trichomonasvirus*	AF325840
Eimeria brunetti RNA virus 1	EbRV1	Unassigned	AF356189
Piscine myocarditis virus	PMCV	Unassigned	HQ339954
Penaeid shrimp infectious myonecrosis virus	IMNV	Unassigned	AY570982
Drosophila totivirus	DTV	Unassigned	GQ342961
Armigeres subalbatus virus	AsTV	Unassigned	EU715328
Omono river virus	OMRV	Unassigned	AB555544

methods showed similar results to the maximum-likelihood method (data not shown).

Electron microscopic analysis

Fig. 5(a–c) shows transmission electron micrographs of double-stained ultrathin sections of a *C. yamaokai* ovary, an egg cell in the ovary and a *C. nawai* ovary. Virus particles were observed in the cytoplasm of the cell. The virus particles had an icosahedral, symmetrical structure with a mean diameter of ~30 nm, which is within the size range of the family *Totiviridae*. Similar virus particles were not observed in a *C. nawai* ovary.

Caste specificity, developmental stage specificity and tropism

Reverse transcription (RT)-PCR analysis revealed that CYV was detected in all castes and developmental stages of *C. yamaokai*, including eggs, larvae, pupae, adult workers, alates (male and female) and queens. Among queen body parts, abdomens constituted the source of the largest proportion of CYV genomes (75.0 ± 26.6 % of CYV genomes in whole queen bodies; $n=6$, Fig. 6). The head and the thorax contained similar levels of CYV genomes, with 13.4 ± 22.1 and 11.6 ± 6.3 %, respectively.

DISCUSSION

In this study, a new totivirus in arboreal ants was identified and characterized. Before IMNV was isolated in shrimp, totiviruses had been detected only in protozoa and fungi. However, totiviruses have been reported in flies, mosquitoes and salmon following the isolation of IMNV. The novel totivirus CYV was found in *C. yamaokai*. To our knowledge, CYV is the first totivirus in a hymenopteran.

The previously known arthropod totiviruses show some similarities to each other in genomic structure (Isawa *et al.*, 2011). Indeed, our phylogenic analysis of four arthropod totiviruses (AsTV, DTV, IMNV and OMRV) revealed that these viruses were closely related. It has been suggested that these viruses should be classified into the new genus denominated as *Artivirus* (Zhai *et al.*, 2010). However, although CYV was found in ants, CYV is not closely related to other arthropod totiviruses. These results suggest that CYV has had a unique evolutionary pathway among arthropod totiviruses.

Table 2. Identities and similarities between amino acid sequences of CYV and other totiviruses

Virus	CP		RdRP	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
ScV-L-A	6.2	44.5	12.3	53.8
ScV-L-BC	7.3	45.7	9.4	50.7
UmV-H1	6.5	45.6	6.4	44.1
GLV	6.9	49.0	9.6	46.3
PMCV	6.2	47.2	17.1	57.3
LRV1-1	5.4	46.3	8.0	45.6
LRV1-4	8.0	49.3	10.4	51.0
LRV2-1	6.4	43.8	11.9	50.2
BfTV1	6.7	45.3	10.4	51.3
CeRV1	8.2	45.8	8.1	44.5
CmRV	5.4	47.2	10.8	55.2
EfV1	5.7	47.1	6.4	44.5
GaRV-L1	6.7	46.9	6.1	44.9
GaRV-L2	5.7	49.1	6.2	44.6
HmTV1-17	4.4	49.1	13.4	51.9
Hv190SV	5.4	46.0	6.5	43.5
MoV1	7.8	50.7	8.1	47.0
MoV2	7.1	48.1	12.3	50.0
SsRV1	7.9	48.0	7.4	44.2
SsRV2	5.3	45.4	8.9	41.9
TVV1	8.5	46.5	10.5	51.7
TVV2	6.3	46.2	5.7	45.0
TVV3	5.9	46.2	8.1	46.1
EbRV1	7.5	52.0	8.8	48.8
IMNV	6.0	46.2	8.1	48.3
DTV	5.8	46.0	7.4	44.9
AsTV	5.7	49.8	9.3	46.7
OMRV	6.8	45.4	7.6	45.2

Horizontal gene transfer is suggested to be widespread from dsRNA viral genes to eukaryotic nuclear genomes, as well as eukaryotic nuclear genes to viral genomes (Liu *et al.*, 2010). In this study, the amino acid sequence of CYV ORF1 shows significant similarities to those of *C. floridanus* and *D. yakuba*. In addition, the amino acid sequence of ORF2 shows similarity to that of *D. yakuba*. These amino acid sequence similarities between CYV and the arthropods may be due to horizontal gene transfer. Totiviruses are found in *Drosophila* flies and *Camponotus* ants, namely DTV and CYV, respectively (Wu *et al.*, 2010 and this study). Thus, DTV and CYV are seemingly involved in gene transfer. However, BLAST analysis revealed that DTV ORFs do not show significant similarity/homology to genes in *Drosophila* flies. Moreover, CYV ORF1 does not show significant homology to the DTV genome. Thus, DTV is unlikely to be a donor or recipient of gene transfer. These results suggest that *Drosophila* files acquired viral genes through biological interactions with *Camponotus* ants or that an unknown CYV-like virus in *Drosophila* flies is a donor of the gene transfer, although the virus could be extinct.

In this study, CYV tissue tropism and existence in immature ants were analysed. CYV infection was systemic, although the viral genome was more abundant in abdomens than heads or thoraces. The viral genome was detected from ants at all developmental stages. A similar tropism was detected and suggested in *Solenopsis invicta* virus 3 (SINV-3) (Valles & Hashimoto, 2009). However, Valles *et al.* (2014) suggested that SINV-3 in immature ants was a contaminant from workers. CYV in immature ants might also be a contaminant from workers However, microscopic analysis detected virus particles in eggs and these results suggest that CYV detected in immature ants is not caused by contamination from workers.

C. yamaokai, in which CYV is found, was first recorded as a cryptic species of *C. nawai* (Terayama & Satoh, 1990). *C. nawai* is a monogynous species with a single queen in a colony and it inhabits warm regions, whereas *C. yamaokai* is polygynous with multiple queens and inhabits relatively cold areas. Although *C. yamaokai* is polygynous, very high within-colony relatedness is observed (Satoh *et al.*, 1997). This high relatedness is caused by *C. yamaokai* queens mating with genetically related males within or near their nest (Satoh, 1989). As some viruses affect host traits (Roossinck, 2011), CYV may influence some of these features of *C. yamaokai*. Future studies are needed to reveal the effects of CYV on host ants.

METHODS

Ant collection. *C. yamaokai* ants were collected from Hachioji (Tokyo, Japan). Nests of *C. nawai* were collected in Shimoda (Shizuoka, Japan). The colonies were kept on a semiweekly provision of 10 % sucrose solution, commercial protein diet for honeybees (Bee Hatcher: protein 9.7 %, carbohydrate 1.0 %, fat 0.1 %, fibre 0.1 %; Nippon Shiryo) and honeybee drones (*Apis mellifera*). The ants were frozen at −80 °C before analysis.

Electron microscopy observations. Queens of *C. yamaokai* and *C. nawai* were dissected and the ovaries removed. The ovaries were pre-fixed in 0.1 M cacodylate buffer with 2.0 % paraformaldehyde and 2.5 % glutaraldehyde at 4 °C overnight. Tissue fragments were washed in 0.1 M cacodylate buffer with 2 % sucrose for 4 h and post-fixed in 1 % osmic acid for 2 h at 4 °C. Following fixation, the fragments were dehydrated with an ethanol series by the conventional method and embedded in Quetol 812 (Nissin EM) by Luft's method. These tissues embedded in Quetol 812 were cut into thick sections (1–2 μm), which were single-stained with 0.1 % Toluidine blue and subjected to light microscopic examination. These tissues embedded in Quetol 812 were also sectioned on a Reichert Ultracut E (Leica) microtome to obtain ultrathin sections (80 nm), which were double-stained with uranyl and lead, and examined under a JEM 1230 (Nihon Denshi) transmission electron microscope.

Preparation and analysis of viral dsRNA. Total nucleic acids were extracted from ants using a phenol/chloroform method following the procedure of Sasaki *et al.* (1995). For nuclease treatment, the nucleic acids were incubated at 37 °C with 5 U DNase I (TaKaRa) in the reaction buffer. The nucleic acids were also incubated at 37 °C with 100 ng RNase A (Machrey-Nagel) in 0.1 × or 2 × saline sodium citrate buffer, or 15 U S1 nuclease (TaKaRa). Samples were electrophoresed on a 1 % agarose gel, followed by ethidium bromide staining.

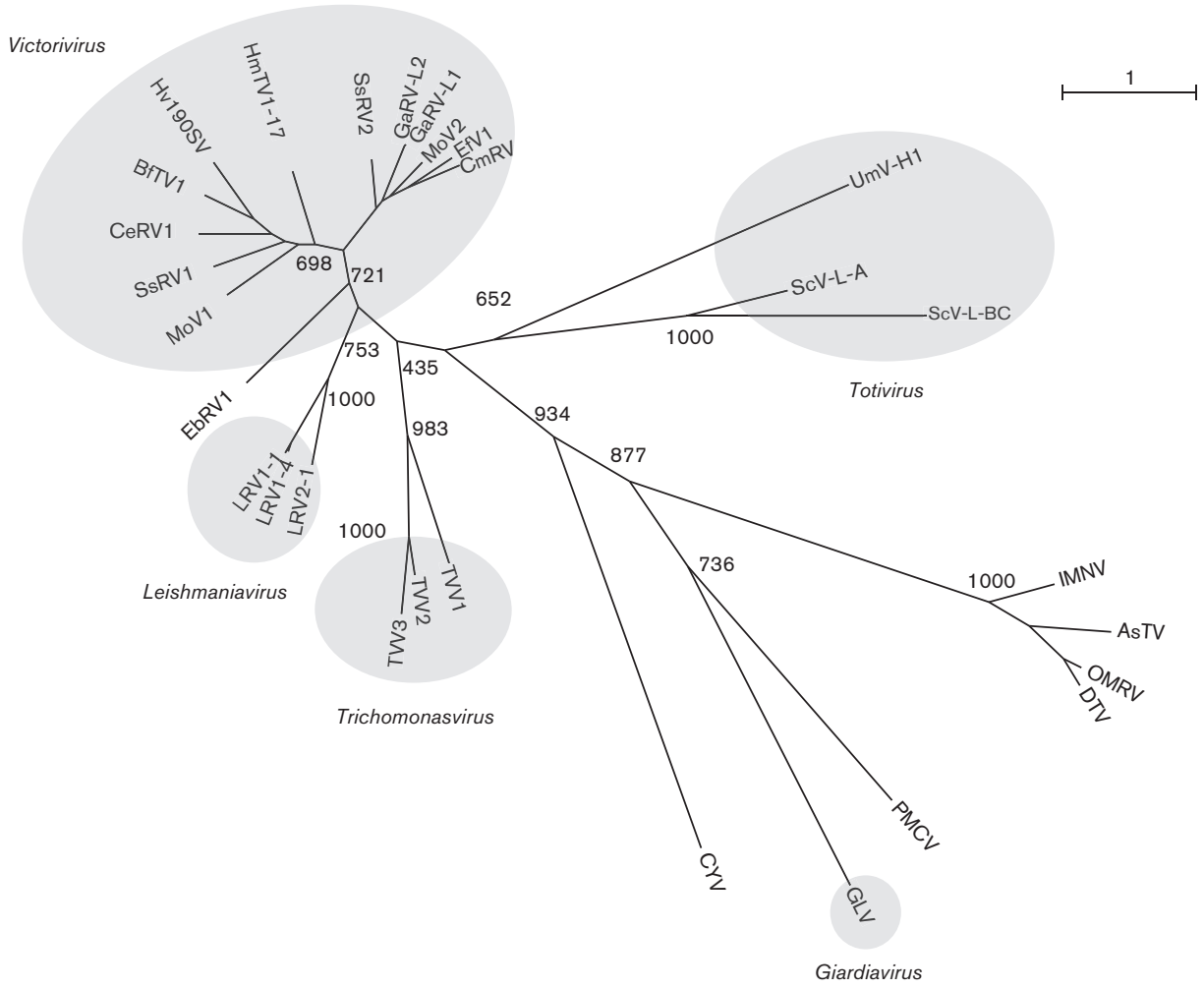

Fig. 4. Phylogenic tree of the family *Totiviridae* reconstructed using a maximum-likelihood algorithm. Numbers indicate bootstrap values.

Viral genome sequence. The total nucleic acids extracted from ants were purified by chromatography on cellulose powder (Advantec) as described by Morris & Dodds (1979). The dsRNA fractions were further purified with RNase-free DNase I (TaKaRa) to eliminate contaminating host DNA. Purified dsRNA molecules were used as a template for cDNA synthesis. Random hexamers were used as primers for the first-strand synthesis. After second-strand synthesis, cDNA fragments were cloned and sequenced. DNA sequencing was performed on both strands by an ABI 3730 automated sequencer with ABI BigDye Terminators version 3.1 Cycle Sequencing. To obtain PCR products that corresponded to the terminal regions of the dsRNA, the 5′ end regions of the dsRNA were amplified using the 5′-Full RACE Core Set (TaKaRa) and a SMARTer RACE cDNA Amplification kit (Clontech) following the manufacturer's protocols with oligo primers (Table S2). The PCR products were also cloned and sequenced on both strands. The overlapping sequences were assembled using GENETYX-ATSQ (version 6.0) to obtain the entire cDNA sequences of CYV. To confirm the CYV genome sequence, a series of cDNA fragments were generated by using oligo primers (Table S3) and sequenced directly.

The complete nucleotide sequences of CYV genomes were analysed to determine the ORFs. These ORFs were then translated into amino acid sequences using GENETYX 9.1.0. The amino acid sequence identity and similarity of viral proteins between CYV and other non-segmented dsRNA viruses were analysed using GENETYX 9.1.0.

Phylogenetic analysis. Phylogenetic trees were reconstructed with the totiviruses listed in Table 1, based on the amino acid sequences (52–399 aa of ORF2) containing conserved RdRp motif domains. The multiple sequence alignment was conducted using CLUSTAL_X 2.1 (Larkin *et al.*, 2007) and MEGA6 (Tamura *et al.*, 2013). The aligned matrix data were analysed by the maximum-likelihood algorithm PhyML 3.1 (Guindon *et al.*, 2010) with the best-fit models of amino acid substitution selected by ProtTest 3.4 (Guindon & Gascuel, 2003; Darriba *et al.*, 2011), as judged by the Akaike information criterion (Posada & Buckley, 2004). Statistical support for splits in the tree was determined using bootstrap testing with 1000 replications. The tree was graphically represented using SeaView4 (Gouy *et al.*, 2010). Phylogenetic trees were also constructed by a neighbour-jointing method with MEGA6 (Tamura *et al.*, 2013) and a Bayesian method with MrBayes (http://mrbayes.sourceforge.net/).

Quantitative analysis of viral dsRNA. Six mated queens were used for the analysis. They were each cut into three parts: head, thorax and

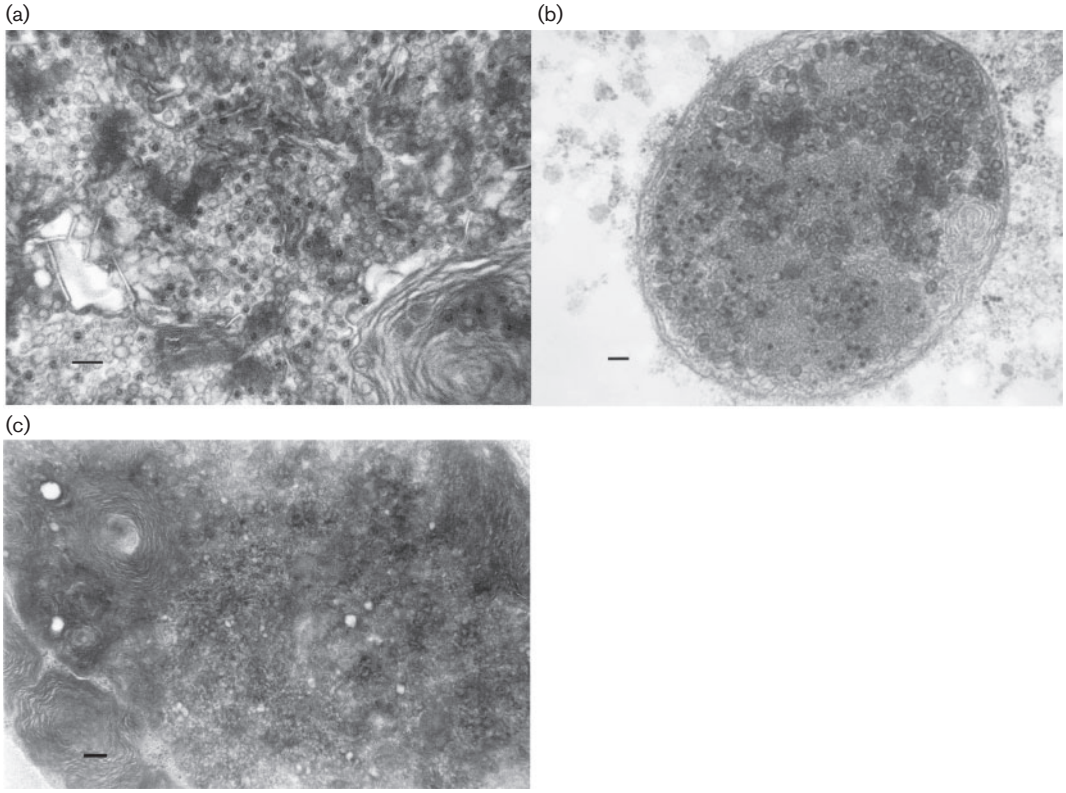

Fig. 5. Electron micrograph of *C. yamaokai* and *C. nawai* ovaries. Virus-like particles are observed in a *C. yamaokai* ovary but not in a *C. nawai* ovary. (a) Ovary of an unmated *C. yamaokai* queen. (b) Egg in an unmated *C. yamaokai* queen ovary. (c) Ovary of an unmated *C. nawai* queen. Bar, 100 nm.

abdomen. The nucleic acid was extracted from each part with RiboZol RNA Extraction Reagent (AMRESCO) and then treated with RNase-free DNase I (TaKaRa). The first-strand cDNA was synthesized with a SuperScript III Transcription System (Invitrogen) using random primers with DMSO-mediated chemical denaturation. Real-time quantitative PCR was carried out using TaKaRa Ex *Taq* Hot-Start Version (TaKaRa), following the manufacturer's instruction with one modification, in which 1 µl SYBR Green was added to the reaction mix. An oligonucleotide primer pair (forward, 5′-ATAACAACAACAATGGCATC-3′; reverse, 5′-CGAACCGTTCACCGTCAATA-3′) based on conserved motifs in the RdRp was used for the amplification. The reactions were conducted in triplicate using a LightCycler Nano (Roche). The reactions were: one cycle of 94 °C for 2 min, and 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by dissociation curve analysis. A standard curve was constructed from RT-PCR products of the RdRp region using a copy number range of $10–1 \times 10^8$. The proportion of CYV genomes in each body part was calculated against the CYV genomes in a whole body.

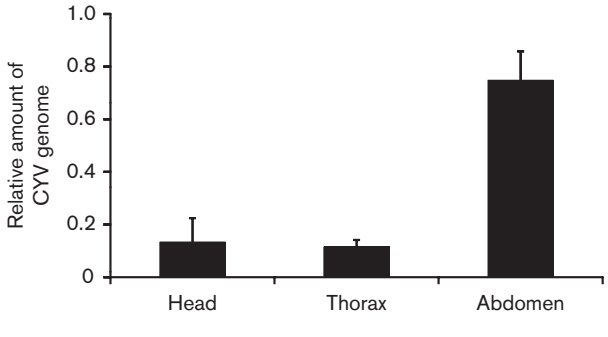

Fig. 6. Tropism of CYV in *C. yamaokai* queens. Values represent mean ± SEM.

Caste and developmental stage specificity of CYV. To detect caste and developmental stage specificity of CYV, up to 10 individuals were pooled for each caste [reproductive queen, alate (male and female), major worker, minor worker] and developmental stage (egg, larva, pupa). The nucleic acid extraction and reverse transcription were conducted as described above. The oligonucleotide primer pair (forward, 5′-ACGGCTGTCCCTGTGTCATCGGACATCG-3′; reverse, 5′-TCGTCGAGTGTAAATTTGTGGCTTCGAAAC-3′) was used for PCR with Ex *Taq* (TaKaRa), following the manufacturer's instruction. The reactions were: one cycle of 94 °C for 2 min, and 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. PCR products were electrophoresed on a 1 % agarose gel, followed by ethidium bromide staining.

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