First Report of the Complete Genome Sequences of Anemone Mosaic Virus and Ranunculus Mild Mosaic Virus Isolated From Anemone Imported From the Netherlands

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Research Article

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Abstract

The anemone mosaic virus (AnMV) and ranunculus mild mosaic virus (RanMMV) infect the anemone plant with characteristic mosaic patterns on leaves. Two complete genome sequences of the two viruses imported from the Netherlands, were determined based on deep sequencing for the first time. Each of AnMV and RanMMV had 9,698 and 9,537 nucleotides (nt), excluding the poly(A) tail. They shared 80.0% nt/amino acid (aa) sequence identities or more, which are above the species demarcation value, with only AnMV and RanMMV reported previously in coat protein region, but having 68.0% nt/aa sequence identities or less with other potyviruses in each coding region of the complete sequences. Additionally, phylogenetic analysis showed that AnMV and RanMMV were included in other known potyviruses. These results suggest that both of AnMV and RanMMV were independent species belonging to the genus Potyvirus.

Background

Anemone, a genus of ornamental bulbous plants in the family Ranunculaceae, is native to the Mediterranean northern coast [1]. Its bulbs are subject to post-entry quarantine in Japan. Today, anemones of various cultivars have been produced and cultivated worldwide. Anemone has been reported to be infected with many plant viruses including anemone mosaic virus (AnMV), cucumber mosaic virus, ranunculus mild mosaic virus (RanMMV), raspberry ringspot virus, tobacco necrosis virus, tobacco rattle virus (TRV), tobacco ringspot virus, tomato spotted wilt virus, and turnip mosaic virus (TuMV) [2–5]. Of those, AnMV is known to cause flower breaking and distortion with leaf mottling, is transmitted by aphid, and is classified in the turnip virus 1 group containing brassica nigra virus, cabbage black ringspot virus based on its biological properties described in 1957 [4]. Both AnMV and these viruses are synonym for TuMV in datasheet of CABI Invasive Species Compendium in 2019 [6]. Alternatively, the coat protein (CP) region sequence of AnMV registered in NCBI databases was detected in anemone from the Netherlands in 2011 and which was not similar to known potyviruses [5]. In addition, AnMV is classified as “Related, unclassified potyvirus” in ICTV 10th report [7]. Therefore, the relationship between this sequence information and biological information of AnMV is not clearly organized. On the other hand, RanMMV that is a member of the genus Potyvirus was first found from Ranunculus asiaticus in Italy and has been known to cause leaf mosaic in ranunculus. Moreover, the transmission of this virus by aphids and host range is limited to the family Ranunculaceae [8]. So far, only CP region sequence of RanMMV has been reported from anemone [5]. Today, the CP region sequences of RanMMV isolated from China, Israel, Italy, and Korea are deposited in NCBI databases [8,9]. Accordingly, the complete genome sequences of AnMV and RanMMV have not been clarified yet. Therefore, we carried out to analyse the complete genome sequences of AnMV and RanMMV based on deep sequencing in this study.

In March 2019, an anemone plant (cv. Charmer), originated from bulbs imported from the Netherlands, showed mosaic on leaves (Supplementary Fig. 1) during a field inspection at post-entry quarantine farm in Japan. To identity the potential viruses involved in the disease, total RNAs were extracted from a 0.1 g symptomatic leaf tissues of one anemone using SpectrumTM Plant Total RNA Kit (Sigma-Aldrich, St.
Louis, MO, USA), according to the attached manual. A next generation sequencing (NGS) library was constructed from the extracted RNA and sequenced on an Ion Personal Genome Machine (PGM) system (Thermo Fisher Scientific, Tokyo, Japan), and then sequence data acquisition and contig preparation were done using CLC Genomics Workbench ver.8.0 (QIAGEN, Hilden, Germany), according to Yanagisawa et al. [10]. We analysed the identities between obtained contigs and the sequence of known viruses registered in NCBI databases by using BLAST search analysis.

To verify the complete genome sequence of AnMV-like virus and RanMMV-like virus, we designed new primers based on the sequence of four potyvirus-related contigs and one contig related to a partial genome sequence of RanMMV (accession no. EU042755.1) considering results of above analysis, respectively (Supplementary Table 1 and Supplementary Fig. 2). cDNAs were constructed using PrimeScript™ Reverse Transcriptase (Takara Bio, Shiga, Japan) with a random primer (N) 6 and an oligo(dT)15 primer (Takara Bio). Polymerase chain reaction (PCR) was performed using KOD -Plus- Neo (TOYOBO, Osaka, Japan), according to the manufacturer's protocol. cDNAs of the 5′- and the 3′-terminal ends were amplified using a SMARTer® RACE 5'/3' Kit (Takara Bio) and a 3′-Full RACE Core Set (Takara Bio), respectively, according to the manufacturer's protocol. Each of the above PCR products were direct-sequenced using a SeqStudio Genetic Analyzer (Thermo Fisher Scientific). Pair-end sequence data of each PCR products were assembled with MEGA X program [11]. Furthermore, NGS reads were mapped to the verified complete genome sequence of AnMV-like virus and RanMMV-like virus using CLC Genomics Workbench ver.8.0.

Open reading frames (ORFs) in the full-length sequence were identified using Open Reading Frame Finder (https://www.ncbi.nlm.nih.gov/orffinder/). We predicted putative cleavage sites of each ORFs by sequence comparison to those of other known potyviruses. To investigate the phylogenetic relationship between AnMV-like virus, RanMMV-like virus, and other potyviruses, a phylogenetic tree was constructed based on the complete polyprotein and amino acid (aa) sequence of CP of these viruses using the maximum-likelihood method with 1,000 bootstrap replications using the MEGA X program. We compared separately the complete genome sequence and the deduced aa sequences encoded by each ORFs of AnMV-like virus and RanMMV-like virus with the viruses closely related to AnMV-like virus and RanMMV-like virus based on the result of phylogenetic analysis (Fig. 1 and Supplementary Fig. 3) and other potyviruses randomly selected using the BLAST program.

To test whether RanMMV-like virus could be detected from the above extracted RNAs, Reverse transcription polymerase chain reaction (RT-PCR) was performed with RanMMV-specific primer sets RanMMV-F/RanMMV-R [12], using QIAGEN OneStep RT-PCR kit (QIAGEN). We performed direct-sequence analysis of these PCR products. We also carried out RT-PCR with each of specific primer sets of TRV [13] and opium poppy mosaic virus (OPMV) [14].

In total, 422,337 reads were generated by NGS analysis. A total of 57 contigs (longer than 500 nucleocides (nt)) were assembled, with a size ranging from 535 to 9,436 nt. BLASTx research analysis showed that 21 contigs were related to virus sequence. Of those, four contigs (893, 1,110, 1,476, and
3,478 nt) had 94.3, 94.9, 98.2, and 94.9% nt sequence identity with some TRV strains (GQ903771.1, AJ250488.1, and KT033412.1), four contigs (953, 1,133, 2,804, and 2,931 nt) had 83.2, 92.7, 89.1, and 83.6% nt sequence identity with some OPMV strains (EU151723.4, KJ372735.1, and LT595034.1), one contig (9,436 nt) had 96.3% nt sequence identity with RanMMV (EU684747.1), and four contigs (535 ~ 4,833 nt) had low sequence nt identity (<81.0%) with potyviruses. One contig (1,339 nt) had also 98.3% nt sequence identity with AnMV reported previously (EU042755.1), but this contig was not related to the AnMV-like sequence in this study. Seven contigs (607 ~ 1,550 nt) which were related to known potyviruses had no significant similarity to database sequences.

The complete genome sequence of AnMV-like isolate (AnMV-NL) and RanMMV-like isolate (RanMMV-NL) had 9,698 nt (ORF; 110–9,376 nt, 3,088 aa) (Accession No. LC604019) and 9,537 nt (ORF; 123–9,347 nt, 3,074 aa) (Accession No. LC604020) excluding the 3’ poly(A) tail constructed as resulting sequence analysis and then nine putative cleavage sites of these viruses were identified (Supplementary Fig. 2), respectively. In addition, each sequence obtained by direct sequence analysis based on PCR products constructed using new designed primer sets matched the sequence of contigs obtained from NGS analysis. A total of 63,691 reads mapped to the genome sequence of RanMMV-NL (mean coverage 666.95) and 6,066 reads mapped those of AnMV-NL (mean coverage 70.93) were obtained. In addition, 2,564 and 17,031 reads were mapped to those of TRV (mean coverage 42.02) and OPMV (mean coverage 502.24).

Phylogenetic tree based on the complete genome sequences of potyviruses showed that each of AnMV-NL and RanMMV-NL were closely related to plum pox virus and beet mosaic virus (Fig. 1). Based on CP gene region, AnMV-NL and RanMMV-NL in this study were most closely related to previous reported AnMV and RanMMV isolates, respectively (Supplementary Fig. 3).

The complete genome sequence of AnMV-NL had 57.0–63.0% nt and 44.0–57.0% aa similar sequence identity with those of other potyviruses. All ORFs of AnMV-NL virus also had low identity to those of selected potyviruses (~67.0% nt sequence identity, ~69.0% aa sequence identity) (Table 1). In contrast, the identity between CP sequences of AnMV-NL and that of AnMV reported previously was high (>80.0% identity for both nt and aa sequences).

Similarly, the complete genome sequence of RanMMV-NL had 58.0–62.0% nt and 47.0–57.0% aa sequence identity with those of other potyviruses. All ORFs of RanMMV-NL also had low identity to those of selected potyviruses (~68.0% nt sequence identity, ~70.0% aa sequence identity) (Table 1). In contrast, the identity between CP sequences of RanMMV-NL and that of RanMMV isolated from ranunculus was high (>97.0% identity for both nt and aa sequences).

We could obtain PCR products of expected size with RanMMV-specific degenerate primer sets. Sequence of a 657 bp product completely matched the CP gene sequence of RanMMV-NL. TRV and OPMV were also detected in anemone using each virus specific primer sets. We could not perform RT-PCR assay to detect AnMV because its specific primer set was not previously reported.
For the first time, we were able to reveal the full genome of these two viruses based on the above results. Although AnMV and RanMMV were only characterized based on the partial genome sequence including CP region in previous study [5,8,9], we showed in this study that nt and aa sequence identity of the complete genome and each ORFs of these two viruses with other potyviruses (Table 1) are far below the current species demarcation criteria for the family Potyviridae [7]. Comparison of the complete genome sequence also revealed that AnMV reported in 2011 [5] was apparently distinguished from TuMV (Table 1). Additionally, phylogenetic analysis showed that AnMV and RanMMV were included in the known potyviruses group (Fig. 1). These results suggest that each of AnMV and RanMMV are independent members of the genus Potyvirus.

AnMV-NL and RanMMV conserved 2,900GAA AAA A2,906 and 2,862GAA AAA AA2,869 motifs (Supplementary Fig. 2), respectively. These motifs could enable PIPO within the P3 region protein in the +2-reading frame and were found in Potyviridae [15]. Our study also revealed that these viruses had some motifs highly conserved among potyviruses. In particular, we focused on the motifs (R/KITC, PTK, and DAG), which were required for aphid transmission [7]. RanMMV had 353RITC356 and 611PTK613 motifs in HC-Pro and 2,806NAG2,808 (corresponding to the DAG motif [16]) motif in CP, and AnMV had also 356KLSC359 (corresponding to the KITC motif [17]) and 613PTK615 motifs in HC-Pro and 2,832DAG2,834 motif in CP. Because we found the motifs involved in aphid transmission, the report that RanMMV was transmitted by aphid was strongly supported [8]. In addition, AnMV could also be transmitted by some aphids. Therefore, these viruses might cause the spread of infection to new plants by aphid transmission. On the other hand, although four viruses including AnMV and RanMMV were detected from anemone, it was unclear which of these viruses were involved in the symptoms observed in the anemone plant. Thus, it will be necessary to investigate the involvement of these viruses in the symptoms in the future.

**Declaration**

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**Conflicts of interest**

The authors declare no competing interests.

**Authors’ contributions**

All authors contributed to the study conception and design. Hironobu Yanagisawa supervised its execution. Material preparation was performed by Moritsugu Oishi and Yuji Fujiwara. Data collection and analysis were performed by Yuya Imamura. The first draft of the manuscript was written by Yuya Imamura and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.
Ethical approval

This article does not contain any experiments involving humans or animals that have been performed by any of the authors.

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Table

Due to technical limitations, table 1 xlsx is only available as a download in the Supplemental Files section.