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Pepper veinal mottle virus in Japan is closely related to isolates from other Asian countries, but more distantly to most of those from Africa

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Abstract

Pepper veinal mottle virus (PVMV) is known to infect chilli pepper and belongs to the *Chilli veinal mottle virus* phylogroup of potyviruses. PVMV has recently appeared in Japan. In this study, we report six complete genomic sequences of PVMV isolates from chilli pepper (i.e. *Capsicum annuum*) in Okinawa Islands in Japan, and we determined the evolutionary relationships between Japanese isolates and the isolates reported earlier from African and Asian countries. Complete genomic sequences of the six Japanese PVMV isolates were 9760 nucleotides in length, excluding the nucleotide primer sequences used for amplifying 5′ end of the genomes. The major findings of this study are as follows: (1) all the Japanese isolates of PVMV have similar biological and molecular characteristics, indicating the presence of only one population in Japan; (2) there are at least three major phylogenetic groups of PVMV worldwide; (3) PVMV probably originated in East Africa; and (4) all the Asian isolates are closely related to the Ghanaian isolate.

Keywords *Pepper veinal mottle virus* · Potyvirus · Genome · Evolution · Emerge · Japan

Introduction

Pepper veinal mottle virus (PVMV) is a member of the genus *Potyvirus* in the family *Potyviridae*. PVMV is one of the nine potyviruses known to infect chilli pepper, which include *Chilli veinal mottle virus* (ChiVMV), *Chilli ringspot virus* (ChiRSV), *Potato virus Y* (PVY) and *Tobacco etch virus* (TEV). PVMV belongs to the ChiVMV phylogroup of potyviruses which comprises ChiVMV, ChiRSV, *Tobacco vein banding mosaic virus* (TVBMV), *Wild tomato mosaic virus* (WTMV), *Habenaria mosaic virus* (HaMV), *Gloriosa stripe mosaic virus* (GSMV) and *Yam mild mosaic virus* (YMMV) [1, 2]. Like the other potyviruses, PVMV is transmitted by aphids in a non-persistent manner [3, 4] but there is no report of seed transmission in limited tests with *Capsicum annuum, Nicotiana clevelandii* and *N. megalosiphon* [5].

PVMV infects chilli pepper, bell pepper, tomato, tobacco, petunia and eggplant as natural hosts [5]. The virus was first reported in the Eastern region of Ghana from chilli peppers (*Capsicum annuum* and *Capsicum frutescens*) [6]. PVMV has been described as one of the biotic factors that affects the yield losses in solanaceous crops in Africa including Ghana, Nigeria, Cote d'Ivoire and Mali [5–15] and in Asia

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The nucleotide sequences of PVMV OKP2, OKP3, OKC25, OKP30, OKP39 and OKP41 isolates reported in this paper are available in the GenBank/EMBL/DDBJ database as accessions numbers LC438540–LC438545.

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including China, Taiwan and Japan [4, 16, 17]. Japanese PVMV isolates show rugosity, mosaic and malformation on chilli pepper (*Capsicum annuum*) leaves, and were first found in the early of 2010s [4]. Three full genomic sequences from Ghana [8], China [17] and Taiwan (Accession number FJ617225), and 19 partial genomic sequences of PVMV have been reported to date, which provides insufficient sequences to assess the population structure of this virus especially the isolates in Asian countries.

Okinawa Islands, at the southwest tip of Japan, are known as one of chilli pepper growing areas for commercial purpose and home consumption, and PVMV has been reported to occur recently in Ishigaki Island [4], one of the Okinawa Islands of Japan. Hence, it is important to know more about the biological and molecular characteristics of Japanese epidemic of PVMV. We report here the full genomic sequences of six PVMV isolates, collected from chilli peppers in Ishigaki and other islands, and compare the evolutionary relationships between Japanese isolates and the isolates reported earlier from other Asian countries and also African countries, including the published complete genomic sequence of the original Ghanaian isolate [8].

Materials and methods

Virus isolates and host tests

Symptomatic chilli and bell pepper leaves of the 52 samples were collected in the growing seasons between 2010 and 2015 from Ishigaki, Iriomote and Yonaguni Islands of Okinawa Islands (Fig. 1). All the samples were examined for PVMV infection by reverse-transcription and polymerase chain reaction (RT-PCR) using potyvirus-specific primers (POTYNIbNOT4P; 5′-GGGGCGGCCGCATA TGGGGTGAGAGAGGT(A/C/G/T)TG(C/T)GT(A/C/G/T)GA(C/T)GA(C/T)TT(C/T)AA(C/T)AA-3′ and Tu3T9M; 5′-GGGGCGGCCGCT$_{30}$-3′) [4]. RT-PCR products from the nuclear inclusion b protein (NIb) coding region to 3′ end of the genome of approximately 2100 bp were amplified from pepper leaves when the primer pairs were used. We further confirmed PVMV infection by sequencing the RT-PCR products using POTYNIb5P primer (5′-CGCATA TGGGGTGAGAGAGG-3′), which is a part of POTYNIb-NOT4P primer, (underlined). The details of RNA extraction and sequencing are described below.

Single chlorotic lesions were collected from inoculated *Chenopodium amaranticolor* 10 days after inoculation, and then the isolated viruses were propagated in *N. benthamiana*, petunia and bell pepper plants. We used saps from these symptomatic leaves to inoculate leaves of test plants because chilli pepper sap has a high content of virus inhibitor [4]. The biologically cloned isolates, which we call OKP2, OKP3, OKC25, OKP30, OKP39 and OKP41 isolates, and those reported in earlier studies, are shown in Table 1. The plants infected with the Japanese isolates were homogenized in 0.01 M potassium phosphate buffer (pH 7.0) and inoculated mechanically onto the first true leaves of plants that belong to the families Solanaceae, Brassicaceae, Chenopodiaceae, Cucurbitaceae and Fabaceae. Details of plant species inoculated in this study are shown in Table S1.

Viral RNA extraction and sequencing

Total RNAs were extracted from PVMV-infected *N. benthamiana* or pepper leaves using Isogen II (Nippon Gene, Tokyo, Japan), following the manufacturer's instructions. The RNAs were then reverse transcribed and amplified using PrimeScript II High Fidelity One Step RT-PCR kit (TaKaRa Bio, Ohtsu, Japan). The RT-PCR products were

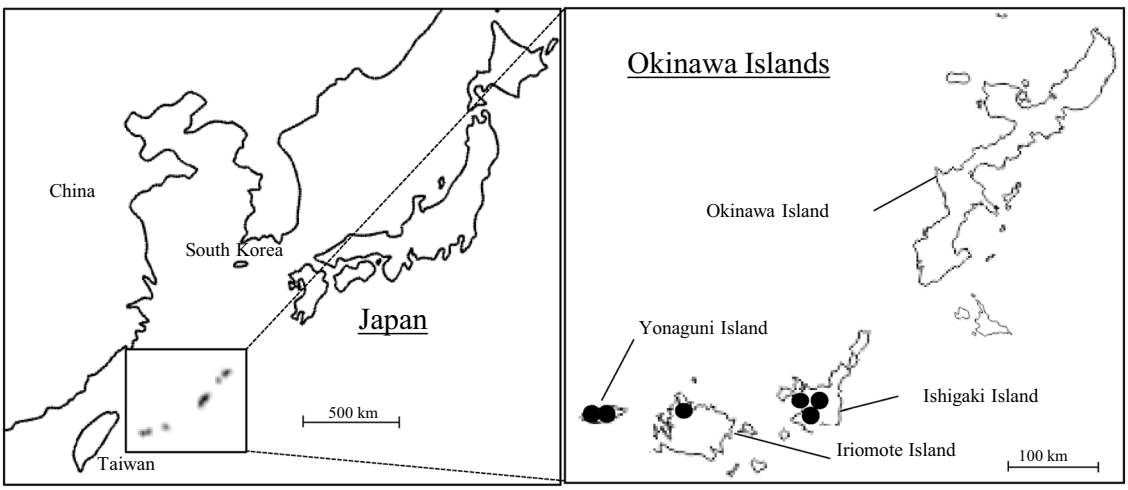

Fig. 1 Collection of *Pepper veinal mottle virus* isolates in Okinawa Islands. Dots indicate the sampling sites

Table 1 Pepper veinal mottle virus isolates used in this study

Isolate	Original host	Country	Year of collection	Accession No. (Reference)	
				Coat protein coding region	Full genome
Asia					
OKP2	*Capsicum annuum* cv. Kahat-ace	Ishigaki, Japan	2010	LC062895 [4]	This study, LC438540
OKP3	*C. annuum* cv. Kahat-ace	Ishigaki, Japan	2012		This study, LC438541
OKC25	*C. annuum* L. *var grossum* cv. unknown	Iriomote, Japan	2014		This study, LC438542
OKP30	*Capsicum frutescens* cv. Shima-togarashi	Yonaguni, Japan	2015		This study, LC438543
OKP39	*C. annuum* cv. Kahat-ace	Ishigaki, Japan	2015		This study, LC438544
OKP41	*C. frutescens* cv. Shima-togarashi	Yonaguni, Japan	2015		This study, LC438545
HN	*C. annuum*	China	2014		KR002568 [17]
Lis1	*Eustoma russellianum*	Taiwan	2004	FJ843065	
ns1	*Solanum nigrum*	Taiwan	2008		FJ617225
Pep1	*C. annuum*	Taiwan	2006	EU719646	
Tom1	*Solanum lycopersicum*	Taiwan	2006	EU719647 [16]	
Africa					
1SPno7-3	*C. annuum*	Mali	2009	GQ918274	
2SPno2	*C. annuum*	Mali	2009	GQ918275	
5SPno3	*S. lycopersicum*	Mali	2009	GQ918276	
CAC2	*Capsicum chinense*	Senegal	< 1999	AJ780966 [5]	
F-Bot	*C. annuum*	Cameroon	< 1999	AJ780967 [5]	
IC	*Capsicum* sp.	Ivory Coast	Unknown	[16]	
P	*C. frutescens*	Ghana	1971		NC_011918 [8]
Potyvirus E	*C. annuum*	Ethiopia	< 1999	AJ780970 [5]	
R1	*Capsicum* sp.	Rwanda	2016	MG470801	
S31	*Solanum integrifolium*	Ghana	< 1999	AJ780968 [5]	
To	*S. lycopersicum*	Tunisia	Unknown	[14]	
Tu1	*Capsicum* sp.	Tunisia	Unknown	[14]	
Tu2	*Capsicum* sp.	Tunisia	Unknown	[14]	
Y90/34	*C. annuum*	Yemen	1990	AJ780969 [5]	

purified by electrophoresis in agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen, K.K, Japan).

The genomic sequences from each of the six isolates were determined using four overlapping independent RT-PCR products to cover the entire PVMV genomes. At least 200-bp overlapping regions (Table S2) between the amplified products were sequenced to confirm that they came from the same genome. The genomic sequences were determined by primer walking using BigDye Terminator v3.1 Cycle sequencing Ready Reaction kit (Applied Biosystems, USA) and an Applied Biosystems Genetic Analyzer DNA model 3130. The resulting sequence data of six PVMV Japanese isolates together with sequences previously reported (Table 1) were managed using BioEdit version 5.0.9 [18]. Only three nearly complete genomic sequences of PVMV isolates (HN, ns1 and P isolates from China, Taiwan and Ghana, respectively) were available from the International public nucleotide sequence databases. The nucleotide sequences of the polyprotein coding region or coat protein (CP) coding regions were aligned using CLUSTAL X2 [19] and TRANSALIGN (kindly supplied by Georg Weiller) to maintain the degapped alignment of the encoded amino acids. The nucleotide sequences of the genomes were then reassembled and formed the 9752 nucleotides (nts) genomes by joining the aligned 5′ and 3′ non-coding region (NCR) to the aligned full genomic sequences.

Recombination analysis

The assembled genomic sequences (9752 nts in length) of six Japanese PVMV isolates and three from the International public nucleotide sequence database were assessed for evidence of recombination. Furthermore, because some unusual nucleotides were found in Ghanaian P (NC_011918) and Chinese HN isolates (KR002568) (Fig. S1), the full genomic sequences were aligned by eye (9761

nts, excluding the 35 nt PVM5T16P primer sequences used to amplify 5′ ends of the genomes) without outgroup sequences. They were also assessed for evidence of recombination. The aligned CP coding sequences (807 nts in length) of six Japanese PVMV isolates with 19 retrieved were also assessed for evidence of recombination. Possible recombination events in the PVMV genomes was identified using RDP [20], GENECONV [21], BOOTSCAN [22], MAXCHI [23], CHIMAERA [24] and SISCAN [25] implemented in RDP4 [26]. The analyses were done using default settings for each detecting program and 0.01 Bonferroni-corrected *P value* cut-off.

Phylogenetic analysis

The polyprotein coding sequences of nine PVMV isolates and CP coding region sequences of 25 isolates were used for phylogenetic analysis. The best-fit model of nucleotide substitutions for each dataset was determined using jModelTest version 2.1.7 [27]. The general time-reversible (GTR) model of nucleotide substitution with rate variation among sites modelled using a gamma distribution and a proportion of invariable sites (GTR + I + G) was used. The genomic sequences of ChiVMV, TVBMV, ChiRSV, WTMV and YMMV were used as outgroup to construct the polyprotein phylogenetic tree, since all these viruses belong to the same potyvirus phylogenetic group. The CP sequences of two ChiVMV were used as an outgroup to construct the CP phylogenetic tree because BLAST searches showed that ChiVMV was the most closely related species to PVMV. Branch support in the tree was evaluated by bootstrap analysis based on 1000 pseudoreplicates. The TREVIEW software [28] was employed to display the calculated trees. We calculated the nucleotide and amino acid identities and similarities using Sequence demarcation tool (SDT) version 1.2 [29], EMBOSS Needle [30] and SIMPLOT [31].

Population structure analysis

The STRUCTURE version 2.3.4 [32] software was used to test for evidence of genetic structure among PVMV subpopulations and to identify individuals that were admixed or had migrated among PVMV populations. The CP coding region of 25 PVMV isolates were used. To select the number of clusters that best represented population structure, the analyses with 1–10 subpopulations ($K = 1–10$), sampling from 10^6 Markov chain steps after a burn-in of 10^5 steps, were performed. The maximum delta-K value was identified to determine the best-supported number of subdivisions in the populations.

Results

Host reaction tests

Thirteen out of 52 symptomatic chilli and bell pepper leaves collected in Okinawa Islands proved to be positive for PVMV infections by RT-PCR. These plant leaves were also checked by RT-PCR using virus-specific primer pairs or the antisera for the infection of *Beet mild yellowing virus*, ChiVMV, *Cucumber mosaic virus, Pepper mild mottle virus* (PMMoV) and *Pepper vein yellows virus*. Some plants were infected with PMMoV (data not shown). Six PVMV isolates (OKP2, OKP3, OKC25, OKP30, OKP39 and OKP41) were chosen considering the collection area and host plants. All the six biologically cloned isolates infected chilli pepper, bell pepper, tomato, petunia, *N. benthamiana, N. clevelandii* and *N. glutinosa* that belong to the family Solanaceae (Table S1). The symptoms on chilli pepper plants were observed approximately 10 days after inoculation, showing mosaic and rugose (Fig. 2). OKP3 isolate showed milder

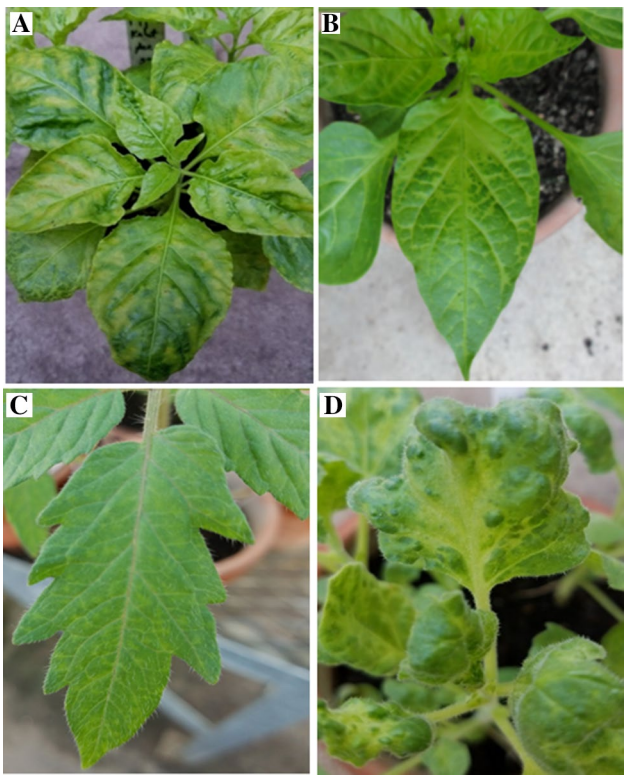

Fig. 2 Symptoms on solanaceous plants infected with *Pepper veinal mottle virus*. **a** Mosaic, rugose and vein banding on uninoculated upper leaves of chilli pepper cv. Kahat-ace, **b** severe mosaic and rugose on uninoculated upper leaves of bell pepper cv. Fruipy yellow, **c** mild mosaic on uninoculated upper leaves of tomato cv. Kyoryoku-beijyu, and **d** severe mosaic and malformation on uninoculated upper leaves of *Nicotiana benthamiana*

symptoms on chilli and bell peppers compared to other five isolates. On the other hand, tomatoes and *N. benthamiana* showed mild and severe mosaic, respectively. Although some PVMV isolates were reported to infect eggplant in some earlier studies, the six Japanese isolates did not infect any of the three cultivars (Chikuyo, Kokuyo and Shoya-onaga). No PVMV infections were observed in the inoculated plants of the families Brassicaceae, Cucurbitaceae and Fabaceae. Details of plant species inoculated in this study are shown in Table S1.

The genomic sequences of Japanese isolates

We determined the complete genomic sequences of six Japanese PVMV isolates. The genomic sequences were 9760 nt in length, excluding the 35 nt primer sequences used to amplify the RT-PCR products at the 5′ ends of the genomes (PVM5T16P, Table S2). A large open reading frame (ORF) consisting of 9222 nts (3074 amino acids), with an AUG start codon and a UGA stop codon, was identified in all the six sequences.

When the deduced amino acid sequences of cylindrical inclusion (positions from nts 3660 to 5591 in the ns1 isolate genome) and nuclear inclusion b (positions nts 7049 to 8606) protein coding regions between Ghanaian P isolate and the other seven isolates were compared, three T residues and one C residue in the Ghanaian P isolate were missing at positions nts 5296, 5311, 5315 and 7266 and one extra G residue was at position nt 7317 (Fig. S1AB). These deletions and an insertion affected the encoded amino acid sequences as they caused frameshifting. Furthermore, when the deduced amino acid sequences of the P1 protein (positions from nts 195 to 1094) coding region between Chinese HN isolate and the other seven isolates were compared, one A residue in Chinese HN isolate was missing at position nt 893. This deletion also caused frameshifting (Fig. S1C).

Recombination analysis and phylogenetic relationships

The nearly complete genomic sequences of six Japanese PVMV isolates with three retrieved from the International public nucleotide sequence databases were assessed for evidence of recombination using RDP4 package. No evidence of clear recombination signals was obtained from the genomes of Ghanaian P, Taiwanese ns1 and Chinese HN isolates nor in six Japanese isolates (data not shown).

Maximum-likelihood (ML) phylogenetic trees of the CP coding regions of 25 isolates and complete genomes of nine isolates are shown in Fig. 3. In the tree of the ORFs encoding the complete polyproteins, all the Japanese PVMV isolates belonged to one tight cluster with the Chinese, Taiwanese and Ghanaian isolates (Fig. 3a). The CP coding region sequences are available from a more diverse set of PVMV isolates, especially many from Africa so the phylogenetic tree constructed from them was more diverse and had two more groups absent from the polyprotein tree (Fig. 3b). Isolates from African countries of Rwanda (MG470801), Yemen (AJ780969) and Ethiopia (AJ780970) belonged to a basal central/east African Group (I) Isolates from Ghana (AJ780968), Cameroon (AJ780967) and Senegal (AJ780966) belonged to west African Group (II) Isolates from Mali (GQ918274-GQ918276), Tunisia (To, Tu1 and Tu2) [14], Ivory Coast (IC), Ghana (NC_011918), Taiwan (EU719646, EU719647, FJ617225 and FJ843065), China (KR002568) and Japan (OKP2, OKP3, OKC25, OKP30, OKP39 and OKP41) belonged to Group (III) The Group III further split into three subgroups; Mali isolates belonged to Subgroup A, Tunisian and Ivory Coast isolate belonged to Subgroup B, and Ghanaian, Taiwanese, Chinese and Japanese isolates belonged to Subgroup C.

Percent nucleotide identity and similarity

The percent identical nucleotides have been calculated using EMBOSS Needle. The percent identical nucleotides in the polyprotein coding regions of the six Japanese isolates were 98.2–99.7% and with those previously reported PVMV isolates were 98.2–98.7% (Table S3A). The percent identical nucleotides in the CP coding regions between six Japanese isolates were 98.6–99.9%, and those between all 25 isolates including previously reported were 80.8–100% (Table S3B). The nucleotide identities in the three major phylogenetic groups of Group I, Group II and Group III were 82.7–86.2%, 90.7–97.1% and 95.8–100%, respectively. The nucleotide similarities calculated by SDT showed the same major groupings as those obtained in the phylogenetic tree (Fig. S2). The nucleotide similarity differences in CP coding regions were mostly seen in the N-terminus as reported earlier for potyviruses (Fig. S3).

Population structure

To identify the genetically homogeneous groups of individuals in PVMV populations, a cluster-based method implemented in STRUCTURE software was used (Fig. 4). After testing 10 successive values of K, our analysis has shown a strong support to three subpopulations of PVMV in CP coding region, at the modal value of $\Delta K = 432.39$, which reveals a clear congruence with the results obtained by CP phylogenetic tree and nucleotide identities. The isolates from Rwanda, Ethiopia and Yemen (green colour) belonged to Group (I). The Cameroon, Senegal and one of two Ghanaian isolates belonged to Group (II). Three Mali isolates, three Tunisian, one Ivory Coast, one Ghanaian, four Taiwanese, one Chinese and six Japanese isolates belonged to Group

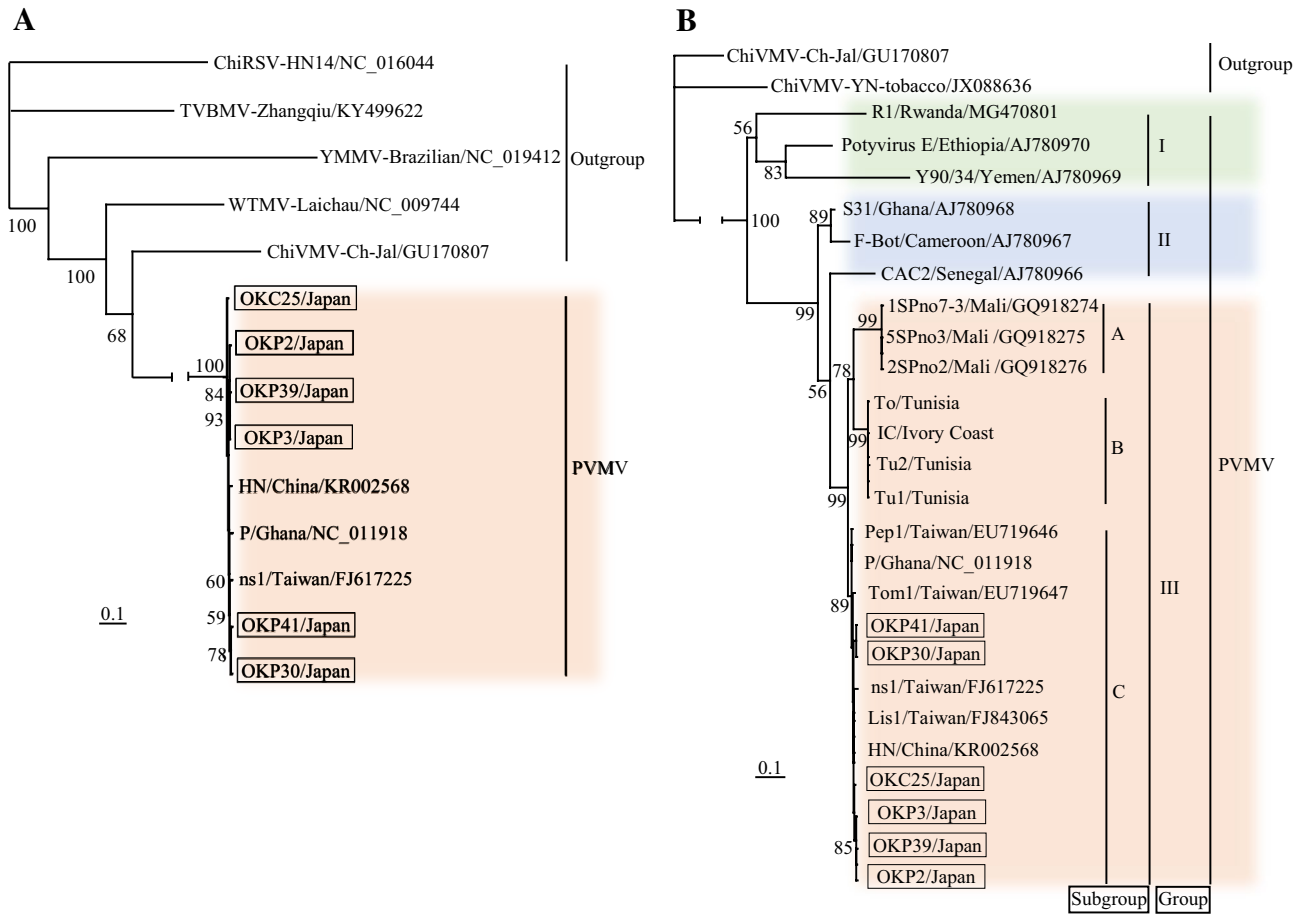

Fig. 3 Maximum-likelihood (ML) phylogenetic tree showing the relationship of *Pepper veinal mottle virus* (PVMV) isolates. Trees were constructed using **a** nine polyprotein coding sequences and **b** 25 coat protein (CP) coding sequences of PVMV genome. Numbers at each node indicate bootstrap values based on 1000 pseudoreplicates (values greater than and equal to 50 are shown). The horizontal branch length is drawn to scale with the bar that indicates 0.1 nucleotide substitutions per site. The nucleotide sequences of polyprotein coding regions of *Wild tomato mosaic virus* (WTMV), *Yam mild mosaic virus* (YMMV), *Chilli ringspot virus* (ChiRSV), *Tobacco vein banding mottle virus* (TVBMV) and *Chilli veinal mottle virus* (ChiVMV), and those of CP coding regions of ChiVMV were used as outgroup. The PVMV isolates in CP phylogenetic tree were divided into three major groups of I, II and III and three subgroups of **a, b** and **c**

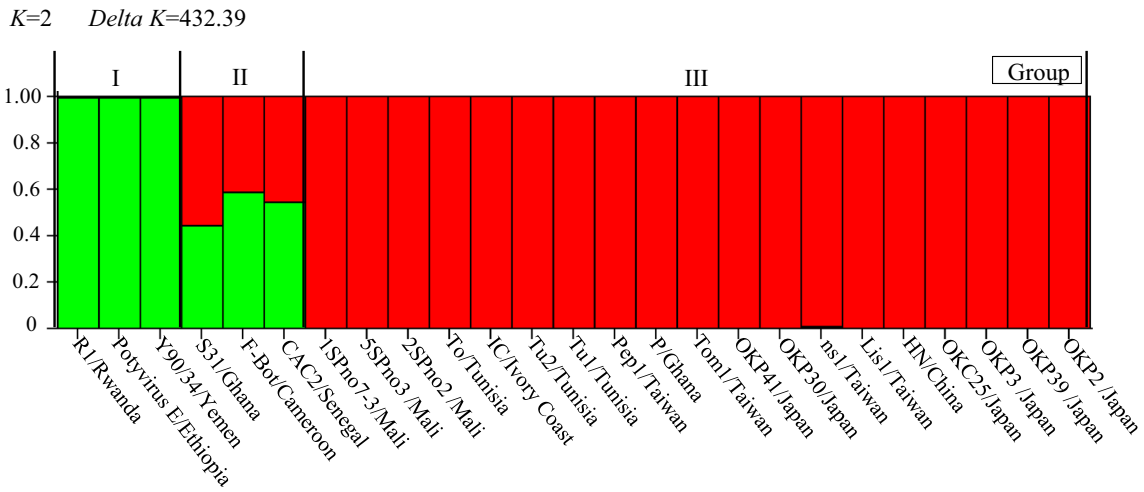

Fig. 4 Population structure of *Pepper veinal mottle virus* isolates inferred by model-based clustering using STRUCTURE [32]. Results of $K=2$ are shown. Each isolate is represented by a single vertical broken into K coloured segments according to each of the K inferred clusters

(III). This may indicate that a part of Asian subpopulation had relationship to African, especially to Ghanaian subpopulation in the past. Furthermore, our results also showed that Africa has three major populations whereas Asia has one.

Discussion

The molecular traits of viruses represent a powerful tool when aiming to investigate the evolutionary relationships between populations. Such studies provide valuable information regarding the design of better strategies for controlling viruses [33, 34]. Over the last 2 decades, many studies described the evolutionary relationships within species of potyviruses, especially *Turnip mosaic virus* [35–38], *Potato virus Y* [39–41], *Soybean mosaic virus* [42] and *Zucchini yellow mosaic virus* [43, 44]. We have collected PVMV isolates recently emerged in Japan and determined the full genomic sequences of Japanese PVMV in this study and compared the evolutionary relationships between Japanese, Asian and African populations. Our analysis shows that all the African, Asian and Japanese isolates of PVMV for which complete genome sequences are available belong to one lineage. However, the analysis of a larger number of the available coat protein sequences of PVMV isolates indicates three different lineages.

The six PVMV Japanese isolates did not show any differences in the host reactions of plants belonging to the families Solanaceae, Brassicaceae, Cucurbitaceae and Fabaceae (Table S1), and thus all Japanese isolates seemed to be biologically similar. The isolates belonged to a group in both polyprotein and CP phylogenetic trees and showed high pairwise nucleotide sequence identities. Furthermore, evidence of recombination was not found in all the genomes of Japanese isolates, indicating all the Japanese isolates were genetically similar. PVMV has been classified into the two major groups of East Asia and Africa based on the CP phylogenetic relationships of the isolates in the earlier studies [5, 17]. The present study added a new group (Fig. 3b) of three subgroups from African and Asian isolates. This grouping was also supported by population structure analysis (Fig. 4). The close relationships of Japanese, Taiwanese and Chinese isolates from the neighbouring countries in East Asia were seen in both polyprotein and CP phylogenetic trees. The distance between Yonaguni Island in Japan and Taiwan is approximately 110 km. This distance may indicate the possibility that PVMV was introduced to Yonaguni Island by winged aphids flying from Taiwan, which is within the range of flying viruliferous aphids. However, we cannot exclude the possibility that the virus may have been introduced by transportation of planting material. Our biological and molecular characterization of PVMV isolates recently emerged in Japan showed that those seemed to be introduced as one population from a foreign country. However, it is not likely that the very close relationship between the Asian isolates and the original Ghanaian isolate is explained by flying aphids. PVMV was first isolated in Ghana [6]. The East Asian isolates and a Ghanaian isolate belonged to one group or one subgroup in the polyprotein and CP phylogenetic trees, indicating that East Asian PVMV probably came from Africa by transportations. We cannot deny the possibility that the seedlings of PVMV-infected solanaceous crops would be a reservoir of this virus. However, seed transmission has not been reported for PVMV yet, but most past attempts to check for seed transmission were done with too few seeds, and would only detect the most readily seed-borne viruses.

PVMV has been found firstly in Ishigaki Island of Japan, which is one of the Okinawa Islands located at the southeast end of Japan [4]. We were interested to determine when PVMV entered Japan, and therefore a timescale analysis was done using BEAST software [45]. Unfortunately, the PVMV CP sequences did not pass date-randomization tests that check for adequate temporal structure, and also even with the most closely related species of ChiVMV CP sequences were added and analysed (data not shown). More isolate sequences, with wide collection dates, are necessary to calculate the PVMV timescales. However, our study confirmed not only the entrance to Japan but also the spreading of PVMV around the neighbouring islands except the main island of Okinawa Islands. Although one cannot ignore the risk of the disease migrating to other parts of Okinawa district and Japan, there is a need to take measures aiming to prevent the virus from spreading to the rest of the country.

The major findings of this study are as follows: (1) all the Japanese isolates of PVMV have similar biological and molecular characteristics, indicating the presence of only one population in Japan; (2) there are at least three major phylogenetic groups of PVMV worldwide; (3) PVMV probably originated in East Africa; and (4) all the Asian isolates are closely related to the Ghanaian isolate.

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Author contributions KO designed the study, JAL, KM, SK and KO conducted the experiments, JAL and KO analysed the data, and KO and JAL wrote the manuscript. All authors reviewed the manuscript.

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Compliance with ethical standards

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

Research involving human or animal participants This article does not contain any research involving human or animal participants.

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