Short Communication

FIRST REPORT AND MOLECULAR IDENTIFICATION OF CHILLI VEINAL MOTTLE VIRUS IN ITALY

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SUMMARY

Chilli veinal mottle virus (ChiVMV), a member of the genus *Potyvirus*, family *Potyviridae*, has recently been reported as a prevailing virus of chilli pepper in eastern Asia causing severe losses. The virus is easily transmitted by several aphid species in a non-persistent manner. In Italy, an increasing interest in this crop has led to an intensified germplasm introduction and exchange of seeds, cultivars and plantlets from all over the world, often overlapping human migration flows, and *de facto* without any phytosanitary control. A two-year survey, aimed at investigating viral diseases spreading in chilli pepper, allowed to identify for the first time in Italy two alien viruses, first pepper vein yellows virus (PeVYV - *Polerovirus*) then ChiVMV, that, on the basis of their epidemiological behaviours, represent a hypothetical risk for cultivations of chilli pepper and other solanaceous plants. In the present work, an Italian ChiVMV isolate has been identified by sequencing the gene coding for the coat protein. Evolutionary and phylogenetic inference revealed that the Italian isolate clustered close to isolates from continental China, showing a lower environmental pressure. Although this first outbreak was in a singular spot in a metropolitan area, the risk that viruliferous aphids could rapidly spread the stylet-borne ChiVMV to neighboring non-commercial gardening and farming is high. This communication also aims to highlight that phytosanitary measures are difficult to apply to prevent the introduction of alien pathogens by vegetable material of minor crops either by trade or amateur exchanges as well as by people communities.

Keywords: chilli pepper, *Capsicum* spp., ChiVMV, molecular characterization.

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Chilli veinal mottle virus (ChiVMV), a member of the genus *Potyvirus*, family *Potyviridae* (King *et al.*, 2012), characterized by a positive-sense single-strand RNA genome of 9.7 kb, has recently been reported as a prevailing virus of chilli pepper (*Capsicum* spp.) (Moury *et al.*, 2005; Gao *et al.*, 2016) all over eastern Asia, causing severe losses in sweet and chilli pepper production. From its first identification in Malaysia by Burnett in 1947 (Brunt and Kenten, 1971), ChiVMV is now widely spread in many countries in Asia and to a lesser extent in Australia and Africa (CABI, 2015). ChiVMV-infected plants show severe symptoms including dark green mottle with vein banding and necrotic rings or spots, leaf distortion and shoestring, reduced leaf size and defoliation (Siriwong *et al.*, 1995; Latifah *et al.*, 2008; Tsai *et al.*, 2008), bearing no fruits or a few malformed and reduced in size (Shah *et al.*, 2001). ChiVMV was reported to infect chilli pepper species such as *C. annuum*, *C. frutescens* and *C. chinense* and, in addition, other plants of the family *Solanaceae*, including *Nicotiana tabacum*, *Solanum lycopersicum* and *S. melongena*, causing similar symptoms, and several weeds (Shah *et al.*, 2008; Arongudade *et al.*, 2012; Yang *et al.*, 2013; Banerjee *et al.*, 2014; Zhao *et al.*, 2014). The virus is transmitted by several species of aphids in a non-persistent manner: *Myzus persicae*, *Aphis gossypii*, *A. craccivora*, *A. spiraecola*, *Rhopalosiphum maidis*, *Toxoptera citricida* and *Hysteroneura setariae*. No seed transmission was reported, so far.

On the basis of the above epidemiological behaviour, and in view of world trade and germplasm exchanges between the main producing countries in South America, Africa and Asia, ChiVMV represents a hypothetical risk for chilli pepper cultivation worldwide. In Italy, despite this crop having experienced a rising interest for its spiciness and nutraceutical properties, there is little information about its phytosanitary status. Over the last decade, uncontrolled exchanges of plant germplasm from both domestic and foreign sources have occurred in order to assemble field collections of different species and varieties. This increases the risk of introduction of exotic

Table 1. Isolates included in the analysis and comparison, retrieved from GenBank. Each isolate was grouped on the basis of their origin.

Isolate	Accession No.	Group
Indonesia	DQ854960	South-East
Taiwan	DQ854947	South-East
Thailand	DQ854956	South-East
South_Korea	AM909717	South-East
Vietnam	DQ925443	South-East
India	EF213677	South-East
India	EF213692	South-East
India	EF213703	South-East
India	EF221615	South-East
India	GU170808	South-East
China	HQ218936	Continental China
China_To	JX088636	Continental China
China	KC693766	Continental China
China	KC711055	Continental China

Table 2. Mean interpopulation diversity (p-distance; Kimura 2-parameters model) computed by MEGA software, where it is possible to observe the lower distance between ChiVMV Italian and Continental China (China Cont) isolates.

Species 1	Species 2	Dist	Std. Err
China Cont	South-East	0.083	0.033
China Cont	Italian	0.050	0.021
South-East	Italian	0.093	0.037

pathogens which once introduced may become established and spread by vector species already present.

In confirmation of what above-mentioned, in a two-year survey, aimed to investigate viral diseases in chilli pepper in Italy (Tiberini *et al*., 2015), an exotic virus, pepper veinal yellows virus (PeVYV), was detected for the first time in Italy, in two distant sites (Tomassoli *et al*., 2016). In addition, in an outdoor germplasm collection field survey in 2015, a severe symptomatology was observed on several plants, consisting of leaf mottling and necrosis, leaf malformation and shoestring and knobs on fruits reduced in size. These symptoms were never found in previous surveys and inspections and our study aimed to identify the causal agent of the disease described above.

Firstly, total RNA extracted from leaf tissue of three symptomatic plants belonging to different *Capsicum* spp. was assayed by specific reverse transcriptase polymerase chain reaction (RT-PCR) against viruses which were identified in a previous study (Tiberini *et al*., 2015): Broad bean wilt virus (BBWV), Cucumber mosaic virus (CMV), Potato virus Y (PVY), Pepper mild mottle virus (PMMoV), Pepper vein yellows virus (PeVYV) and Tomato spotted wilt virus (TSWV).

Since negative results were obtained, the samples were analyzed by one-step RT-PCR assay using a degenerate primer set (CPUP/P9502) targeting the coat protein CP and 3'-terminal untranslated region (3'UTR) sequences of several potyviruses (Vlugt *et al*., 1999). All three samples gave a PCR product of *ca*. 700 bp; the amplicons were purified, cloned (pGEM®-T Easy Vector, Promega, Madison, WI, USA), sequenced and analyzed by BLASTn search. The sequences showed an identity ranging from 92% to 90% versus Chinese isolates of ChiVMV in the amplified region.

More leaf samples were collected in the same site, identifying totally 12 infected plants by the use of specific primers by Tsai *et al.* (2008), covering a genomic region from nuclear inclusion b (NIb) gene to 3'UTR. Molecular assay was performed under the following conditions: two microliters of total RNA were submitted to one step-one tube RT-PCR in a 25 µl volume containing 2.5 µl 5× buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 2.5 mM of each dNTP, 0.5 mM of each primer, 20 U of RNaseOUT (Invitrogen, California, USA) 1.25 U of AMV RT (Promega), 0.75 U of GoTaq® DNA polymerase (Promega). Reverse transcription was at 42°C for 60 min, followed by denaturation at 95°C for 5 min and by amplification consisting of 35 cycles with the following steps: 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension of the amplification products for 10 min at 72°C. The DNA product of the expected size (about 1100 bp) was checked by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide.

The three PCR amplicons from the first sampling and three from the second one were purified, cloned (pGEM®-T Easy Vector, Promega, Madison, WI, USA), sequenced and analyzed by alignment showing a nucleotide (nt) identity of 100% each other. For this reason, only one sequence was deposited in GenBank (accession No. KX889918).

The comparison of the Italian isolate sequence (1035 nt) versus the closest published sequence (BLASTs) showed a nucleotide identity of about 90% with Chinese ChiVMV isolate (KC711055). When only the complete CP sequence (855 bp) was compared with isolates from foreign countries, identities of 90-92%, 90-98% were observed at nucleotide and amino acid levels, respectively.

To better investigate the phylogenetic relationship, the CP nucleotide sequence of the Italian isolate was compared with other CPs sequences of different isolates mainly collected in southeast of Asia and continental area of China (Table 1). Although ChiVMV has been recently reported in Africa and Oceania, no sequences are actually available in GenBank, making it impossible to include them in the present study.

The preliminary analysis was performed by MEGA software, calculating the nucleotide composition, amino acidic substitutions relative to the most closely related Chinese isolate and mean inter-population diversity. The Italian isolate showed a nucleotide composition of U (22.8%) C (19.5%) A (32.5%) G (25.1%) and four differences in the amino acid sequence (21 [2823] E/D; 36 [2840] R/Q; 248 [3015] S/N; 270 [3072] S/T, where the first number represents the position in the ChiVMV Italian isolate partial sequence, and the second represents the position in closest

ChiVMV Chinese complete sequence). Only one resulted in a non-synonymous substitution (position 36 [2840]) where a glutamine (apolar uncharged) had been substituted by an arginine (positively charged), in comparison to the closely related reference sequences (KC693766/KU987835). Regarding the mean inter-population diversity (p-distance; Kimura 2-parameters model) (Table 2), it is possible to observe that the ChiVMV Italian isolate is the closest to the continental China group sharing the lower genetic distance. In support of this view, a phylogenetic tree, generated by the neighbour-joining method (5000 bootstrap replications) and maximum likelihood method (MEGA Software), independently showed that the Italian isolate grouped with Chinese isolates within a distinct clade (data not shown).

The same result was obtained by a Bayesian analysis using BEAST 1.8.2 under the GTR+G_4 substitution model selected by jModelTest according to the suggestions of Gao *et al.* (2016). To establish convergence of all parameters, 5×10^8 generations were run. Convergence and effective sample size of the parameters were checked with Tracer 1.6. The resulting trees were summarized using a maximum clade credibility topology from Tree Annotator 1.8.0 with a burn-in of the first 10% of sampled trees. The topology of the trees (Fig. 1), where node branches were visualized by the use of Figtree, overlaps the previous phylogenetic inference by the use of neighbour-joining analysis. In particular, the node branches have high probability value (PPs value > 0.60). Moreover, the different colours visualized in the branches represent the evolutionary rates, where the higher the rate (brown-darkest colour) the lower is the environmental pressure. Among the clade represented by Chinese continental isolates, it is possible to observe that the Italian isolate has a high rate meaning a lower environmental pressure that cannot moderate an in-site molecular evolution. These data were confirmed by the estimation of selection pressure using Codon-based Test of Neutrality (MEGA 6), by analyses conducted using the Nei-Gojobori method, over 285 positions. The P value obtained for the Italian isolate, less than 0.05, considered significant at the 5% level, represents the probability of rejecting the null hypothesis of strict-neutrality, thus indicating that a positive selection has been establishing [dN = dS; average number of non-synonymous substitution per non-synonymous site (dN); average number of synonymous substitution per synonymous site (dS)].

ChiVMV, although wide-spread in Asia and Africa, has never been reported in Europe. This is the first report of ChiVMV in Italy and Europe in chilli plants belonging to the main cultivated species *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum* but not in plants of the wild species C. *rhomboideum* (syn *C. ciliatum*) that were in the same site. On the other hand, this first outbreak was in a singular spot where all chilli plants, whether infected or not, from collection-field were destroyed. The following year, new inspection was carried out in the same area and a few chilli plants were found infected and destroyed, whereas

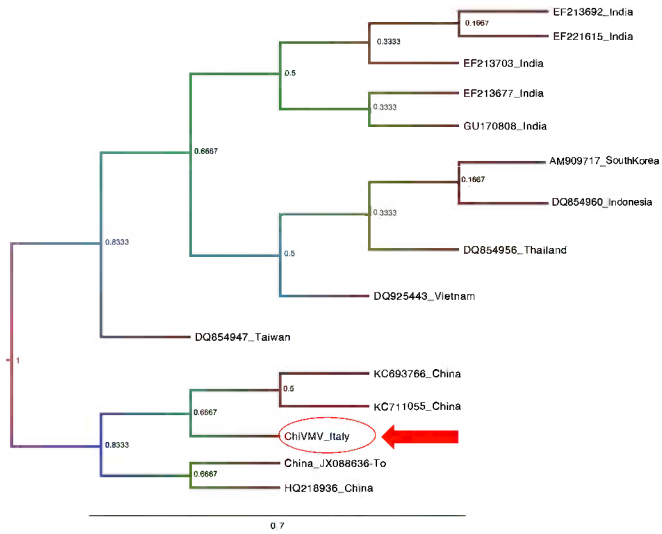

Fig. 1. Bayesian phylogenetic trees of the 14 ChiVMV isolates based on coat protein sequences (identified by their GenBank accession numbers in the figure) and the Italian isolate. For each node, the Bayesian posterior probabilities (PP C 0.70) are indicated. The distance unit is substitutions/site.

no infection of solanaceous weeds (*S. nigrum* and *S. dulcamara*) and ornamental plants (*Physalis alkekengi*) grown next to infected chilli was detected. Nevertheless, there is a risk that viruliferous aphids could spread the stylet-borne ChiVMV to neighbouring gardening and farming plants. Surveys and sampling are underway to monitor the area for elimination and total eradication.

ChiVMV associated with management of a minor crop, here identified in chilli pepper, is an example of how the phytosanitary measures are difficult to apply to prevent the introduction of alien pathogens through vegetable material either by trade or exchanges between individuals, as well as by immigrant communities carrying fresh food and spices from their countries of origin. In particular, the introduction of ChiVMV could have a high economic impact on commercial solanaceous crops as it has been reported affecting tomato and sweet pepper (Zhao *et al.*, 2014). This the first report of ChiVMV in Italy and Europe and more studies are in progress to complete the molecular characterization of this isolate.

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