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Research

e-Xtra*

Biological and Genetic Characterization of Physostegia Chlorotic Mottle Virus in Europe Based on Host Range, Location, and Time

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Abstract

Application of high throughput sequencing (HTS) technologies enabled the first identification of Physostegia chlorotic mottle virus (PhCMoV) in 2018 in Austria. Subsequently, PhCMoV was detected in Germany and Serbia on tomatoes showing severe fruit mottling and ripening anomalies. We report here how prepublication data-sharing resulted in an international collaboration across eight laboratories in five countries, enabling an in-depth characterization of PhCMoV. The independent studies converged toward its recent identification in eight additional European countries and confirmed its presence in samples collected 20 years ago (2002). The natural plant host range was expanded from two to nine species across seven families, and we confirmed the association of PhCMoV presence with severe fruit symptoms on economically important crops such as tomato, eggplant, and cucumber. Mechanical inoculations of selected isolates in the greenhouse established the causality of the symptoms on a new indexing host range. In addition, phylogenetic analysis showed a low genomic variation across the 29 near-complete genome sequences available. Furthermore, a strong selection pressure within a specific ecosystem was suggested by nearly identical sequences recovered from different host plants through time. Overall, this study describes the European distribution of PhCMoV on multiple plant hosts, including economically important crops on which the virus can cause severe fruit symptoms. This work demonstrates how to efficiently improve knowledge on an emergent pathogen by sharing HTS data and provides a solid knowledge foundation for further studies on plant rhabdoviruses.

Keywords: biological characterization, data sharing, emergent viruses, European distribution, high throughput sequencing, mechanical inoculation, PhCMoV

High throughput sequencing (HTS) technologies have drastically increased the pace of new virus discoveries (Adams et al. 2018). Following genome identification, biological characterization is essential to evaluate the scientific, commercial, and regulatory impact of plant pathogens (Massart et al. 2017). Biological characterization of a new virus requires comprehensive knowledge on host range, vector, transmission, symptomatology, and general understanding of the epidemiology (Massart et al. 2017). It requires studying of the virus to be done under controlled conditions, e.g., through mechanical inoculation or grafting (bioassays) (Roenhorst et al. 2013). This is a long and complex process that does not follow the current pace of virus discoveries by HTS (Hou et al. 2020). In this context, HTS data sharing across laboratories before publication can speed up the characterization of emerging viruses in plants, avoid duplication of effort, and accelerate a more accurate pest risk analysis (Hammond et al. 2020). For example, it could describe the natural host range and symptoms associated with a new pathogen more extensively and identify crops that may have been impacted or crops that could serve as a reservoir. Merging HTS data from different sources (regions or countries) and data collected at different times (including historical samples) provides a better view of the spatial and temporal status and distribution of viruses while improving knowledge on epidemiology from phylogenetic analyses. Additionally, historical samples and nucleic acids can be used to obtain valuable information on the viral origin, and gathering data from different sources about the conditions of discovery (host range, symptoms, etc.) can help to identify a possible route of invasion (Jones et al. 2021).

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Table 1. Sample references with collection year, localization (country and town if known), original host, symptoms, detection or confirmation method, sequencing strategy, and bioinformatics pipeline used; NCBI GenBank accession numbers for each sequenced isolate and coinfection with other viruses are also presented[z]

Isolate name	Collection date	Origin: country (region or city)	Site (farm)	Original host (laboratory host if sequenced)	Symptoms on fruits	Symptoms on leaves (laboratory host if sequenced)
Fr_SM1	2002	France (Provence-Alpes-Côte d'Azur)	Site B	*Solanum melongena*	Deformed	Vein clearing, deformation
KY706238	2003	Germany, (State of Hess)	Site N	*S. lycopersicum*	Unknown	Unknown
Fr_SL1	2011	France (Corse)	Site C	*S. lycopersicum*	Deformed, uneven ripening, mottled	Dwarf, mottled
232-12	2012	Serbia (Rasina District)	Site Q	*S. lycopersicum*	Mottled, uneven ripening	Mottled
238-12	2012	Serbia (Rasina District)	Site R	*S. lycopersicum*	Mottled, uneven ripening	Mottled
323-12	2012	Serbia (Jablanica District)	Site S	*S. lycopersicum*	Mottled	N/S
Fr_SM2	2013	France, (Maine et Loire)	Site D	*S. melongena*	Deformed, uneven ripening, mottled	Vein clearing, plant: dwarf
Fr_SM3	2013	France, (Maine et Loire	Site D	*S. melongena*	Deformed, uneven ripening, mottled	Yellowing
Fr_SL2	2014	France (Provence-Alpes-Côte d'Azur)	Site E	*S. lycopersicum*	Deformed, uneven ripening, mottled	Severe necrosis, dotted tasks (apical leaves)
KX636164	2014	Austria	Site O	*Physostegia virginiana*	N/A	Deformed, chlorosis, mottled
KY859866	2015	Germany, (State of Hess)	Site N	*S. lycopersicum* (*N. benthamiana*)	Marbling, discoloration	N/S
Nd_SL1	2017	Netherlands	Site F	*S. lycopersicum* (*N. benthamiana*)	N/A	Deformed, vein clearing
Ru_SL1	2017	Russia	Site G	*S. lycopersicum* (*N. benthamiana*)	Uneven ripening, mottled	Mottled
MK978541	2017	Germany, (State of Hess)	Site N	*S. lycopersicum* (*N. benthamiana*)	Marbling, discoloration	Distortion, mild yellow spots
MW848528	2017	Germany, (State of Hess but different site)	Site P	*S. lycopersicum* (*N. benthamiana*)	Marbling, discoloration	Mild yellow spots
Nd_CS1	2018	Netherlands (Zélande)	Site H	*Cucumis sativus*	Pointed, deformed, vertical chlorotic stripes	Interveinal chlorosis, sunken veins (rugosity)
Nd_H1	2018	Netherlands, (Gelderland)	Site I	*Helleborus*	N/A	Vein clearing, chlorotic patterns, rings
Nd_H2	2018	Netherlands (South Holland)	Site J	*Helleborus*	N/A	Chlorosis next to veins, mosaic
Fr_SM4	2018	France (Nouvelle Aquitaine)	Site K	*S. melongena*	Deformed, uneven ripening, mottled	Vein clearing, deformed
Be_SL1	2018	Belgium, (Gembloux)	Site A	*S. lycopersicum*	Deformed, uneven ripening, mottled	Vein clearing on apical leaves
Be_SM1	2019	Belgium, (Gembloux)	Site A	*S. melongena*	N/A	Vein clearing
Sl_SL1	2019	Slovenia	Site L	*S. lycopersicum*	Deformed, uneven ripening, mottled	Severe leaf curling, mottling, plant: dwarf
Ro_SL1	2019	Romania	Site M	*S. lycopersicum* (*N. benthamiana*)	Uneven ripening, mottled	N/A
Be_IB1	2019	Belgium (Kruisem)	Site U	*Ipomoea batatas*	N/A	Chlorosis, purple pattern
Be_SA1	2019	Belgium (Putte)	Site T	*Stachys affinis*	N/A	N/S
Ge_CS1	2020	Germany, (State of Hess)	Site N	*C. sativus*	N/A	Mosaic, leaf curling, chlorotic spots, yellowing
Be_GP1	2020	Belgium, (Gembloux)	Site A	*Galinsoga parviflora*	N/A	Vein clearing
Be_PM1	2020	Belgium (Putte)	Site T	*Persicaria maculosa*	N/A	N/S
Be_IB2	2020	Belgium, import from Portugal	Site V	*I. batatas*	N/A	Vein clearing, mosaic, stunting

(*Continued on next page*)

[z] a = protocol used by NVWA; b = protocol used by ILVO; c and d = protocol used by Uliege; e = protocol used by JKI; f = protocol used by NIB. All the samples were sequenced on the Illumina platform, except for * = MinION. N/A = nonapplicable (for example, in the case that there is no fruit when the symptoms were recorded), and N/S = no symptoms observed.

Table 1. (*Continued from previous page*)

Detection method (D)/confirmation (C) (protocol used)	Sequencing strategy (protocol used)	Coinfection with other viruses: Bioinformatic (B) or PCR results (PCR)	Bioinformatic pipeline (assemblers/analyses)	Reference	GenBank accession
D: RT-PCR + sequencing (Alfaro-Fernández et al. 2011)	Total RNA, a	B: no	CLC workbench / Geneious	This study	MW934551
C: RT-PCR (Gaafar et al. 2018)	Total RNA + ribodepletion (Gaafar et al. 2018)	B: no	Geneious	Gaafar et al. 2018	KY706238
D: RT-PCR + sequencing (Alfaro-Fernández et al. 2011)	Total RNA, d	B: Potato virus Y	SPAdes / Geneious	This study	MZ574100
RT-PCR (Vučurović et al. 2021)	Small RNA sequencing (Vučurović et al. 2021)	B: no	CLC workbench / Geneious (Vučurović et al. 2021)	Vučurović et al. 2021	MT269810
RT-PCR (Vučurović et al. 2021)	Small RNA sequencing (Vučurović et al. 2021)	B: no	CLC workbench / Geneious (Vučurović et al. 2021)	Vučurović et al. 2021	MT269811
RT-PCR (Vučurović et al. 2021)	Small RNA sequencing (Vučurović et al. 2021)	B: Southern tomato virus	CLC workbench / Geneious (Vučurović et al. 2021)	Vučurović et al. 2021	MT269812
D: RT-PCR + sequencing (Alfaro-Fernández et al. 2011)	Total RNA, d	B: no	SPAdes / Geneious	This study	MZ574102
D: RT-PCR + sequencing (Alfaro-Fernández et al. 2011)	Total RNA, d	B: no	SPAdes / Geneious	This study	MZ574103
D: RT-PCR + sequencing (Alfaro-Fernández et al. 2011)	Total RNA, d	B: Pepino mosaic virus + squash mosaic virus	SPAdes / Geneious	This study	MZ574101
RT-PCR (Gaafar et al. 2018)	Total RNA + ribodepletion (Gaafar et al. 2018)	B: no	Geneious	Menzel et al., 2018	KX636164
C: RT-PCR (Gaafar et al. 2018)	Total RNA (Gaafar et al. 2018)	B: no	Geneious	Gaafar et al. 2018	KY859866
D: same as seq strategy, C: mechanical inoculation	Total RNA, a	B: no	CLC workbench / Geneious	This study	OK646027
D: same as seq strategy, C: mechanical inoculation	Total RNA, a	B: no	CLC workbench / Geneious	This study	OK646028
C: RT-PCR (Gaafar et al. 2018)	dsRNA (Gaafar et al. 2019)	B: no	Geneious	Gaafar et al. 2019	MK978541
D: ELISA using JKI-2051	Total RNA + ribodepletion (Gaafar et al. 2019)	B: no	Geneious	Gaafar et al. 2021	MW848528
D: same as seq strategy, C: mechanical inoculation	Total RNA, a	B: no	CLC workbench / Geneious	This study	OK646030
Same as seq strategy	Total RNA, a	B: no	CLC workbench / Geneious	This study	OK646029
D: same as seq strategy, C: mechanical inoculation	Total RNA, a	B: no	CLC workbench / Geneious	This study	OK646031
D: RT-PCR + sequencing (Alfaro-Fernández et al. 2011)	Total RNA, d	B: no	SPAdes / Geneious	This study	MZ574104
C: RT-PCR (Gaafar et al. 2018)	VANA, c	B: no	SPAdes / Geneious	This study	MZ501244
D: same as sequencing strategy, C: RT-PCR (Gaafar et al. 2018)	VANA, c	B: no	SPAdes / Geneious	This study	MZ501245
C: RT-PCR (Gaafar et al. 2018)	Total RNA, f	B and PCR: Tomato mosaic virus, potato virus Y	CLC Genomics Workbench / SPAdes	This study	MW366749
D: same as seq strategy, C: mechanical inoculation	Total RNA, a	B: no	CLC workbench / Geneious	This study	OK646026
C: RT-PCR (own primers)	Total RNA, b	B: Sweet potato feathery mottle virus	Own pipeline + VirusDetect + BWA/QUASR	This study	MZ389081
C: RT-PCR (Gaafar et al. 2018)	Total RNA, b	B: no	Own pipeline + VirusDetect + BWA/QUASR	This study	MZ322957
C: RT-PCR (Gaafar et al. 2018)	dsRNA, e*	B: no	Minimap2 / Geneious	This study	MW081210
C: RT-PCR (Gaafar et al. 2018)	Total RNA, d	B: no	SPAdes / Geneious	This study	MZ574099
C: RT-PCR (own primers)	Total RNA, b	B: no	Own pipeline + VirusDetect + BWA/QUASR	This study	MZ389082
C: RT-PCR (own primers)	Total RNA, b	B: Sweet potato feathery mottle virus, sweet potato chlorotic stunt virus, potato virus Y	Own pipeline + VirusDetect + BWA/QUASR	This study	MW834321

Proving a causal relationship between a virus and a disease is one of the first steps in evaluating the risks associated with a new disease agent. However, complying with Koch's postulates is a time-consuming process that requires extensive bioassays (Adams et al. 2018; Fraile and García-Arenal 2016). To accelerate this characterization, Fox (2020) proposed a new approach based on epidemiological studies and statistical analysis that provide valuable insights into causal relationships. In that context, bringing together HTS data and bioassay results from various research laboratories offers a possibility to optimize the study of causal associations between a disease and a potential viral or virus-like agent.

Physostegia chlorotic mottle virus (PhCMoV) was first identified on *Physostegia virginiana* collected from Austria by Illumina HTS in 2014 (Menzel et al. 2018). Subsequently, PhCMoV was detected in Germany and Serbia on tomatoes showing severe fruit marbling and ripening anomalies (Gaafar et al. 2018; Vučurović et al. 2021). PhCMoV has a negative-sense, single-stranded RNA (−ssRNA) genome of 13,321 nucleotides and belongs to the genus *Alphanucleorhabdovirus* of the family *Rhabdoviridae* (Kuhn et al. 2020). Plant rhabdoviruses are believed to originate from insect viruses (Dolja et al. 2020; Whitfield et al. 2018); they are insect-vector-transmitted in a persistent and propagative manner (Jackson et al. 2005). Seed or pollen transmission of plant rhabdoviruses has never been described (Jackson et al. 2005).

Phylogenetic analyses of alphanucleorhabdoviruses revealed a close relationship between PhCMoV and eggplant mottled dwarf virus (EMDV), potato yellow dwarf virus (PYDV), constricta yellow dwarf virus (CYDV), and joá yellow blotch-associated virus (JYBaV) (Bejerman et al. 2021; Dietzgen et al. 2021). Those five alphanucleorhabdoviruses share the same genome organization, which contain seven canonical open reading frames (ORFs) encoding (from 3′ to 5′) nucleoprotein (N), unknown function protein (X), phosphoprotein (P), putative movement protein (Y), matrix protein (M), glycoprotein (G), and large polymerase protein (L) (Dietzgen et al. 2021). These viruses infect dicotyledonous plants, and three of them (EMDV, PYDV, and CYDV) are transmitted by leafhoppers. Vectors are still to be identified for the two most recently discovered viruses (JYBaV and PhCMoV). As genetically close plant rhabdoviruses are transmitted by a particular type of vector (Dietzgen et al. 2020), PhCMoV and JYBaV are quite likely transmitted by a leafhopper, similar to their close alphanucleorhabdovirus relatives.

Recent discoveries of PhCMoV in several European countries on various host plants, associated with severe symptoms in some cases, suggest that it is an emerging virus potentially harmful to economically important crops. Therefore, efficient and rapid characterization is required to establish proper risk assessment and to manage the disease. In that context, eight European laboratories worked together to improve knowledge on PhCMoV biology, epidemiology, and genetic diversity.

Materials and Methods

The PhCMoV isolates that are reported here were independently detected and studied in different laboratories. PhCMoV was detected and identified from different plants during virus surveillance programs and plant pathogen diagnostic processes. For the detection, HTS and conventional sequencing (polymerase chain reaction [PCR] and Sanger sequencing) approaches were conducted. To confirm the presence of the virus after HTS detection, RT-PCR or mechanical transmission tests were performed. Ribodepleted total RNA, double-stranded RNA (dsRNA), and virion-associated nucleic acids (VANA) were used as extraction and virus enrichment strategies prior to HTS on Illumina or Oxford Nanopore Technologies MinION platforms.

Host plant species, geographical location, date of collection, symptoms, and the sequencing method for each sample are indicated in Table 1. All the sequences were deposited in the GenBank database, and the corresponding accession numbers are indicated in Table 1. The number of reads generated and horizontal coverage for each sample is indicated in Supplementary Table S1. PhCMoV was detected from samples collected as part of surveys in Germany, Belgium, France, the Netherlands, and Slovenia and from symptomatic plants of different origins (the Netherlands, Russia, and Romania) submitted to the national reference laboratory in the Netherlands for diagnostics. The context of sample discovery is descripted for each sample in the following section, but the different sequencing methods and bioinformatic analyses are detailed in Supplementary Method S1.

Samples Be_SL1, Be_SM1, and Be_GP1. During a survey on Solanaceae in 2019 in Belgium, one plant of *Solanum lycopersicum* (Be_SL1) was collected in a tomato production tunnel where multiple plants were showing deformed, mottled, and discolored fruits (Supplementary Fig. S1). During this survey, the leaves of five plants of *Solanum melongena* (Be_SM1) showing strong vein clearing were collected in another tunnel and pooled together. The virus enrichment method VANA and the library preparation was performed on these two samples prior to HTS (Supplementary Method S1), revealing the presence of PhCMoV.

A year later, multiple eggplant and tomato plants exhibited similar symptoms to those that were observed in 2019 within the same site we collected. Additionally, while inspecting *Capsicum annuum* grown in one of the tunnels, a plant of *Galinsoga parviflora* (Be_GP1) showing vein clearing was collected (Fig. 1H). RNA was extracted following the method described by Oñate-Sánchez and Vicente-Carbajosa (2008), and the detection of PhCMoV in these samples was confirmed by RT-PCR using the primers published by Gaafar et al. (2018). The sample related to a new host (Be_GP1) was sequenced by Illumina after total RNA extraction, DNase treatment, and ribodepletion (Supplementary Method S1).

Samples Be_SA1, Be_IB1, Be_IB2, and Be_PM1. In the framework of a study on the phytosanitary risk of viruses in newly introduced crops in Belgium (PRONC, FPS project), eight samples of *Stachys affinis* (crosne) and 91 samples of *Ipomoea batatas* (sweet potato) from imported vegetatively propagated starting material and seeds were collected in 2019 and 2020 in different production sites, including two community-supported agriculture farms. The samples were taken randomly and not specifically based on the presence of symptoms. In a follow up survey, asymptomatic plants of several common weeds, including *Persicaria maculosa* (lady's thumb), *Chenopodium album* (lamb's quarters), *Solanum nigrum* (black nightshade), grasses (e.g., *Digitaria sanguinalis* [hairy crabgrass] and *Echinochloa crus-galli* [cockspur grass]), and some other crops (*Physalis philadelphica* [tomatillo] and *Sechium edule* [chayote]) growing around the crosne plants were sampled. The samples were sequenced by Illumina after total RNA extraction, DNase treatment, and ribodepletion (Supplementary Method S1).

Sample Ge_CS1. During a survey in July 2020, nine cucumber samples (*Cucumis sativus* L.) showing mosaic leaf curling, chlorotic spots, and yellowing symptoms were collected in an organic farm in Hesse State, Germany, where the previously published PhCMoV isolates KY706238, MK948541, and KY859866 had been discovered (Gaafar et al. 2018). Using immunosorbent electron microscopy, cucumber mosaic virus was identified in five samples, while bacilliform particles were observed in one sample (Ge_CS1), suggesting the presence of a rhabdovirus. To identify the virus, dsRNA extraction followed by MinION sequencing were performed (Supplementary Method S1).

Sample SL_SL1. In Slovenia, a survey of viruses in tomatoes and surrounding weeds was conducted in summer 2019. Thirty-five plant samples were collected within greenhouses at one farming site (10 tomato plants with symptoms resembling viral infection [which include, but are not limited to, leaf curling and mosaic and yellowing leaves], 10 tomato plants without any visible disease symptoms, and 15 samples from 12 wild species growing as weeds). The samples were sequenced by Illumina after total RNA extraction, DNase treatment, and ribodepletion (Supplementary Method S1).

Samples Nd_SL1, Ru_SL1, Nd_H1, Nd_H2, Ro_SL1, and Nd_CS1. From 2017 to 2019, symptomatic plant samples from the Netherlands, Russia, and Romania were submitted to the NPPO of the Netherlands for diagnostic purposes. The samples were sequenced by Illumina after total RNA extraction, DNase treatment, and ribodepletion (Supplementary Method S1).

Samples Fr_SL1, Fr_SL2, Fr_SM2, Fr_SM3, Fr_SM4, and Fr_SM1. A survey conducted on cucurbits viruses in the south of France (Provence-Alpes Côte d'Azur) in summer 2008 revealed one cucumber sample with mosaic and yellowing leaf symptoms (sample: 'C08-119'). An enzyme-linked immunosorbent assay (ELISA) performed with antisera produced for detecting the cucurbit-infecting viruses EMDV, zucchini yellow mosaic virus, watermelon mosaic virus, cucurbit aphid-borne yellows virus, cucumber mosaic virus, melon necrotic spot virus, Moroccan watermelon mosaic virus, papaya ringspot virus, and Algerian watermelon mosaic virus only revealed the presence of EMDV (pers Eric Verdin).

In 2018, eggplant samples collected in Nouvelle-Aquitaine (Lot-et Garonne department) with vein clearing and deformed leaf symptoms were simultaneously analyzed in two French research institutes (ANSES and INRAE) by RT-PCR with primers published by Alfaro-Fernández et al. (2011). Sanger sequencing was performed on amplicons of eggplant samples as well as cucumber samples collected in 2008. A BLASTn homology search revealed the presence of PhCMoV for two samples (Fr_SM4, 'C08-119').

From 2002 to 2018 in Southeastern France, several eggplant and tomato plants showing dwarfing, bumpy, and marbling fruits and leaves, as well as deformations and vein clearing, were collected. Dip preparations that were prepared from young symptomatic tomato or eggplant leaves, negatively stained with 1% phosphotungstic acid, and observed by transmission electron microscopy revealed the presence of characteristic bullet-shaped particles, suggesting the presence of a rhabdovirus. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions and tested by RT-PCR with a set of primers designed for the detection of EMDV (Alfaro-Fernández et al. 2011). The PCR products showed 78 to 81% nucleotide sequence identity with EMDV, but since the PhCMoV sequence was not available at the time of detection (2002, 2011, 2013, and 2014), the virus in the samples was categorized as "unknown nucleorhabdovirus" and set aside. Recently, these sequences were BLAST searched to the updated NCBI database, and the infection with PhCMoV was confirmed (96 to 98% of nucleotide sequence identity). Thereafter, the samples have been sequenced by HTS, Fr_SL1, Fr_SL2, Fr_SM2, Fr_SM3, and Fr_SM4 following the same methods described for Be_GP1 and Fr_SM1 following the same method described for Nd_SL1 (Supplementary Method S1). Since 'C08-119' is the only sample that was not fully sequenced, the sequence of the amplicon generated with the primers of Alfaro-Fernández et al. (2011) and obtained by Sanger sequencing is available in the Supplementary Method S2 and on NCBI under the accession 'RYS_C08-119-A2021'.

Since mechanical transmission assays were performed in two distinct laboratories (JKI and NPPO-NL), the methods differ.

Sample isolate: KY882264 (JKI). PhCMoV-infected *Nicotiana benthamiana* fresh leaves containing the MW848528 isolate were used to inoculate *Chenopodiastrum murale*, *Chenopodium quinoa*, *Datura metel*, *Datura stramonium*, *Hyoscyamus niger*, *Medicago sativa*, *N. benthamiana*, *N. occidentalis* 'hesperis', *N. occidentalis* 'P1', *N. tabacum* 'samsun', *Petroselinum crispum*, *Petunia* sp., *Physalis floridana*, *S. lycopersicum* 'harzfeuer', and *S. lycopersicum* 'linda'. Four plants per species were inoculated. The method used for inoculation was described before by Gaafar et al. (2019). Briefly, symptomatic leaves were homogenized in Norit inoculation buffer (50 mM phosphate buffer, pH 7, containing 1 mM ethylenediaminetetraacetic acid, 20 mM sodium diethyldithiocarbamic acid, 5 mM thioglycolic acid, 0.75% activated charcoal, and 30 mg Celite). Using a glass spatula, the homogenate was gently rubbed onto the leaves which were then rinsed with water. The inoculated plants were kept under greenhouse conditions (at 22°C; photoperiod of 16 h light [natural daylight with additional growth light Phillips IP65, 400 W] and 8 h dark). Symptoms were observed 4 weeks post-inoculation, and the presence of PhCMoV was confirmed by RT-PCR with the primers of Gaafar et al. (2018).

Sample isolates: Ru_SL1, Nd_SL1, Ro_SL1, Nd_CS1, and Nd_H2 (NPPO-NL). In the Netherlands, different PhCMoV isolates were tested on selected herbaceous indicators including *C. quinoa*, *D. stramonium*, *N. benthamiana*, *N. glutinosa*, *N. occidentalis* 'P1', *N. tabacum* 'WB', *P. floridana*, and *S. lycopericum*. Not all the plants were tested for all isolates, but the combinations are presented in Table 2. Three plants per species were inoculated. The method

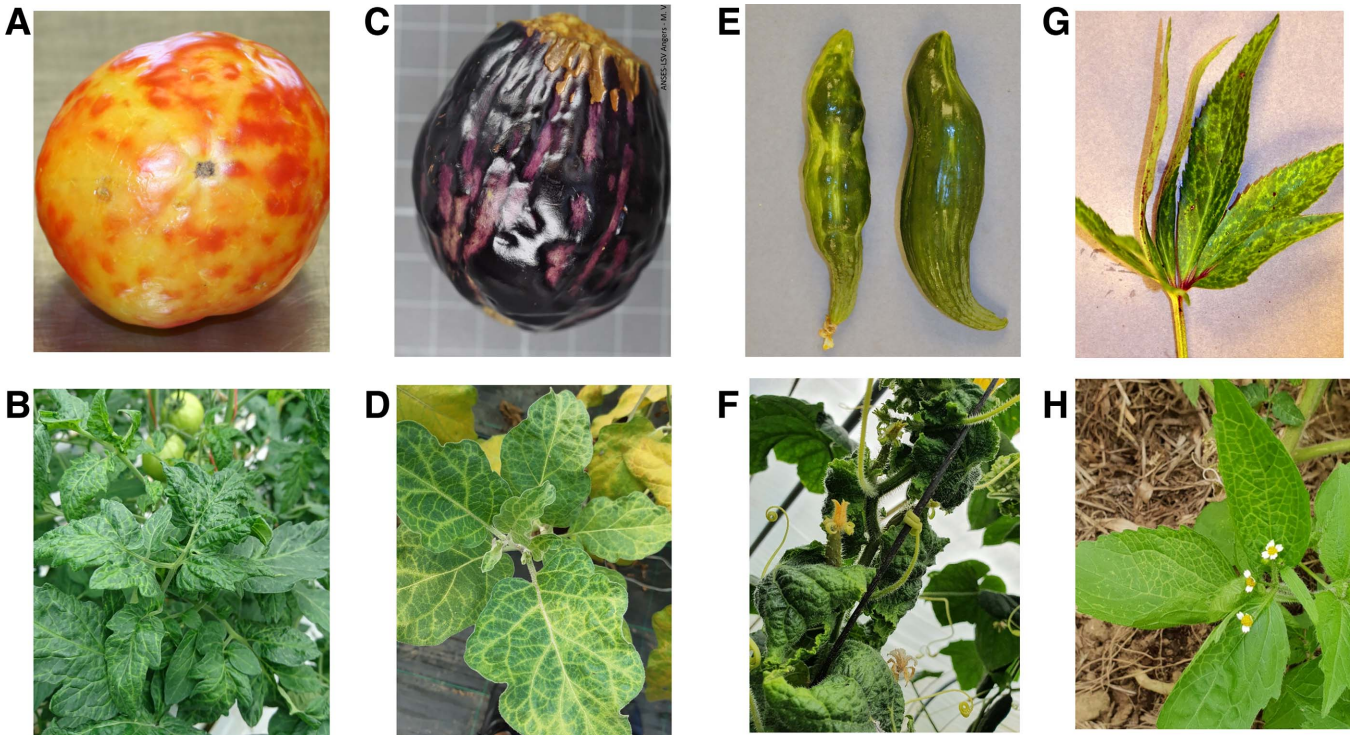


Fig. 1. Pictures of natural Physostegia chlorotic mottle virus (PhCMoV)-infected plants. **A and B,** symptoms of PhCMoV on infected *Solanum lycopersicum* fruits (Ro_SL1) and leaves (Nd_SL1); **C and D,** *Solanum melongena* fruit (Fr_SM4) and leaves (Be_SM1); **E and F,** *Cucumis sativus fruits* (Nd_CS1) and leaves (Ge_CS1); and **G and H,** *Helleborus* leaves (Nd_H1) *Galinsoga parviflora* (Be_GP1). No coinfections with other viruses occurred in these samples.

used for the inoculation protocol is described by Verhoeven and Roenhorst (2000). Briefly, 1 g of infected frozen leaf material (*N. benthamiana* for Ru_SL1 and Nd_SL1 and the original host for Ro_SL1, Nd_CS1, and Nd_H2) was ground in 10 ml of inoculation buffer (0.02 M phosphate buffer pH 7.4, 2% [wlv] polyvinylpyrrolidone [MW 10000]). Plants were inoculated at a young stage (three to six leaves) by gently rubbing the inoculum onto carborundum-dusted leaves. After inoculation, plants were rinsed with water and placed in a glasshouse at 18 to 25°C with supplementary illumination for a day length of at least 14 h. Each isolate was inoculated to at least two plants per plant species and inspected visually for symptoms during the following 7 weeks. The virus infection was confirmed by ELISA in all the inoculated plants (pers Marleen Botermans and Ruben Schoen).

For the phylogenetic analyses, all the known PhCMoV sequences to date were used. This includes PhCMoV sequences published by Gaafar et al. (2018), Gaafar et al. (2021), Menzel et al. (2018), and Vučurović et al. (2021) and the 21 new PhCMoV sequences generated in this study.

Prior to genome analysis, PhCMoV genomes were all trimmed to start at the sequence "CATGAGACT" (position 40 on genome KX636164) and end after "TGCACCTA" (position 13,275 on genome KX636164). Phylogenetic analysis was carried out using the MEGA-X software (v10.1.8) (Kumar et al. 2018). Sequence alignments were performed on near-complete genomes using MUSCLE, and the best DNA model was applied to the maximum-likelihood analysis (GTR + G + I model). Support for the branching patterns in the phylogenetic trees was determined by analyzing 1,000 bootstrap replicates. For graphical representation, SIMPLOT software (version 3.5.1) was used to compare similarity of the genomic sequences of selected PhCMoV isolates to the reference query KX636164 (Window: 200 bp, Step: 20 bp, Gapstrim: On, Hamming). To improve the graphical representation, the analysis was limited to 16 PhCMoV isolates, including the most divergent ones (Nd_SL1 and Nd_H2). The KX636164 genome has been chosen as a reference because it is the first discovered PhCMoV isolate and has the longest genome (Menzel et al. 2018).

Finally, to compare the genetic similarity between the different isolates for different genomic regions, the sequence of the N, X, P, Y, M, G, and L ORFs were extracted using Geneious software for all the isolates indicated in Table 1. Pairwise nucleotide and amino acid sequence identities were calculated for all isolates based on MUSCLE alignment (Muscle 3.8.425 by Robert C. Edgar).

Results

Natural host range and symptoms. In addition to the detection of PhCMoV in new host species belonging to the Lamiaceae (*S. affinis*) and Solanaceae (*S. melongena*) families, this study expands the natural host range of PhCMoV to five new plant families: Cucurbitaceae (*C. sativus*), Ranunculaceae (*Helleborus* sp.), Convolvulaceae (*I. batatas*), Polygonaceae (*P. maculosa*), and Asteraceae (*Galinsoga parviflora*) (Table 1). These detections enabled the description of PhCMoV-related symptoms on several hosts (Fig. 1, Table 1). Only samples with single infection by PhCMoV are shown in Fig. 1 (eggplant: Be_SM1, Fr_SM1, Fr_SM2, Fr_SM3, and Fr_SM4; cucurbits: Nd_CS1 and Ge_CS1; *Helleborus*: Nd_H1 and Nd_H2; *G. parviflora*: Be_GP1; tomato: Nd_SL1, Ru_SL1, Ro_SL1, Be_SL1, and Be_PM1).

As described previously by Gaafar et al. (2018) and Vučurović et al. (2021), infected tomato fruits were unevenly ripened and mottled (Ru_SL1, Be_SL1, and Ro_SL1) (Fig. 1A). In this study, some of the infected tomato fruits were also deformed (Supplementary Fig. S1). All PhCMoV-infected tomato plants that bore mature fruit at the time of collection showed symptomatic fruits regardless of their growing conditions. The symptoms observed on tomato leaves were more variable: no symptoms were observed on the leaves of Be_SL1 and Ro_SL1, mottled leaves were observed on Ru_SL1, and vein clearing and deformed leaves were observed on Nd_SL1 (Fig. 1B).

Like infected tomato, PhCMoV-infected eggplants showed deformed, unevenly ripened, and mottled fruit (Fr_SM2, Fr_SM3, and Fr_SM4) (Fig. 1C). Fr_SM1 showed deformed fruit. On the leaves, Be_SM1 and Fr_SM2 showed vein clearing (Fig. 1D), and

Table 2. Physostegia chlorotic mottle virus indexing host range study across different laboratories (DSMZ, JKI, and NVWA)[z]

	DSMZ - KX636164 (Menzel et al. 2018)		JKI - KY859866 (Gaafar et al. 2018) - HZ15-192		JKI - MW848528 (this study) - HZ16-558		NVWA - Ru_SL1 (this study)	NVWA - Nd_SL1 (this study)	NVWA - Ro_SL1 (this study)	NVWA - Nd_CS1 (this study)	NVWA - Nd_H2 (this study)
Inoculated test plant	Symptoms	ELISA/ RT-PCR	Symptoms	ELISA/ RT-PCR	Symptoms	ELISA/ RT-PCR	Symptoms	Symptoms	Symptoms	Symptoms	Symptoms
Chenopodium quinoa	–	–	y, m	+	–	–		–			
C. sativus	–	–									
Chenopodiastrum murale					–	–					
Datura stramonium	–	–			y	+	–	–			
D. metel	–	–			y	+					
Hyoscyamus niger					–	–					
Medicago sativa					–	–					
Nicotiana benthamiana	m	+	y, m	+	y, vc	+	m, r, g (5 weeks p.i., 3/3)	m, r, g (5 weeks p.i., 3/3)	m, r, g (4 weeks p.i. (1/2))	vc, m, r, g (5 weeks p.i. 2/3)	m, r, g (7 weeks p.i., 3/3)
N. glutinosa	–	–			–	–		–			
N. occidentalis 'P1'	–	–	–	–	–	–	vc (4 weeks p.i., 3/3)	vc, g (cl) (4 weeks p.i., 3/3)	(0/2)	c (7 weeks p.i., 1/3)	vc, g (7 weeks p.i., 1/3)
N. tabacum samsun					–	–					
N. tabacum 'WB'	vc	+					–	–			
N. clevelandii	m	+	y, m	+							
N. glutinosa '24A'	–	–			–	–					
N. hesperis	–	–			–	–					
N. occidentalis '37B'	vc	+	–	–							
Physalis floribunda							–	–			
Petroselinum crispum					–	–					
Petunia					–	–					
Physalis floridana	–	+			–	–					
Solanum lycopersicum					–	–		–			

[z] c = chlorosis; cl = chlorotic lesions; g = growth reduction; ic = interveinal chlorosis; m = mottle; nl = necrotic lesions; r = rugosity; vc = vein clearing; y = yellowing; () = symptoms observed occasionally; – = no symptoms; empty space = not tested; weeks p.i. = number of weeks after inoculation before the observation of the first systemic symptom; x/x = number of plants showing symptoms/number of inoculated plants; + = positive.

Fr_SM3 showed yellowing. Fr_SM4 and Fr_SM1 exhibited vein clearing and deformed leaves. Fr_SM2 showed dwarfism. Sample Be_SM1 grouped five eggplants, all of which showed vein clearing in new leaves. No mixed infection occurred in this bulk sample, which strongly suggests that PhCMoV was the causal agent of the symptoms observed on all the plants. No fruit was present at the time of sampling.

Infected cucumber fruits were pointed, deformed, and showed vertical chlorotic stripes (Nd_CS1) (Fig. 1E). The leaves exhibited interveinal chlorosis and sunken veins (Supplementary Fig. S1), leaf curling, chlorotic spots, and yellowing symptoms (Fig. 1F).

Finally, *G. parviflora* (Be_GP1) and *Helleborus* sp. (Nd_H1 and Nd_H2) leaves showed vein clearing (Fig. 1G and H). No symptoms were observed on *S. affinis* or *P. maculosa* at the time of collection.

Experimental host range and symptoms. We conducted independent experiments to investigate the indexing host range of PhCMoV. The results of Menzel et al. (2018) (isolate KX636164) and Gaafar et al. (2018) (isolate KY859866) were grouped with our own present results to have a more complete overview (Table 2).

At JKI, PhCMoV (MW848528) was mechanically transmitted to *D. stramonium*, *D. metel*, and *N. benthamiana* and induced yellowing and vein clearing 4 weeks after inoculation. Inoculation of the other 13 tested plant species failed (Table 2). This result differs from previous published reports, where *C. quinoa* and *P. floribunda* were successfully inoculated, whereas inoculation of *D. stramonium* and *D. metel* failed.

In the Netherlands, five PhCMoV isolates where single infection occurred (Nd_SL1, Nd_CS1, Nd_H2, Ru_SL1, and Ro_SL1) were mechanically transmitted to different indicator plants (*D. stramonium*, *N. benthamiana*, *N. occidentalis* 'P1', *N. tabacum* 'WB', *P. floribunda*, and *S. lycopersicum*). An overview is presented in Table 2.

In all experiments, *N. benthamiana* displayed systemic symptoms 4 to 7 weeks postinoculation (Table 2), and Nd_SL1, Nd_CS1, Nd_H2, and Ru_SL1 induced systemic symptoms in *N. occidentalis* 'P1' 4 to 7 weeks postinoculation.

Extended distribution across Europe since 2002. This study provides an overview of the wide European geographical distribution of PhCMoV; its presence is confirmed in six additional countries besides Germany, Austria, and Serbia, which is where the virus was previously reported (Gaafar et al. 2018; Menzel et al. 2018; Vučurović et al. 2021): Russia, Romania, Slovenia, the Netherlands, Belgium, and France (Table 1).

Although most of the detections are recent, reanalysis of historic *S. melongena* samples (Fr_SM1) showed that PhCMoV was present in France as early as 2002. A cucumber sample collected in France in 2008 that was originally diagnosed as EMDV by ELISA using in-house antiserum was reanalyzed and diagnosed as PhCMoV by RT-PCR. This shows that some EMDV antiserums used by ELISA can cross-react with PhCMoV and lead to incorrect diagnosis.

Phylogenetic analysis of the genomes. In total, 21 new near-complete PhCMoV sequences were generated during this study, and their evolutionary relationships were investigated alongside all PhCMoV, EMDV, and PYDV complete genomes available from the GenBank database on a maximum-likelihood tree (Supplementary Fig. S2). Supported by bootstrap values of 1,000, the analysis did not show any clustering according to host plant, country of origin, or year of collection (Fig. 2). However, isolates collected from the same site (same farm) A, B, N, or T grouped together regardless of the

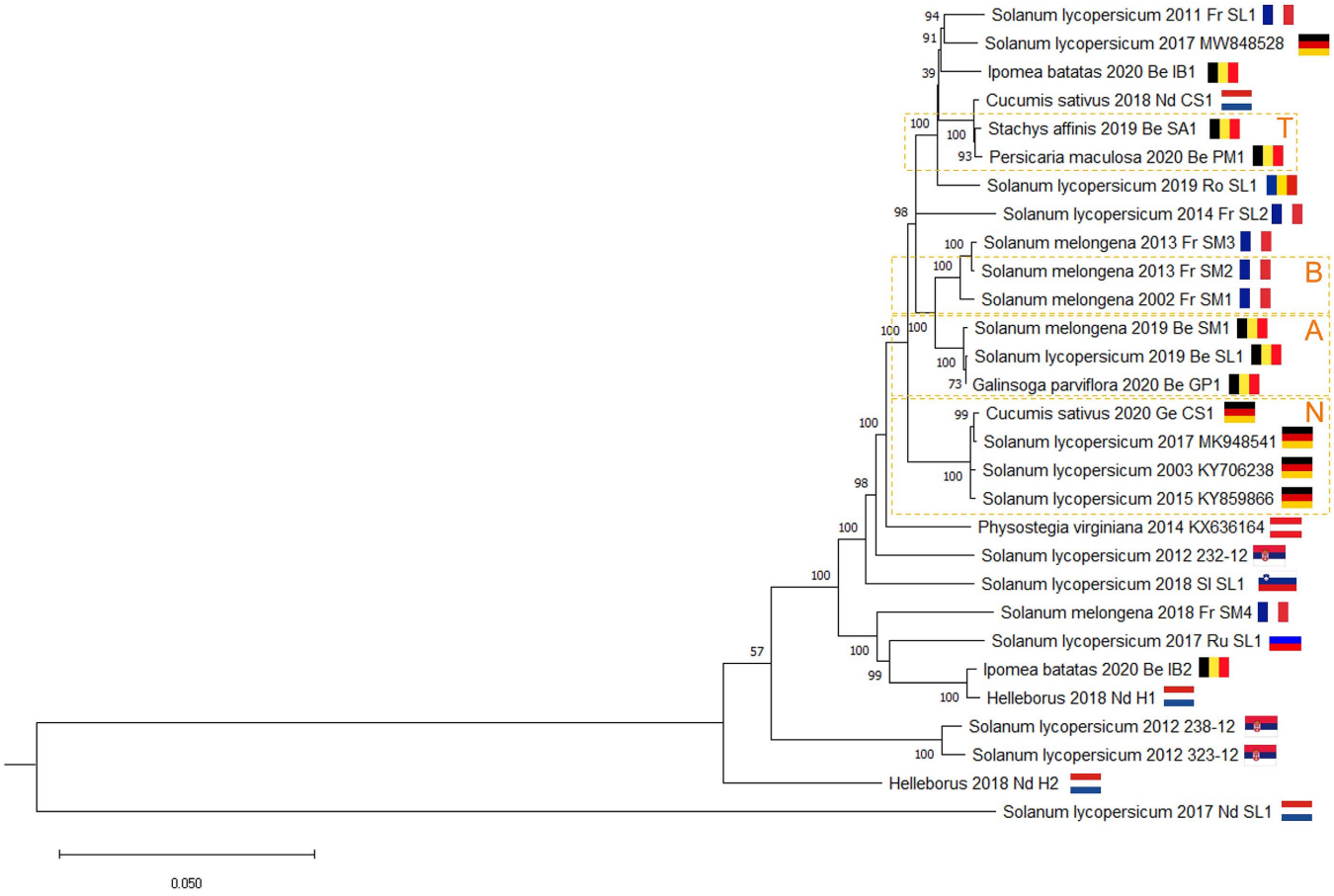


Fig. 2. Phylogenetic tree inferring relationships of 29 Physostegia chlorotic mottle virus (PhCMoV) isolates (among which were 21 new genomes published in this study) based on nucleotide alignment of near-complete genomic sequences. The phylogenetic tree was inferred using the maximum likelihood method (GTR + G + I model) based on the full genome sequence MUSCLE alignment (nucleotides) of all the PhCMoV isolates known at this date. Each isolate is labeled with its name and the information of the collection: country (flag), host, and year. Orange squares and letters highlight identical collection sites (farm). The values on the branches show the percentage of support out of 1,000 bootstrap replications, and the scale bar indicates the number of nucleotide substitutions per site.

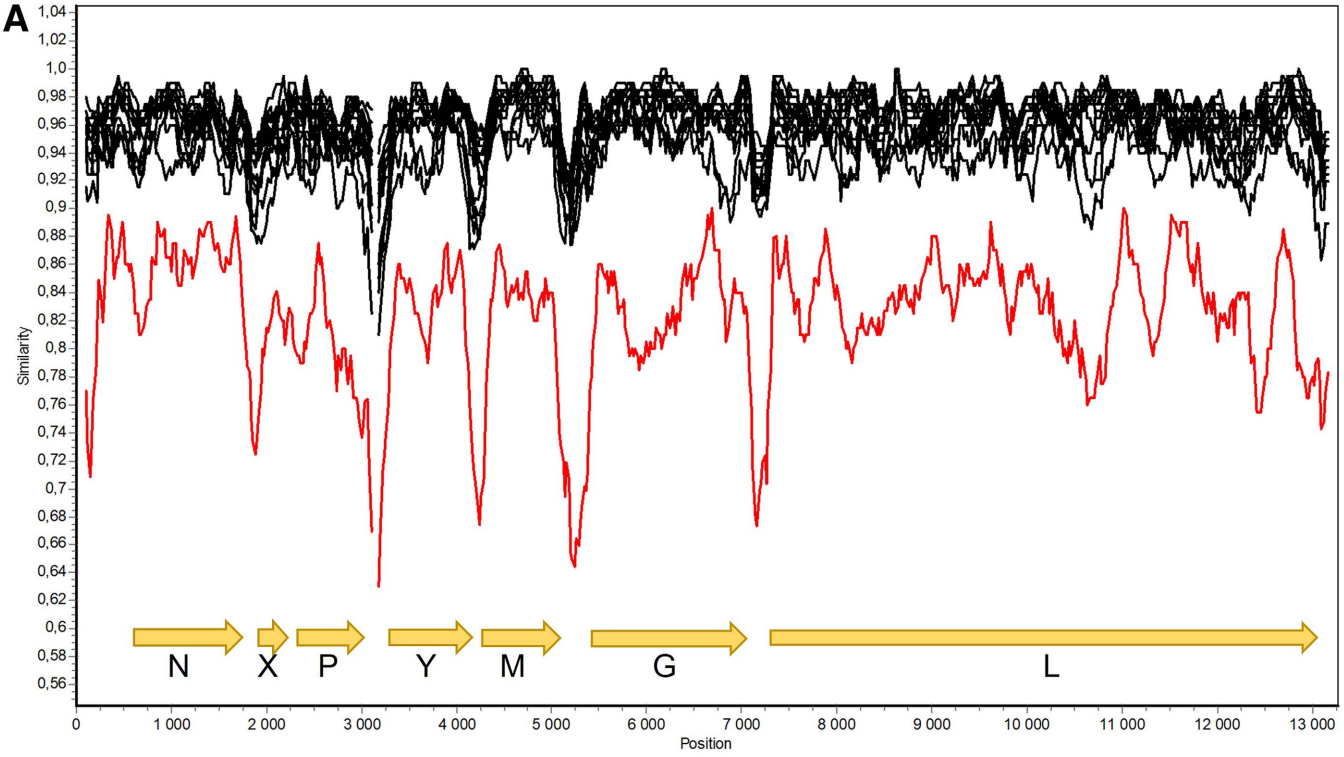
collection date or host plant (Fig. 2). This was particularly obvious for some of the samples from Germany, namely Ge_CS1, KY706238, KY859866, MK978541, and MW848528. They were collected at the same site (Hesse State) and grouped together regardless of their collection date (from 2003 to 2020) and host plants (cucumber, tomato). Be_SL1, Be_GP1, and Be_SM1 were also collected on the same farm (Gembloux, Belgium) 1 year apart on three distinct host plants but have almost identical genome sequences (100% nucleotide identity; Supplementary Fig. S3). Similarly, Fr_SM2 and Fr_SM3 were collected at the same location and clustered together (Fig. 2). Interestingly, Be_SA1 and Be_PM1 sampled from the same farm also clustered together, along with Nd_CS1, which was isolated from a different country and host family (Fig. 2). Overall, all the sequences from samples collected on a same site clustered together, but the clusters did not all represent a geographical point.

To better understand the evolutionary relationships among PhCMoV isolates, nucleotides and amino acid identities were calculated from the alignment of nearly complete genome sequences and for each ORF (Fig. 3B). Relatively low genetic variability was observed for the near-complete genomic sequences (>93% nucleotide identity) in 28 out of 29 isolates (Fig. 3B). The Nd_SL1 isolate was the most divergent isolate with 81 to 82% of nucleotide sequence identity compared with the other 28 genomes (Fig. 3B). However, when the amino acid sequence identities (aa id) of the different isolates were compared, the variability of Nd_SL1 ranged among the average pairwise identities of the other isolates for most ORFs (N, P, Y, M, and G) (Fig. 3B). Using Simplot to observe the sequence similarity along the genome, a clear drop was visible in the intergenic regions located in between the coding regions (Fig. 3A). Overall, for all isolates except Nd_SL1, the ORF encoding protein L was the most conserved gene, with a percentage of aa id > 99%. It was followed by the ORF encoding proteins G (aa id > 97%); M, Y, and P (aa id > 96%); N (aa id > 95%); and X (aa id > 88%).

Discussion

By collaborating and sharing data before submitting the results for publication, eight European research groups investigated PhCMoV in detail and characterized its genome and biology.

This study demonstrates the ability of PhCMoV to naturally infect seven host plants (annual and perennial ones) in addition to the two previously known hosts across seven families, including economically important crops (*S. lycopersicum*, *S. melonga*, and *C. sativus*), newly introduced crops in Europe (*I. batatas* and *S. affinis*), wild plants (*G. parviflora* and *P. maculosa*), and ornamentals (*Helleborus* sp.). Similar observations have been made for other alphanucleorhabdoviruses, e.g., EMDV with more than 25 hosts recorded on CABI (2021) (https://www.cabi.org/), including crops and perennial plants such as *Hibiscus* sp., *Hydrangea macrophylla*, *Agapanthus*, or *Pittosporum* sp. This suggests that the host range of PhCMoV is likely to be wider than described here, and additional perennial hosts might help the virus overwinter.



B

		Full genome	N	X	P	Y	M	G	L
PhCMoV isolates genome and ORF size range	nts	13320	1269-1482	306-366	885	864-1104	756-798	1857-1935	5841-5874
	aa	na	422-494	101-121	294-295	287-368	251-265	618-656	1938-1958
Range of sequence identity of Nd_SL1 to all known PhCMoV	nts ID (%)	82	85-87	76-81	79-81	83-85	84-85	82	83-84
	aa ID (%)	na	96-100	73-76	83-87	92-99	93-97	91-94	95-96
Range of sequence identities amongst all known PhCMoV isolates excluding Nd_SL1	nts ID (%)	>93	>94	>89	>93	>93	>95	>94	>94
	aa ID (%)	na	>95	>88	>95	>95	>96	>97	>98

Fig. 3. Differences and similarities between selected Physostegia chlorotic mottle virus (PhCMoV) isolates in different open reading frames (ORFs). **A,** graphic representation of nucleotide identities (%) using SIMPLOT of 16 full genome sequences of PhCMoV (ref query = KX636164; Window: 200 bp, Step: 20 bp; Gapstrim: On; Hamming across the complete genome sequence and its genome organization). In red is the representation of the most divergent isolate Nd_SL1. **B,** nucleotide and amino acid sequence identities calculated for N, X, P, Y, M, G, and L ORFs for all isolates studied. The identities (%) were calculated based on MUSCLE alignment (Muscle 3.8.425 by Robert C. Edgar). The number of base pairs for the full genome sequence is indicated for KX636164.

Our results outline PhCMoV symptomatology on a large range of plants collected in fields, gardens, and greenhouses. Overall, the presence of the virus was associated with virus-like symptoms on leaves (vein clearing, chlorosis, mottling, etc.) and severe symptoms on fruit (deformation, marbling, and uneven ripening). Only two samples (*S. affinis* and *P. maculosa*) did not exhibit any symptoms, suggesting that asymptomatic plants might host the virus. We did not describe the symptomatology of PhCMoV on sweet potato because of coinfection. Considering only the samples single infected with PhCMoV, the symptoms were often variable across plants from the same species. These variations may be due to several biases. First, they could be due to human perception since different people recorded the symptoms. Second, the plants corresponded to different cultivars and were grown under heterogeneous conditions. In addition, symptom expression may be different depending on the plant growth stage at the time of infection. Nevertheless, the presence of the virus was always associated with obvious vein clearing on the leaves of *G. parviflora*, eggplant, and *Helleborus*. This symptom was also described for EMDV on honeysuckle and eggplant (Martelli and Cherif 1987).

The severe symptoms observed on tomato fruit (marbling, mottling, and uneven ripening) confirmed previous reports (Table 1, Gaafar et al. 2018; Vučurović et al. 2021). Even though remarkable, these symptoms were not specific to PhCMoV; similar observations were made in the case of other viral infections (EMDV [Blancard 2009], pepino mosaic virus [Hanssen et al. 2009], tomato brown rugose fruit virus [EPPO Bulletin 2020], and in the case of nutrient disorder mostly referred to as "blotchy ripening" [Adams and Ho 1995]). The symptoms observed on tomato leaves were highly variable (mottling and vein clearing) and sometimes absent. Therefore, tomato leaves do not represent a good indicator of PhCMoV presence.

Vein clearing was observed on the leaves of four out of five eggplant samples. Vein clearing is not specific for PhCMoV, as it is also representative of the presence of EMDV and alfalfa mosaic virus (Martelli and Hamadi 1986; Sofy et al. 2021), but it is generally associated with viral presence on eggplant and can differentiate viral presence from that of other pathogens, abiotic stresses, or nutritional disorders. Interestingly, this symptom can be used to monitor the spread of the virus in a parcel infected by PhCMoV. Finally, the number of samples per species sampled on the other host plants was too low to be associated with a specific symptom.

To confirm the presence of PhCMoV and to study its mechanical transmission, infected leaves collected in various sites were mechanically inoculated on different indicator hosts. In total, 4 out of 18 indicator plant species were successfully inoculated and showed systemic symptoms (Table 2; *D. metel*, *D. stramonium*, *N. benthamiana*, and *N. occidentalis* 'P1'). In the previous studies, *C. quinoa*, *N. occidentalis* '37B', *N. clevelandii*, *N. tabacum* 'WB', and *P. floridana* were also mechanically inoculated (Table 2, Gaafar et al. 2018; Menzel et al. 2018). This host range is similar to that of EMDV which includes: *N. clevelandii*, *N. glutinosa*, *N. rustica*, *N. tabacum*, *P. hybrida*, and *P. floridana* (Katis et al. 2011; Mavrič et al. 2006). No systemic symptom of EMDV infection has ever been reported on *C. quinoa* or *D. stramonium*. Despite the overall high sequence identity of the PhCMoV isolates analyzed in this study, the results were variable across laboratories. Some plants were successfully inoculated in some laboratories but not in others (for example: *N. occidentalis* 'P1' and *D. stramonium*), and the range of observed symptoms on a same host plant species was variable. Inoculation success and symptom expression depend on environmental conditions (Hull 2014) and inoculum sources. In addition, at NPPO-NL, some symptoms were recorded 4 to 7 weeks postinoculation on *N. occidentalis* 'P1' and *N. benthamiana*, which is longer than the recommended period of 3 weeks (Roenhorst et al. 2013). Indexing is very important to maintain and study viruses in controlled conditions, to separate them in case of multiple infection, and to find the best host for virus purification. It would also be interesting to inoculate several plant species in the same experimental conditions to compare the impact of divergent isolates on symptom expression. Overall, all the studies converged toward *N. benthamiana* being the best experimental PhCMoV host. Our study also showed that inoculated plants suspected to host PhCMoV should be kept in a greenhouse for symptom observations for at least 7 weeks.

With the generation of 29 sequences of near-complete genomes, PhCMoV is now the plant rhabdovirus with the highest number of near-complete genomes available. These genomes provided data for studying the virus genetics in relation to host range, geographical location, and time. Despite genetic variability ranging between 82 and 100% of nucleotide sequence identity (for the near-complete genome), the 29 samples did not cluster according to country or host plant.

In addition, there was 100% identity between isolate KY706238 collected on tomato in 2003 and isolate Ge_CS1 collected on cucumber from the same site in 2020. This genome conservation over time was observed in four distinct sites across Europe (yellow boxes in Fig. 2). It suggests that the genome of PhCMoV does not evolve rapidly once established in a suitable ecosystem. This highlights the impact of the geographical dimension on the genetic evolution of PhCMoV and is in line with observations on other plant rhabdoviruses (EMDV and rice stripe mosaic virus [RSMV]) whose phylogenetic clusters correlate with geographical localization but not necessarily with the host plant or the sampling date (Pappi et al. 2016; Tang et al. 2014; Yang et al. 2018). Since plant rhabdoviruses are transmitted from plant to plant by insects in a persistent and propagative manner and no other way of natural transmission is known, insect vectors are likely to be the cause of the strong selective pressure on the genetic diversity of plant rhabdoviruses (Power 2000).

For the 29 isolates analyzed in this study and collected from eight countries and eight host plant species, the genetic diversity was very low (less than 3% at the nucleotide level for the near-complete genome). This low genetic diversity has been observed in other plant rhabdoviruses. For example, Yang et al. (2018) showed that the genome of 13 isolates of RSMV collected in various geographical regions in China showed 99.4% of nucleotide sequence identity. In another study, Samarfard et al. (2018) showed a 99% aa sequence identity of protein N across 13 alfalfa dwarf virus isolates from different regions in Argentina. In our study, between 92 and 99% of nucleotide sequence identity was observed among the 29 available PhCMoV genomes with only one outlier, Nd_SL1, with 81 to 82% of nucleotide sequence identity with the other 28 isolates (Fig. 3). However, this isolate was not an outlier at the protein level; for instance, it had more than 96% aa identity with all the PhCMoV isolates for protein N, while the nucleotide sequence identity ranged between 85 to 87% for the corresponding gene. Similar observations have been reported for the cytorhabdovirus lettuce necrotic yellows virus (LNYV); the ORF encoding protein N of two subgroups were approximatively 80% identical at the nucleotide level and 96% identical at the aa level (Higgins et al. 2016).

Overall, this study brings together some key elements on the genetic diversity of PhCMoV and its potential drivers. It shows the importance of accumulating genomic sequences from diverse isolates to draw robust conclusions. Viral genomes from samples of different origins (new location, new host, or different collection date) support a better understanding of the genetic diversity and evolution of this virus, but the presence of an exception (i.e., isolate Nd_SL1) suggests that the genetic diversity of PhCMoV remains partly uncovered and that the results need to be interpreted carefully. Considering the severity of the symptoms observed on economically important crops, it is unclear why the virus remained unnoticed for at least the past two decades. The lack of appropriate diagnostic tests might be one of the reasons for this delay since cross-reactions occurred with one of the EMDV antibodies in 2008. This suggests that additional infections may have been misdiagnosed. In addition, samples collected in 2002 (Fr_SM1), 2008, 2011 (Fr_SL1), 2013 (Fr_SM2/3), and 2014 (Fr_SL2) were set aside for identification because the PCR products showed 78% nucleotide identity with EMDV, and the PhCMoV sequence was not available at the time. Our research highlights the strength of HTS in plant virus detection, and the wider application of

these technologies for virus detection might explain the sudden simultaneous identifications throughout Europe. Another complementary hypothesis of the recent detections might be that the virus was present in the environment but went unnoticed because it did not cause a problem (low incidence), and a recent change in the environment led to its emergence. Whether the virus is more prevalent nowadays or whether it was overlooked in the past remains unknown. However, the current situation requires rapid characterization and a common response from European countries because simultaneous PhCMoV detections in several European countries over a wide host range including economically important foodstuffs suggests that the virus could be an emerging pathogen. In that context, prepublication data sharing and collaboration have been valuable to improve knowledge about this virus and would be beneficial in the future to efficiently evaluate the risk associated with any emerging disease and to implement management strategies.

One of the next priorities will be to identify the insect vector and its life cycle. EMDV, PYDV, and CYDV are the closest relatives of PhCMoV with a known vector, and those vectors all belong to *Cicadellidae*, which makes leafhoppers prime candidates for transmitting PhCMoV (Dietzgen et al. 2020). Furthermore, according to the transmission tests carried out by Babaie and Izadpanah (2003), EMDV was transmitted by one specific leafhopper (*Agallia vorobjevi*) and not by the other 13 leafhopper species present in and around EMDV-infected fields. This suggests specific virus-insect transmission. A second priority line of research will be to determine in which hosts the virus is present in winter. This ability of plant rhabdoviruses to infect different host plants across families is an important factor to be considered for controlling the disease because a large diversity of plants can serve as a reservoir during the no-crop season. A third axis will be to assess the impact of the virus in terms of yield and economical loss on different cultivars and when the plants are inoculated at different developmental stages.

Finally, understanding the epidemiology of the virus and the reasons for its multiple recent detections in Europe are key elements to be investigated to evaluate if it can present a threat for vegetable production and how to prevent potential outbreaks.

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