Characterisation of a novel virus infecting orchids of the genus Pleione

Petr Komínek[a],[*], Sébastien Massart[b], Khanh Pham[c], Paul van Leeuwen[c], Marcela Komínková[a]

[a] *Division of Crop Protection and Plant Health, Crop Research Institute, Drnovská 507, 16106 Prague 6, Ruzyně, Czech Republic*
[b] *Plant Pathology Laboratory, TERRA-Gembloux Agro-Bio Tech, University of Liège, Passage des Déportés 2, 5030, Gembloux, Belgium*
[c] *Wageningen Plant Research, Wageningen UR, GTB Gewasgezondheid Bodem en Water, PO Box 20, 2665 ZG, Bleiswijk, the Netherlands*

ARTICLE INFO

ABSTRACT

Plants of the genus *Pleione*, originating from hobby growers in the Netherlands and in the Czech Republic, were conspicuous for viral infection, showing symptoms of leaf mosaic or flower breaking. Using Sanger and high throughput sequencing, the full genome sequence of a novel potyvirus was obtained from sequencing data. The genome sequence was annotated and compared to the genome of other potyviruses. The virus was experimentally transmitted by aphids into Pleione and *Chenopodium quinoa* plants. The name Pleione flower breaking virus (PlFBV) was suggested for the new virus. The presence of the virus was confirmed using RT-PCR, with newly designed primers targeting this new species. The incidence of the virus was contrasted between both countries and might have been influenced by the growth conditions and the exposure of the plants to aphids.

Orchids of the genus *Pleione* D. Don (family *Orchidaceae*) are small epiphytic, lithophytic, or terrestrial orchids (Pridgeon et al., 2006). They create annual pseudobulbs with one or two leaves, usually deciduous in winter. Flowers appear either before or after the leaves, and they generally number 1-2 per plant. According to Chen et al. (2009), the genus *Pleione* contains about 26 species and is native to Southeast Asia. The plants have great horticultural importance because of their large flowers, ease of cultivation, and vegetative propagation (Zhu and Chen, 1998).

In 2012, a hobby orchid grower asked virology specialists at the Crop Research Institute (CRI), Prague, Czech Republic to analyse the health status of his large orchid collection of the genus *Pleione*. These *Pleione* plants showed mosaic (100% of plants, see Fig. 2B) or flower breaking (5%, see Supplementary Fig. 1) symptoms and were suspected to be infected by a virus. Similarly, in 2013, a hobby grower from the western Netherlands asked specialists from Wageningen University & Research (WUR) to analyse *Pleione formosana* Hayata (Gibbs et al., 2000) plants cultivated in greenhouse, showing symptoms of flower breaking or streaking of the colour of the flowers (see Supplementary Fig. 2).

In such cases, identification of the viral pathogen present in diseased plants holds both scientific and agronomic interest. There are only a limited number of scientific reports on the pathogens of orchids from the genus *Pleione*. Only one virus species, namely *Pleione virus Y*, has been reported to infect *Pleione formosana* Hayata. Its genome is poorly

characterized, with only one partial sequence of the coat protein (1,645 nt) available in GenBank (acc. no. AF185958). The virome of Pleione orchids is therefore poorly characterized and the presence of a new or an unexpected virus was suspected. Until recently, discovery of a novel plant virus was a long and meticulous task. The recent development of high throughput sequencing (HTS) technologies, also called Next Generation Sequencing (NGS), has provided a powerful alternative for their detection and identification without any *a priori* knowledge. The nucleic acids extracted from the plants can be sequenced at very high throughput, allowing potentially the identification of any virus present in the plant and the sequencing of its full or nearly full genome (Massart et al., 2014).

Therefore, the whole genome of an unknown virus can be sequenced by HTS technologies, but, at this stage, there is no information on the biological significance of this new species - with nothing being known about its particle structure, pathogenicity, host range, transmission, prevalence, epidemiology, *etc*. Therefore, the application of HTS technologies should be completed by the genome annotation followed by a biological characterisation of the newly identified virus using classical methodologies. As it can be a long and complex process, a framework for progressive characterisation has recently been proposed (Massart et al., 2017) in order to prioritize the efforts and the focus.

This paper describes the characterization of a novel potyvirus identified in diseased Pleiones from two countries through observation of viral particles, sequencing of its full genome by HTS technologies and

---

Table 1
High throughput sequencing results from *Pleione* plants. Genome coverage was calculated by Geneious.

Sample (cultivar)	Number of unique reads	Virus	Number of mapped virus reads	Genome coverage in %	Genome sequencing depth
Wharfedale	4,415,108	PlFBV	124,054	99.99	1871 X
Shasta	7,640,276	PlFBV	26,819	99.8	403 X
Slender Loris	1,056,330	PlFBV	33,914	99.6	509 X

Sanger and its preliminary biological characterisation.

For the identification of the agent present in symptomatic Pleiones, electron microscopy was used to reveal any viral particles present in the plant's sap. Plant leaves with symptoms were homogenized with 4% phosphotungstic acid (pH 6.9) at a ratio of approximately 1:5. A small droplet was taken from the homogenate, and formvar coated grids were placed into the droplet for 20 s. The grids were then removed, and any excess of the homogenate was carefully removed by a piece of filtration paper. After air drying, the grids were then examined by a Philips EM 208 S transmission electron microscope, operating at 70 kV. Images were taken with a Veleta digital camera (Olympus, Hamburg, Germany) and processed using iTEM software (EMSIS, Muenster, Germany).

As a result, filamentous particles were observed (see Supplementary Fig. 3) in the sap of symptomatic Pleiones. The modal length of these particles was 720 nm.

As the observed particles were resembling those of potyviruses, RT-PCR approach was used in the virology laboratory of WUR Wageningen to amplify the 3′ terminal region with a potyvirus-specific degenerate primers in combination with an oligo-dT reverse primer (Langeveld et al., 1991). The primers were used according to the mentioned work, PCR product was directly sequenced. Obtained sequence was analysed using web-based BLAST, showing it belongs to a novel virus. It was tentatively named Pleione flower breaking virus, acronym PlFBV, according its host and major symptoms. The sequence of 1,367 bp length was submitted into GenBank under acc. no. KF360240.1. To obtain full-length sequence of the novel virus, high throughput sequencing was applied.

RNA was isolated from symptomatic Pleiones, using a mirVana miRNA isolation kit (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions, starting with 1 g of plant material. RNA quality was assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). On the basis of quality measuring, three RNA samples with highest quality were selected for HTS. They originated from Czech Republic and corresponded to symptomatic *Pleione* hybrids named Wharfedale, Shasta, and Slender Loris. Ribosomal RNA was removed using a RiboMinus Plant Kit for RNA-Seq (ThermoFisher Scientific, Waltham, MA, USA). Total RNA libraries were then prepared using a simplified protocol (no enrichment in poly-A RNA) with a TrueSeq Stranded mRNA kit (Illumina, San Diego, CA, USA). More precisely, ribodepleted RNA entered the process at point 12 of the manufacturer's instructions; a "Make RFP" chapter, and only Fragment, Prime, Finish mix was added. All further steps were performed according to the instructions.

Libraries were prepared at both CRI Prague (*Pleione* cultivar Slender Loris) and the GIGA sequencing centre of Liège University in Belgium (cultivars Wharfedale and Shasta). The libraries were sequenced using a MiSeq machine in the BIOCEV centre in Vestec, Czech Republic (Slender Loris), and on a Nextseq 500 sequencing machine at Liège University in Belgium (Wharfedale and Shasta); all three for 2 × 150 nt.

Data obtained from the 3 samples were analysed using Geneious software, version 11.0.4. (Biomatters, Auckland, New Zealand). Duplicated reads were first eliminated. *De novo* assembling was done using a Geneious assembler with the following parameters: word length 21, maximum mismatches per read 10%, minimum overlap identity 92%, index word length 14, re-analyse threshold 16, maximum ambiguity 16. *De novo* contigs were annotated using the TBLASTX module of Geneious for homology with a local database of viruses and viroids

downloaded from the RefSeq database of NCBI. The contigs with a viral hit were further analysed. Mapping unique reads into reference sequences were done using a Geneious mapper with the following parameters: fine tuning none (fast read mapping), minimum mapping quality 30, word length 17, maximum mismatches per read 2%, do not allow gaps, minimum overlap identity 98%, index word length 9, maximum ambiguity 16, search more thoroughly for poor matching reads set to yes.

Different numbers of unique reads were obtained from individual samples (see Table 1). After *de novo* assembly, the contigs were annotated as described. In all three samples, one of the largest obtained contigs (length of *ca.* 9,900 bp) appeared to be an almost full-length genome of PlFBV. There was no other plant virus present in the analysed data, either known or unknown. The genome of the PlFBV was assembled after mapping reads back to the longest contig. The 5′ and 3′ ends of the PlFBV genome were sequenced by RACE, using a 5′/3′ RACE Kit, 2nd Generation (Roche, Basel, Switzerland).

Table 1 shows the results of mapping reads to the full length of PlFBV, as obtained after RACE. The genome sequencing depth was high: 403X, 509X and 1,871 X. In the three samples the genome coverage reached almost 100% of the complete genome sequence.

Sequencing depth was calculated as number of unique mapped reads * length of reads / length of virus genome, this represent the average coverage of each base by sequencing reads.

The three full-length genome sequences of PlFBV and the partial PlFBV genome sequence from Netherlands were very similar to each others with a total of 20 divergent positions between isolates (see Supplementary Table 1). The full-length sequence of PlFBV from the *Pleione* hybrid Wharfedale was submitted into GenBank under acc. no. MH376747. The PlFBV genome is 9,940 nt long, excluding the poly-adenylated 3′ end.

A large ORF, typical in potyviruses, was identified using the Geneious ORF finder. It is located between nucleotide positions 94 and 9,774. The genomic 5′ non-coding region is 93 nt long; and the 3′ non-coding region is 166 nt long, excluding the polyadenylated end. A further short ORF placed inside the P3 gene, called PIPO (pretty interesting Potyviridae ORF) and described by Chung et al. (2008), was found starting in position 3,321, just after conserved motif GGAAAAAA, which serves as a "slippery site" for -1 frameshift. The PIPO is 231 nucleotides long (= 77 amino acids). The stop codon TAA for the PIPO ORF occurs in position 3,552. The large polyprotein is probably subsequently cleaved, as in the case of other potyviruses. We performed an

Table 2
PlFBV proteins - size, cleavage sites, and comparison to KjMV.

Protein	Length (aa)	Cleavage site at the end	Amino acid identity with KjMV (%)
P1	465	TRHF/S	44
HCPro	455	YKVG/G	68
P3	346	VRLQ/N	53
6K1	52	VRPQ/S	81
CI	634	VRLQ/G	78
6K2	53	VVHQ/G	60
VPg	189	VRQE/G	62
NIa – Pro	242	VFEQ/A	73
Nib	521	VCLQ/A	72
CP	270		71
whole polyprotein	3227		70

NC 006262 Watermelon mosaic virus
NC 002634 Soybean mosaic virus
NC 021196 Calla lily latent virus
NC 007216 Wisteria vein mosaic virus
NC 007728 East Asian Passiflora virus
NC 010954 Fritillary virus Y
NC 004047 Bean common mosaic necrosis virus
NC 004013 Cowpea aphid-borne mosaic virus
NC 009742 Telosma mosaic virus
NC 016441 Yam bean mosaic virus
NC 003397 Bean common mosaic virus
NC 003224 Zucchini yellow mosaic virus
NC 014790 Passion fruit woodiness virus
NC 019415 Blue squill virus A
NC 015394 Hardenbergia mosaic virus
NC 011560 Zantedeschia mild mosaic virus
NC 003537 Dasheen mosaic virus
NC 016159 Keunjorong mosaic virus
MH376747 Pleione flower breaking virus isolate Wharfedale
NC 002600 Peanut mottle virus
NC 009741 Basella rugose mosaic virus
NC 005304 Beet mosaic virus
NC 009745 Banana bract mosaic virus
NC 021197 Donkey orchid virus A
NC 008824 Narcissus degeneration virus
NC 017977 Vallota speciosa virus
NC 007433 Shallot yellow stripe virus
NC 005029 Onion yellow dwarf virus
NC 008028 Daphne mosaic virus
NC 005028 Papaya leaf-distortion mosaic virus
NC 003742 Cocksfoot streak virus
NC 013261 Canna Yellow Streak Virus
NC 003606 Johnsongrass mosaic virus
NC 007147 Pennisetum mosaic virus
NC 018833 Iranian johnsongrass mosaic virus
NC 003398 Sugarcane mosaic virus
NC 004035 Sorghum mosaic virus
NC 003377 Maize dwarf mosaic virus
NC 001671 Pea seed-borne mosaic virus
NC 017967 Hippeastrum mosaic virus
NC 020072 Colombian datura virus
NC 001555 Tobacco etch virus
NC 021065 Sunflower mild mosaic virus
NC 007913 Konjac mosaic virus
NC 018872 Pokeweed mosaic virus
NC 004039 Potato virus A
NC 001768 Tobacco vein mottling virus
NC 007180 Thunberg fritillary virus
NC 004011 Leek yellow stripe virus
NC 005288 Lily mottle virus
NC 003536 Clover yellow vein virus
NC 003492 Bean yellow mosaic virus
NC 004752 Yam mosaic virus
NC 010736 Algerian watermelon mosaic virus
NC 009995 Moroccan watermelon mosaic virus
NC 001785 Papaya ringspot virus
NC 020896 Sweet potato latent virus
NC 015393 Celery mosaic virus
NC 014905 Apium virus Y
NC 003605 Lettuce mosaic virus
NC 018093 Sweet potato virus G
NC 017970 Sweet potato virus 2
NC 001841 Sweet potato feathery mottle virus
NC 001445 Plum pox virus
NC 000947 Japanese yam mosaic virus
NC 002509 Turnip mosaic virus
NC 003399 Scallion mosaic virus
NC 011541 Narcissus yellow stripe virus
NC 023628 Narcissus late season yellows virus
NC 018176 Arracacha mottle virus
NC 017824 Tomato necrotic stunt virus
NC 014536 Brugmansia suaveolens mottle virus
NC 010735 Verbena virus Y
NC 014325 Bidens mottle virus
NC 008393 Pepper severe mosaic virus
NC 001616 Potato virus Y
NC 014038 Sunflower chlorotic mottle virus
NC 014327 Pepper yellow mosaic virus
NC 004573 Peru tomato mosaic virus
NC 004426 Wild potato mosaic virus
NC 004010 Potato virus V
NC 009994 Tobacco vein banding mosaic virus
NC 016044 Chilli ringspot virus
NC 021786 Habenaria mosaic virus
NC 019412 Yam mild mosaic virus
NC 001517 Pepper mottle virus
NC 019409 Ornithogalum mosaic virus
NC 011918 Pepper veinal mottle virus
NC 009744 Wild tomato mosaic virus
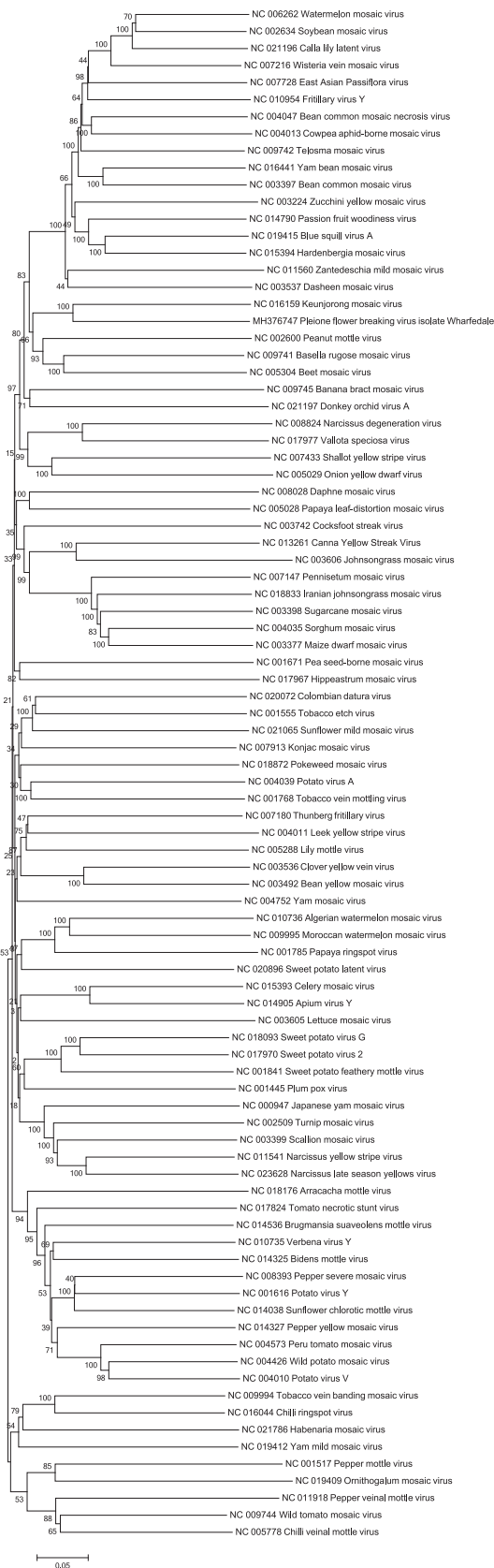NC 005778 Chilli veinal mottle virus

0.05

Fig. 1. Phylogenetic analysis of the polyprotein coding sequences of important members of the genus *Potyvirus*, showing the position of the Pleione flower breaking virus (highlighted in yellow) inside the genus. The evolutionary history was inferred using the Neighbor-Joining method using MEGA7 (Kumar et al., 2016), and the optimal tree is shown. The numbers next to the branches represent the percentage of replicated trees according to the bootstrap test (500 replicates). The evolutionary distances were computed using the p-distance method (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2. The P1/HC-Pro cleavage site was identified at a position similar to other species of the genus *Potyvirus* but presented a unique recognition sequence (TRHF/S) (see Supplementary Figures 4 and 5, and Supplementary File 1).

The DAG triplet, located near the N end of the CP gene, which is considered responsible for potyvirus aphid transmissibility (Atreya et al., 1990), was found starting at nt position 8986 of the full virus sequence.

To confirm the assignation of Pleione flower breaking virus into the genus *Potyvirus*, phylogenetic analysis using the MEGA7 program (Kumar et al., 2016) was conducted. The nucleotide sequence of entire ORF of ninety members of the genus *Potyvirus* was compared and aligned using ClustalW embedded in MEGA7. The Neighbor-Joining method was used for the evolutionary analysis, and bootstrap was calculated from 500 replicates (Fig. 1). The analysis confirmed the position of PlFBV inside the genus *Potyvirus*.

To compare two viruses infecting plants of the genus *Pleione*, we made further phylogenetic analysis, based only on the coat protein gene, and including Pleione virus Y. The resulting tree is available as Supplementary Fig. 6, and it reveals that the two Pleione-infecting viruses are not related. Instead, PlFBV clustered together with another potyvirus, Keunjorong mosaic virus (Nam et al., 2013), which has been described as infecting *Cynanchum wilfordii* (Maxim.) Hemsl. that is a perennial shrub belonging to the family *Asclepiadaceae*, occurring in East Asia where it is important in traditional medicine (Lee et al., 2018; Jiang et al., 2018). PlFBV and KjMV protein sequences were also compared. The amino acid identities of individual proteins of the two viruses ranged from 44% in the case of the P1 protein, to 81% in the case of 6K1. Overall amino acid identity was 70% (see Table 2). This further confirms the difference between PlFBV and KjMV, showing that they are two different virus species; as the criteria given by ICTV put a threshold for species demarcation to 58% amino acid identity for the P1 region, and 82% for the entire polyprotein (Wylie et al., 2017).

In addition, a RT-PCR protocol was developed to detect PlFBV in *Pleione* plants. The primers were designed using Primer3Plus (Untergasser et al., 2007) with the default parameters. Their sequences are 5´ CCGTCCATCATCGTCCAAAC 3´ (antisense primer) and 5´ ATC GCTCAACTGAAATCGCC 3´ (sense primer), expected product length was 703 nucleotides. They were placed on the CP gene and they were also checked by NCBI BLAST to ensure their specificity. To detect the virus, RNA was isolated from 30 plants of the Pleione collection from the Czech Republic, using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, reverse transcription was performed with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), using random hexamers for reaction priming according to the manufacturer's directions. For PCR, the FastStart Taq DNA Polymerase (Roche, Basel, Switzerland) was used. The reaction conditions were as follows: 95 °C/5 min, 40 x (95 °C/35 s, 64 °C/40 s, 72 °C/55 s), 72 °C/7 min. As expected, the product of the reaction was 703 bp (see also Supplementary Fig. 7) and the PlFBV was detected in all 30 tested plants.

Further, aphid transmission experiments with PlFBV were conducted. All plants used for transmission experiments were previously tested by the designed RT-PCR protocol to ensure they are not infected before the inoculation (see also Supplementary Fig. 7). Young aphids *Myzus persicae* Sulz., reared at CRI Prague, were placed onto

analysis of tentative cleavage sites of individual proteins within its amino acid sequence using the web page http://www.dpvweb.net/potycleavage/index.html. Identified cleavage sites are listed in a
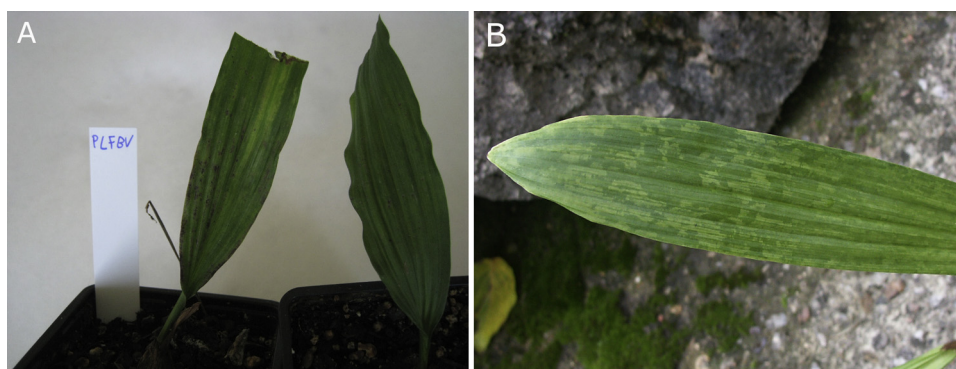
Fig. 2. Symptoms of virus infection on leaves of *Pleione* plants.
A) Mosaic symptoms on *Pleione formosana* Hayata after artificial infection by aphids (left) compared with a healthy plant (right).
B) Mosaic symptoms on a leaf of the *Pleione* hybrid Christianii.

symptomatic leaves of *Pleione* hybrids Wharfedale and Shasta. After 5–10 min of acquisition feeding, they were carefully transferred manually to young plants of *Chenopodium quinoa* Willd., *Nicotiana benthamiana* Domin, and *Nicotiana clevelandii* Gray x *N. glutinosa* L. (10 plants per species), as well as to three plants of *Pleione formosana* Hayata purchased in a market. At least 50 aphids were used per every inoculated plant to ensure successful virus transmission.

The inoculated herbaceous plants showed no symptoms after aphid inoculation. However, plants of *Chenopodium quinoa* Willd., inoculated by aphids from plants of *Pleione* x Shasta, were positive by RT-PCR for PlFBV, when tested 5 weeks after transmission (see also Supplementary Fig. 7). Sequencing of the PCR product confirmed the identity with PlFBV genome (data not shown). Electron microscopy examination also revealed the presence of filamentous virus particles in the *Chenopodium quinoa* Willd. plants (picture not shown).

Three aphid inoculated *Pleione formosana* Hayata plants showed symptoms of mosaic 5 weeks after transmission (see Fig. 2A). The presence of the PlFBV was also confirmed by RT-PCR (Supplementary Fig. 7). Three non-inoculated plants showed no symptoms. Aphid transmission is the main mode of potyvirus transmission, and PlFBV is no exception as was proven by our experiment.

Concerning virus incidence, in the Netherlands the diseased plants were removed and the years afterwards no more disease symptoms were observed. One putative reason is the location of the Pleiones in a greenhouse, which might have prevented or limited the presence of aphid vectors. On the other hand, the virus prevalence in the Pleione collection from the Czech Republic was 100%. This might be related to the grower´s practice growing the plants in open air during summer months, where they are freely accessible to aphids.

We can conclude that a distinct member of the genus *Potyvirus*, family *Potyviridae*, was found infecting plants of the genus *Pleione*. Its entire genome sequence has been characterized, and also its transmissibility by aphids has been demonstrated. We suggest the name Pleione flower breaking virus, and the PlFBV acronym for this virus.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virsres.2018.12.009.

References

Atreya, C.D., Raccah, B., Pirone, T.P., 1990. A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. Virology 178, 161–165.
Chen, X., Cribb, P.J., Gale, S.W., 2009. Pleione D. Don. Flora China 25, 325–333.
Chung, B.Y.W., Miller, W.A., Atkins, J.F., Firth, A.E., 2008. An overlapping essential gene in the Potyviridae. Proc. Natl. Acad. Sci. U. S. A. 105 (15), 5897–5902.
Gibbs, A., Mackenzie, A., Blanchfield, A., Cross, P., Wilson, C., Kitajima, E., Nightingale, M., Clements, M., 2000. Viruses of orchids in Australia; their identification, biology and control. Aust. Orchid Rev. 65, 10–21.
Jiang, H.W., Lin, J., Wang, G.M., Zhang, J.J., Gu, S.S., Cao, L., Chen, Y., Wang, L., Jiao, H., Zhu, W.L., Zhang, H.Y., Zhao, W.M., Li, J.L., 2018. Acetophenone derivatives from the root bark of *Cynanchum wilfordii* as potential neuroprotective agents. Phytochem. Lett. 24, 179–183.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Langeveld, S.A., Dore, J.-M., Memelink, J., Derks, A.F.L.M., van der Vlugt, C.I.M., Asjes, C.J., Bol, J.F., 1991. Identification of potyviruses using the polymerase chain reaction with degenerate primers. J. Gen. Virol. 72, 1531–1541.
Lee, H., Kim, M.H., Choi, Y.Y., Hong, J., Yang, W.M., 2018. Effects of *Cynanchum wilfordii* on osteoporosis with inhibition of bone resorption and induction of bone formation. Mol. Medic. Reports 17, 3758–3762.
Massart, S., Olmos, A., Jijakli, H., Candresse, T., 2014. Diagnosis of viral and high throughput sequencing: towards a paradigm shift? Virologie 18, 247–250.
Massart, S., Candresse, T., Gil, J., Lacomme, C., Predajna, L., Ravnikar, M., Reynard, J.-S., Rumbou, A., Saldarelli, P., Škorić, D., Vainio, E.J., Valkonen, J.P.T., Vanderschuren, H., Varveri, C., Wetzel, T., 2017. A framework for the evaluation of biosecurity, commercial, regulatory, and scientific impacts of plant viruses and viroids identified by NGS technologies. Front. Microbiol. 8, 45.
Nam, M., Lee, J.H., Choi, H.S., Lim, H.S., Moon, J.S., Lee, S.H., 2013. Complete genome sequence of keunjorong mosaic virus, a potyvirus from Cynanchum wilfordii. Arch. Virol. 158, 1817–1820.
Genera orchidacearum volume 4. In: Pridgeon, A.M., Cribb, P.J., Chase, M.W., Rasmussen, F.N. (Eds.), Epidendroideae (Part One). Oxford University Press, Oxford, pp. 696.
Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M., 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35, W71–W74.
Wylie, S., Adams, M., Chalam, C., Kreuze, J., López-Moya, J., Ohshima, K., Praveen, S., Rabenstein, F., Stenger, D., Wang, A., Zerbini, F., ICTV Report Consortium, 2017. ICTV virus taxonomy profile: potyviridae. J. Gen. Virol. 98 (3), 352–354.
Zhu, G.H., Chen, S.C., 1998. Humiles, a new section of *Pleione* (*Orchidaceae*). Novon 8, 461–463.