



Characterisation of a novel virus infecting orchids of the genus *Pleione*

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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 (PIFBV) 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

Abbreviations: HTS, high throughput sequencing; PIFBV, Pleione flower breaking virus; KJMV, Keunjong mosaic virus

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Table 1High 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	PIFBV	124,054	99.99	1871 X
Shasta	7,640,276	PIFBV	26,819	99.8	403 X
Slender Loris	1,056,330	PIFBV	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 PIFBV, according to 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 PIFBV. There was no other plant virus present in the analysed data, either known or unknown. The genome of the PIFBV was assembled after mapping reads back to the longest contig. The 5' and 3' ends of the PIFBV 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 PIFBV, 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.

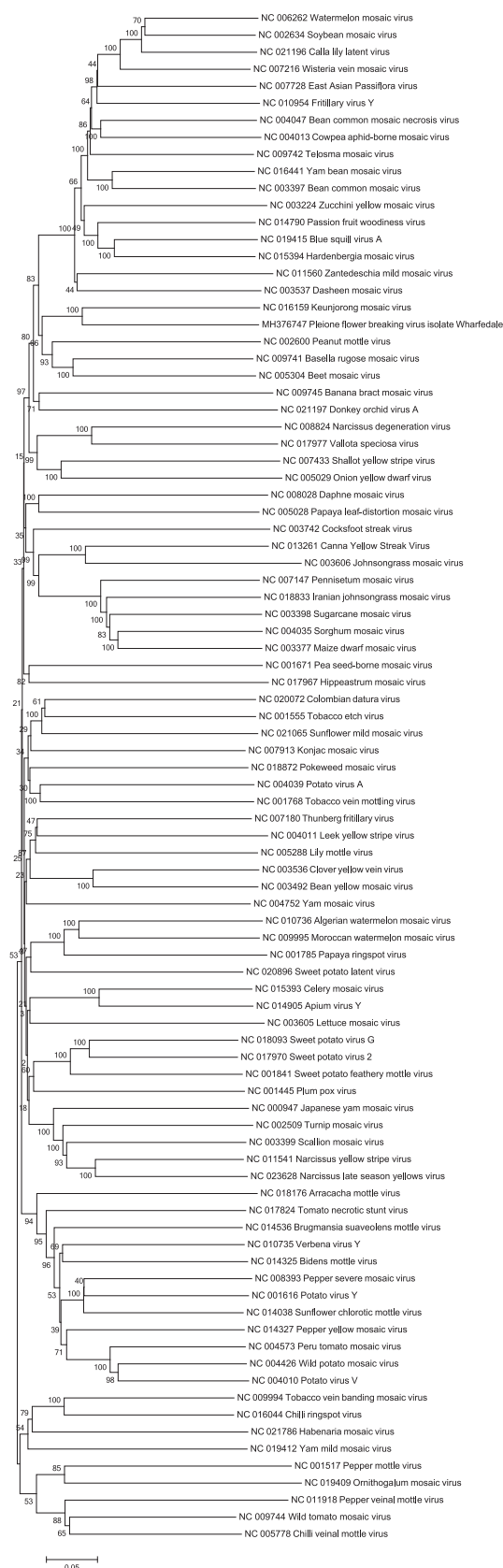
The three full-length genome sequences of PIFBV and the partial PIFBV genome sequence from Netherlands were very similar to each others with a total of 20 divergent positions between isolates (see Supplementary Table 1). The full-length sequence of PIFBV from the *Pleione* hybrid Wharfedale was submitted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

PIFBV 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



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. 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 PIFBV 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, PIFBV clustered together with another potyvirus, Keunjong 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). PIFBV 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 PIFBV 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 PIFBV 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 Transcript 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 PIFBV was detected in all 30 tested plants.

Further, aphid transmission experiments with PIFBV 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

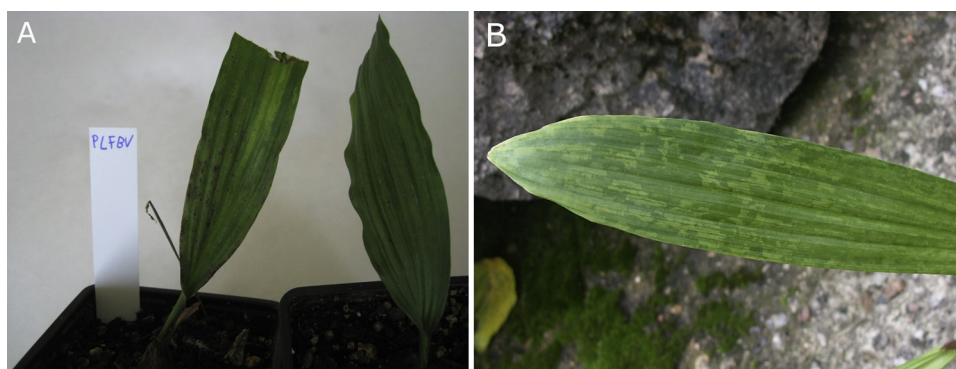


Fig. 2. Symptoms of virus infection on leaves of *Pleione* plants.

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.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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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.virusres.2018.12.009>.

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