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Detection and molecular identification of '*Candidatus* Phytoplasma asteris' associated with muscari virescence of three grape hyacinth species

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Abstract: Grape hyacinths are popular perennial, flowering bulbous plants. In 2018, 2019, and 2020, some plants of three *Muscari* species showed symptoms similar to those associated with phytoplasma infection in commercial ornamental gardens in Hungary. Symptoms included virescence of flowers and yellowing of leaves. Symptomatic and asymptomatic *Muscari* plants were sampled at two locations to detect and identify the pathogens involved. Total DNA was extracted from the plants and used as a template in polymerase chain reaction assays to amplify 16S rRNA gene sequences and housekeeping genes (*tuf*, *secY*) with phytoplasma-specific primers. The resulting PCR products from symptomatic plants were cloned and sequenced bidirectionally. Homology searching of the obtained sequences against the GenBank database indicated the presence of '*Candidatus* Phytoplasma asteris' in the three *Muscari* species. This is the first report worldwide of *C.* P. asteris phytoplasma infecting *M. botryoides* and *M. comosum,* and its first identification in *M. armeniacum* in Hungary.

Keywords: grape hyacinths, phytoplasma, virescence, aster yellows

Muscari is a genus of perennial, small, spring-flowering bulbous plants with bell-shaped flowers carried on a simple, leafless flower stalk on terminal, crowded racemes. Most originate from the Mediterranean region of Europe and South-East Asia. Among the 30 or more species, the most often cultivated are *Muscari armeniacum* and *M. botryoides* (Wraga & Placek 2009).

Phytoplasmas are plant-infecting bacteria known to infect several hundred species, including many commercial floricultural crops. Bellardi et al. (2018) reported several phytoplasmas in ornamental plants belonging to fourteen 16S ribosomal groups and to about 30 ribosomal subgroups, including '*Candidatus* Phytoplasma asteris' which belongs to the 16SrI group and has been recorded on more than 200 plant species (Kaminska 2008).

Plant diseases associated with phytoplasmas typically exhibit a range of symptoms indicative of disturbances in the normal balance of plant hormones (Dermastia 2019). The most characteristic symptoms in ornamental plants are virescence, phyllody, sterility of flowers, witches broom, generalized stunting and yellows (Bellardi et al. 2018). From 2018 to 2022, virescence of flowers and yellowing of the leaves were observed on five *Muscari* cultivars

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belonging to three species in Hungary. Virescence symptoms of *M. armeniacum* were described in a comprehensive survey of phytoplasma diseases of ornamentals in Lithuania (Samuitiene et al. 2007), and the disease was named muscari virescence.

Since phytoplasma infections can cause serious commercial losses, especially in the case of ornamental plants, where their decorative value is affected, there is great interest in controlling the pathogen and/or its insect vector or even in destroying the infected stock to avoid further distribution of the disease.

This study was undertaken to detect and identify the phytoplasma in the symptomatic *Muscari* species exhibiting symptoms of virescence in Hungary.

MATERIAL AND METHODS

Sample collection. Samples were collected in April 2018 and in May 2019 and in May 2020 from three species; *M. armeniacum* 'Valerie Finnis', *M. botryoides* 'Album' and *M. comosum* 'Plumosum' in a garden in Nyársapát (N47.087373 E19.780381), and in May 2022 from two other *M. armeniacum* cultivars; *M. armeniacum* 'Fantasy Creation' and 'Double Beauty' from a commercial ornamental nursery in Püspökladány (N47.31120644752863 E21.12881533517595), both located in south-eastern Hungary. Leaf and flower stalk samples were taken from three symptomatic plants and three asymptomatic plants of each variety, except *M. armeniacum* 'Fantasy Creation' and 'Double Beauty', where samples were taken from ten symptomatic and three asymptomatic plants.

DNA extraction and PCR assay. Total DNA was extracted from 1 g of leaf and flower stalk samples from each cultivar by CTAB method (Ahrens & Seemüller 1992). Phytoplasma 16S rDNA was amplified using universal primers P1 (Deng & Hiruki 1991), P7 (Schneider et al. 1995) and R16F2n/R16R2 (Gundersen & Lee 1996). Translocase protein (*secY)* and elongation factor Tu (*tuf)* genes were amplified with AYsecY_F-46/AYsecY_R1450 (Viczián et al. 2023) and fTuf1/rTuf1 (Schneider & Gibb 1997) primer pairs, respectively. PCR reactions were performed with the following parameters: 2 min of initial denaturation at 94 °C followed by 30 cycles at 94 °C for 60 s, 55 °C for 90 s and 72 °C for 150 s. A final extension was performed for 10 min at 72 °C.

Cloning of PCR products and sequencing. All amplified PCR products of expected sizes for three primer pairs were ligated into the pJET1.2/blunt cloning vector using a CloneJET PCR cloning kit (Thermo Fisher Scientific, Waltham, MA, USA). The cloned PCR fragments (three from each PCR reaction) were sequenced by LGC Genomics (Berlin, Germany) using pJET1.2 forward and reverse primers, and the obtained sequences were deposited in GenBank.

Sequence analysis, phylogenetic tree and *in silico* RFLP analysis. The resulting consensus sequences were BLAST searched against NCBI nucleotide sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and further analysis was performed by either pairwise or multiple alignments using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira et al. 2022). Phylogenetic analysis was performed using the maximum likelihood method implemented in MEGA (version 7.0) (Kumar et al. 2016). The phytoplasma 16S rRNA gene sequences detected from the three *Muscari* species were compared with each other and with 24 other phytoplasma sequences previously published in the NCBI database (www.ncbi.nlm.nih.gov). *Acholeplasma multilocale* (GenBank accession number: NR_042960) and *A. laidlawii* (GenBank accession number: NR_113658) were used as out-groups to root the trees. The similarity coefficients of the obtained sequences were estimated with the *i*PhyClassifier online phytoplasma classification tool (Zhao et al. 2009).

RESULTS AND DISCUSSION

Symptoms of diseased plants. The pedicels of *M. armeniacum* 'Valerie Finnis', 'Fantasy Creation' and 'Double Beauty' have doubled in length, the petals have changed colour to greenish, and the position of the flowers has changed from hanging to upright (Figure 1 a/s). In the inflorescence, the flowers of *M. botryoides* 'Album' became 1.5–2 cm long, tubular, with the edges of the petals green, the flowers standing horizontally instead of hanging (Figure 1 b/s). The flowers of *M. comosum* 'Plumosum', originally pinnate, have become deformed like small, greenish cauliflowers (Figure 1 c/s). Symptoms described above were present in all 55 samples of the cultivar *M. armeniacum* 'Fantasy Creation' and in all 30 samples of 'Double Beauty' in the Püspökladány ornamental nursery, and three to five symptomatic plants were found in a private garden in the case of *M. armeniacum* 'Valerie Finnis', *M. botryoides* 'Album' and *M. comosum* 'Plumosum' varieties.

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Figure 1. Typical virescence symptoms observed in infected *Muscari armeniacum* 'Valerie Finnis' (a/s), *M. botryoides* 'Album' (b/s) and *M. comosum* 'Plumosum' (c/s) varieties Symptomatic plants on the top (a/s, b/s, c/s) and healthy plants on the bottom (a/h, b/h, c/h)

Detection of phytoplasma by PCR assay. Amplicons of the expected sizes (P1/P7: 1 830 bp, R16F2n/R16R2: 1 246 bp, AYsecY_F-46/AYsecY_R1450: 1 520 bp, fTuf1/rTuf1: 1 085 bp) were produced from all symptomatic plants but not from the asymptomatic ones (data now shown).

Sequencing and phylogenetic analysis. The 16S rRNA gene sequences obtained from *M. armeniacum* cultivars ('Valerie Finnis', 'Fantasy Creation' and 'Double Beauty'), *M. botryoides* 'Album' and *M. comosum* 'Plumosum' have been deposited in GenBank under accession numbers ON515747, ON564434 and OR075969, respectively. Identical sequences were obtained from *M. botryoides* 'Album' and *M. comosum* 'Plumosum', sharing 100% identity with onion yellows phytoplasma strain OY-M (GenBank accession number AP006628), which is the alternative reference strain of '*Candidatus* Phytoplasma asteris' (Bertaccini et al. 2022). The 16S rRNA gene sequences of *M. armeniacum* cultivars showed 99.89% similarity to the onion yellows phytoplasma strain OY-M (GenBank AP006628).

The cloned and sequenced PCR products obtained with *secY* gene-specific primers have been deposited in GenBank under the accession numbers ON564431 (*M. armeniacum* 'Valerie Finnis', 'Fantasy Creation' and 'Double Beauty'), ON564430 (*M. botryoides* 'Album') and OR075968 (*M. comosum* 'Plumosum'). The *secY* gene fragments obtained from the three *Muscari* species studied had 100% identity with each other and with the onion yellows phytoplasma strain OY-M (GenBank AP006628).

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A part of the elongation factor Tu (*tuf*) gene was amplified, cloned and sequenced in the cultivars of *M. armenicum* (GenBank accession number ON515745) and the cultivar *M. botryoides* 'Album' (GenBank accession number ON564433). The sequences shared 99.9% of sequence identity with each other. The *tuf* gene fragments obtained from *M. armeniacum* and *M. botryoides* showed 99.8% and 99.9% identity with the onion yellows phytoplasma strain OY-M (GenBank AP006628).

In *i*PhyClassifier analysis, the virtual RFLP pattern derived from the query 16S rDNA was identical (similarity coefficient 1.00) to the reference pattern of 16Sr group I, subgroup B (GenBank accession number AP006628) (Zhao et al. 2009). This differs from the result of Samuitiene et al. (2007) in Lithuania, where the phytoplasma detected in *M. armeniacum* was assigned to the 16SrI-M subgroup. However, among the group 16SrI phytoplasmas in Europe, the subgroup I-B strains have the widest host range, including ornamentals (Marcone et al. 2000).

A phylogenetic tree constructed with the three *Muscari* species 16S rRNA gene sequences obtained in this study (GenBank accession numbers

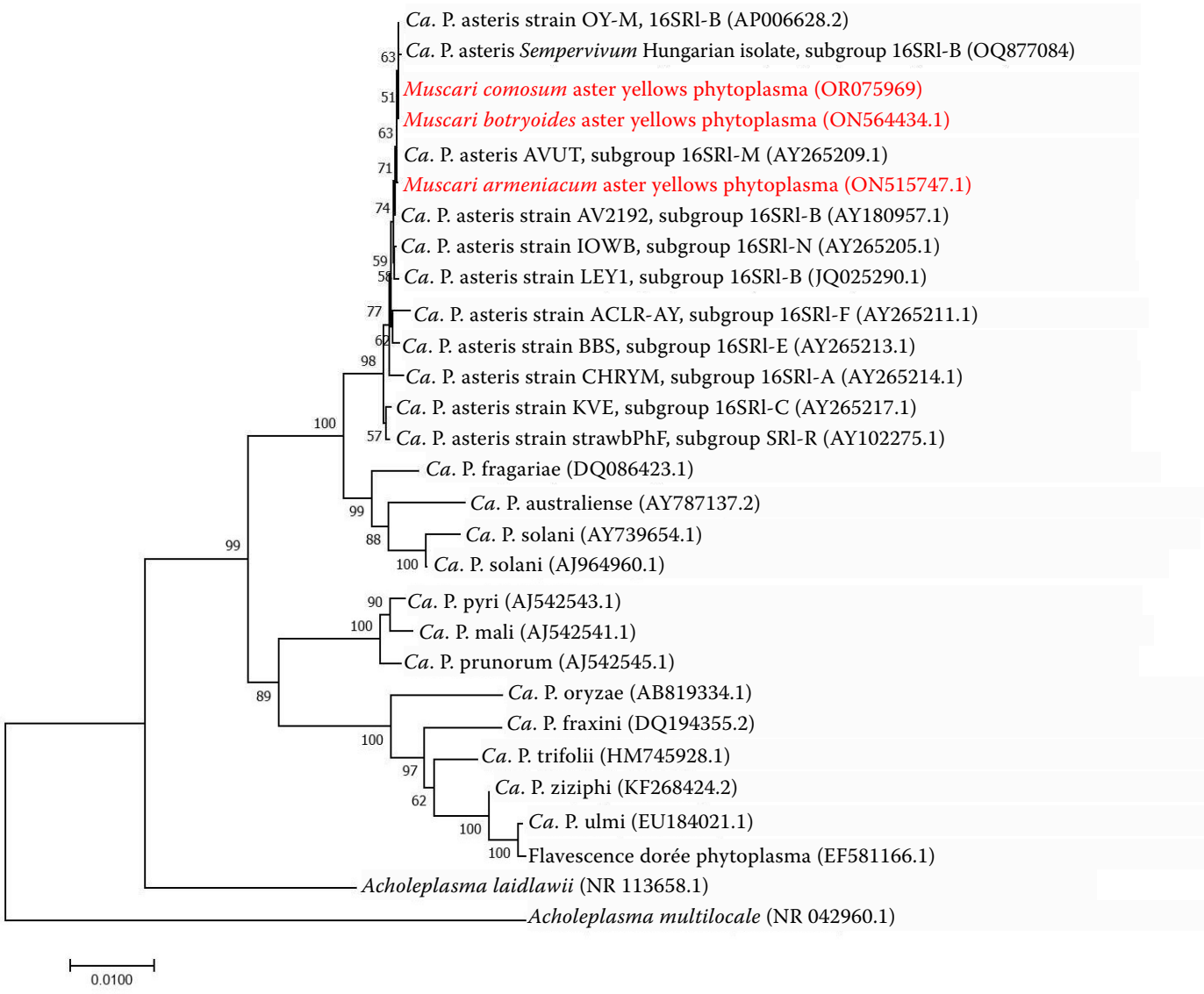

Figure 2. Phylogenetic tree constructed by the maximum likelihood method using partial 1.8 kb 16S rRNA gene sequences of the *Candidatus* Phytoplasma asteris strains of *Muscari armeniacum*, *M. botryoides* and *M. comosum*, highlighted in red and 24 phytoplasmas available in the GenBank database. GenBank accession numbers are given in parentheses. *Acholeplasma laidlawii* and *A. multilocale* sequences were out-groups to root the tree. Phylogenetic analysis was performed using MEGA software (version 7.0); Bootstrap values based on 1 000 replications are indicated at the nodes

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ON515747, ON564434, OR075969) indicated the '*Ca*. P. asteris' strains of the three *Muscari* species clustered well together and with other '*Ca.* P. asteris' strains (Figure 2). The strains obtained were also in a very close relationship with a '*Ca*. P. asteris' strain (GenBank accession number OQ877084) was identified in *Sempervivum tectorum* in Hungary. The incidence of the disease was found to vary from location to location in Hungary. One can suppose that the high incidence of the disease may be attributed to infected propagation material, while the sporadic occurrence of the disease may be more related to insect vectors. The disease can potentially cause economic losses in nurseries with high incidence of symptomatic plants. This is the first identification of '*Ca*. P. asteris' in *M. armeniacum* in Hungary and in *M. botryoides* and *M. comosum* worldwide.

Data availability. The data that support the findings of this study will be openly available in GenBank (Accession numbers: ON515747, ON564434, OR075969, ON564431, ON564430, OR075968, ON515745, ON564433).

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