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Identification of viral and phytoplasmal agents causing diseases in *Gaillardia* Foug. plants in Lithuania

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Abstract. *Gaillardia* plants exhibiting symptoms characteristic of viral and phytoplasmal diseases were collected at botanical gardens and floriculture farms in Lithuania. *Cucumber mosaic cucumovirus* was isolated from diseased plants exhibiting symptoms characterized by stunting, flower breaking and malformation of petals. The virus was characterized based on electron microscopy, serology, reactions of inoculated test-plants, and reverse transcription polymerase chain reaction (RT–PCR). Symptoms of leaf yellowing and general stunting of plants, and virescence and phyllody of flowers were associated with infection by a phytoplasma. Phytoplasmal 16S rRNA gene sequences were amplified in polymerase chain reactions (PCRs) primed by phytoplasma universal primer pairs P1/P7 and R16F2n/R16R2. RFLP analysis of the 1.2 kbp rDNA product, subjected to single enzyme digestions with nine restriction endonucleases, revealed that the *Gaillardia* plants were infected by a phytoplasma (gaillardia yellows, GaiY) belonging to group 16SrI (aster yellows phytoplasma group), subgroup I–C (clover phyllody, CPh, phytoplasma subgroup). 16S rDNA sequence alignments and phylogenetic analysis confirmed that the GaiY phytoplasma was closely related to CPh phytoplasma. *Gaillardia* can be naturally infected by a broad biodiversity of phytoplasma strains belonging to subgroups 16SrIII–B (clover yellow edge subgroup), 16SrI–A (aster yellows subgroup), and 16SrI–C in Lithuania.

Key words: *Gaillardia, Cucumber mosaic cucumovirus,* 16SrI–C subgroup phytoplasma

INTRODUCTION

During recent years, increasing attention has been devoted to the development of field floriculture in Lithuania, particularly for the benefit of small family farming businesses that produce seedlings of perennial ornamental plants for the domestic market and for export to neighboring countries. Like other segments of agriculture, this enlarging sector is threatened by plant diseases; both the quality and quantity of ornamental plants including the object of this study is affected by viral and phytoplasmal diseases. Since viral and phytoplasmal infections are systemic in diseased plants, propagation by division, movement of plants and plant parts could contribute to geographical spread of these pathogens. To aid growers to control plant diseases and to facilitate growth of the floriculture industry, the scientific staff of Plant Virus Laboratory in the Institute of Botany carries out regular surveys to monitor the

phytosanitary status of ornamental plants grown at Botanical gardens, as well as other locations, and in response to requests from floriculture farms and parterres in cities.

In surveys over the past two years, we found that plants of *Gaillardia* frequently expressed symptoms characteristic of viral and phytoplasmal diseases. The genus *Gaillardia* Foug., native of North America, belongs to the family *Asteraceae* Dumort. Two species, *G. aristata* Purch. and *G. pulchella* Foug., are popular ornamentals grown commercially for cut flowers and as garden plants in Lithuania. Few published data are available concerning viral diseases of *Gaillardia*. M. Klinkowski (1968) described white flower breaking disease of *Gaillardia* caused by *Turnip mosaic potyvirus*. R/ L/ Wick (2003) reported *Impatiens necrotic spot virus* and *Tomato spotted wilt virus* infection on *Gaillardia.* Phytoplasmal diseases have been investigated to a greater extent, including investigations in Lithuania. During surveys of ornamental plants in prior decades, *Gaillardia* plants showing symptoms of stunting, general yellowing, and flower virescence and phyllody were frequently observed in different locations in Lithuania. The symptoms were first attributed to phytoplasmas based on the frequent association of such symptoms with known phytoplasma infections of other plant species, and based on results from studies involving ultrathin section electron microscopy (EM) (Makutėnaitė–Navalinskienė, 1981; Staniulis, 1988). Later, molecular methods were employed to identify and differentiate phytoplasmas associated with diseases of vegetables, legumes, forest trees, weeds, cereals and ornamental plants in Lithuania (Jomantiene et al., 2000, 2002; Valiunas et al., 2000; Valiūnas, 2003; Urbanavičienė et al., 2004; Samuitiene et al., 2006). Those studies revealed that *Gaillardia* plants were natural hosts for phytoplasmas belonging to group 16SrIII, subgroup 16SrIII–B (Jomantiene et al., 2002) and group 16SrI, subgroup 16SrI–A (Samuitiene et al., 2006). The objective of the present study was to determine the possible association of both virus and phytoplasma with diseases in *Gaillardia* and to increase knowledge on the biodiversity of these two classes of disease agents in Lithuania.

MATERIALS AND METHODS

Plant samples. Plant material of *G. aristata* and *G. pulchella* was collected in Botanical gardens of Vilnius, Kaunas Vytautas Magnus, Klaipėda Universities, and the Experimental Station of Field Floriculture, and at three floriculture farms located in the Vilnius and Ukmergė regions. Diseased *Gaillardia* plants were collected in Vilnius city's parterre during the growing seasons in 2004 and 2005.

The experimental work was carried out at the Plant Virus Laboratory of the Institute of Botany, Lithuania, and at the Molecular Plant Pathology Laboratory, USDA-Agriculture Research Service, Beltsville, MD, USA.

Virus identification. Viruses were identified by using the test–plant method (Francki et al., 1979; Brunt et al., 1996), electron microscopy (EM) negative staining technique, agarose gel double–diffusion serological test (Dijkstra & de Jager, 1998), and reverse transcription polymerase chain reaction (RT–PCR) (de Blas et al., 1994). The inocula for mechanical inoculation of test-plants were prepared by triturating tissues from diseased plants in 0.1 M phosphate buffer (pH 7.0) containing as virus–stabilizing additives 0.2% 2-mercaptoethanol or 0.01 M sodium diethyldithio-carbamate. The following test–plants were used: *Amaranthus caudatus* L.*, Atriplex*

hortensis L.*, Chenopodium amaranticolor* Coste et Reyn*, C. hybridum* L.*, C. quinoa* Willd.*, Cucumis sativus* L.'Delikates', 'Rodničok', *Gomphrena globosa* L.*, Nicandra physalodes* (L.) Gaertn.*, Nicotiana alata* Link et Otto*, N. debneyi* Domin., *N. glutinosa* L.*, N. rustica* L.*, N. tabacum* L. 'Samsun', *Petunia hybrida* Vilm., *Tetragonia expansa* Murr., *Zinnia elegans* Jack.

Virus particles were examined in leaf dip preparations negatively stained with 3 % uranyl acetate electron microscopically using a JEM–100S electron microscope, at magnification 25000.

RT–PCR was accomplished using the primers designed from non-coding intergenic region in the coat protein gene. An upstream primer: 5'–GTAGACATCTGTGACGCGA–3' homologous to nucleotides 114–132 and a downstream primer: 5'–GCGCGAAACAAGCTTCTTATC–3' complementary to position 633–653 were selected, resulting in a 540 bp amplification product (Quemada et al., 1989).

Total RNA was extracted from symptomatic test–plant material stored frozen at –20°C using QuickPrep™ Total RNA Extraction Kit (Amersham Biosciences UK). Extraction procedure was carried out according to the manufacturer's instructions.

All PCR procedures were carried out in Eppendorf Master Cycler Personal. For RNA denaturation mixture of 10 µl RNA (for each sample) and 1 µl of downstream primer was incubated 5 min at 70° C and 5 min at 4° C.

Reverse transcription (RT, cDNA synthesis) reaction mixture (for one sample): 4 µl 5x PCR buffer; 1 µl RNasin; 2 µl 10 mM dNTPs; 1 µl MulRev Transcriptase and 11 µl of denatured RNA mix. Reaction was performed incubating at 37^0 C for 60 min, at 70° C for 10 min and at 4° C for 5 min.

PCR reaction mixture contained (for one sample): 11 µl c DNA; 34.75 µl PCR water, 4 µl 2 mM dNTPmix, 1 µl upstream primer, 1 µl downstream primer, 5 µl 10xPCR buffer without Mg, 3 µl $MgCl_2$, 0.25 µl Taq DNA polymerase. Reaction mixtures were incubated at 94°C for 4 min (for first step), 40 cycles of 94° C for 1 min, 52°C for 2 min, 72° C for 2 min, and at 72° C for 10 min (final step).

Resulting PCR products were analyzed by electrophoresis through 1% agarose gel, stained with ethidium bromide, and DNA bands visualized using a UV transilluminator. DNA fragment size standard was Gene Ruler 1 kbp DNA Ladder # SMO311, fragment sizes (from top to bottom): 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp (MBI Fermentas).

DNA extraction and polymerase chain reaction (PCR) amplification of rDNA. Nucleic acid, to be used as a template in PCR, was extracted from frozen plant tissues using Genomic DNA Purification Kit (MBI Fermentas, Vilnius, LT) according to the manufacturer's instructions. Ribosomal (r) DNA was amplified in nested PCRs using phytoplasma–specific primer pairs P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) and R16F2n/R16R2 (Gundersen & Lee, 1996). In nested PCR, rDNA amplified in PCR primed by P1/P7 was diluted 1:50 with sterile water and used as the template in PCR primed by R16F2n/R16R2 under conditions as described by Jomantiene et al., (1998), Taq DNA (recombinant) polymerase was used (MBI Fermentas, Vilnius, Lithuania).

Restriction fragment length polymorphism (RFLP) analysis of amplified phytoplasmal 16S rDNA. Products from the nested PCR primed by R16F2n/R16R2 were analysed by single enzyme digestion according to manufacturer's instructions

with restriction endonucleases: *Alu*I, *Mse*I, *Kpn*I, *Hha*I, *Hpa*II, *Hae*III, *Rsa*I, *Hinf*I, *Taq*I (MBI Fermentas, Vilnius, LT). RFLP profiles of digested DNA were analysed by electrophoresis through 5% polyacrilamide gel, staining with ethidium bromide, and visualization using an UV transilluminator. The RFLP profiles were compared with previously published data (Lee et al., 1998; Marcone et al., 2000).

Nucleotide sequencing and sequences analysis. Products from PCR primed by R16F2n/R16R2 were cloned in *Echerichia coli* using the TOPO–TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and sequenced using automated DNA sequencing. Nucleotide sequences determined in this study were deposited in the GenBank database. Putative restriction site maps were constructed by use of DNASTAR software MapDraw program of the sequence analysis software suite Lasergene (DNASTAR, Madison, WI). For calculations of sequence similarities, sequences were aligned by using the MegAlign program in the same software package.

Phylogenetic analysis. 16S rRNA gene sequences (1.2 kbp in size, representing the R16F2n/R16R2 PCR product) from 23 phytoplasma strains and *Acholeplasma palmae* were aligned using Clustal X version 1.63b (Thompson et al., 1997). A phylogenetic tree was constructed by the Neighbor-Joining method, and the tree was viewed by using TreeViewPPC (Page, 1996). *A. palmae* was selected as outgroup to root the tree. Accession numbers of sequences are given in Fig. 4.

RESULTS AND DISCUSSION

Diseased plants of Gaillardia were observed in all locations surveyed. During the growing seasons of 2004 and 2005, all plants of *G. aristata* (15–20) growing in Vilnius city's parterre were affected by disease.

Virus identification. *Cucumber mosaic cucumovirus* (CMV) was isolated from *Gaillardia aristata* and *G. pulchella* plants bearing conspicuous viral symptoms on flowers. Petals were distorted and displayed variously shaped and colored spots and streaks. Plants generally were stunted.

CMV was identified on the basis of symptom expression in mechanically inoculated test–plants. The results are presented in Table 1. The most characteristic symptoms of CMV infection developed in the following test–plants: *Cucumis sativus* (local diffused spots and systemic mottling, chlorotic rings); *Nicotiana debneyi* (local chlorotic spots and systemic chlorotic and necrotic ringspots); *N. glutinosa* (systemic chlorotic mottling, distortion of leaves). EM revealed isometric virus particles about 30 nm in diameter in negatively stained preparations made from naturally infected *Gaillardia* plants and from inoculated test–plants (Fig. 1). Identification of CMV was confirmed by a positive serological test reaction in agarose gel double–diffusion test.

CMV identification by test–plant reactions, EM and serological tests were verified in RT–PCR using as samples test plants inoculated with virus isolate from *G. aristata*. Total RNA was extracted from frozen leaf tissue of infected test–plants. Leaf tissue from healthy *N. rustica* plant was used as negative control. CMV isolate from *Delphinium cultorum* was used as positive control. Specific for CMV PCR products were obtained with all investigated isolates and positive control, but not with negative control. Specific bands in agarose gel of analyzed products after electrophoresis at a position corresponding to the expected size of amplification product of 540 bp were obtained, confirming CMV identity (Fig. 2).

Table 1. Test–plant reaction to inoculation by *Cucumber mosaic cucumovirus* (CMV) isolated from *Gaillardia.*

Test-plant	Test-plant reaction
Amaranthus caudatus	L:NLL
Atriplex hortensis	L:NLL
Chenopodium amaranticolor	L:ClLL
C. hybridum	L: NLL
C. quinoa	L: LL
Cucumis sativus 'Delikates'	L: DifSp; S: YSp, Mo
C. sativus 'Rodničok'	L: ClDifSp; S: ClRi
Gomphrena globosa	L: LL
Nicandra physalodes	L: NLLSp; S: LeDis,ClSp, NDot, LeTN
Nicotiana alata	L: NSp; S: ClGrRiSp, NRi
N. debneyi	L: DifClSp; S: ClGrRiSp, NRi
N. glutinosa	S: ClGrMo, LeDis
N. rustica	L: NRiSp; S: ClGrMo
N. tabacum 'Samsun'	S: Mo, ClGRiSp, N
Petunia hybrida	L:ClSp; S:M,Mo
Tetragonia expansa	L:RNSp
Zinnia elegans	S: Mo

Abbreviations: L – local reaction, LL – local lesions, M - mosaic, Mo – mottling, Cl – chlorotic, chlorosis, S – systemic reaction, N – necrotic, necrosis, Ri – ring, Sp – spots, spotting, Le - leaf, T – top, Dis - deformation, Dif - diffuse, Dot – dots, G - gray, Y – yellow, Gr – green, R - red.

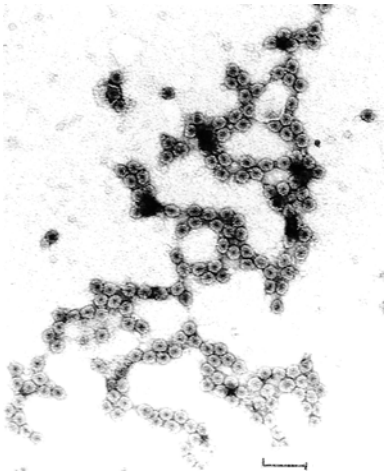

Fig. 1. Electron micrograph of negatively stained (uranyl acetate) particles of *Cucumber mosaic cucumovirus* (CMV) isolated from diseased *Gaillardia*. Bar represents 100 nm.

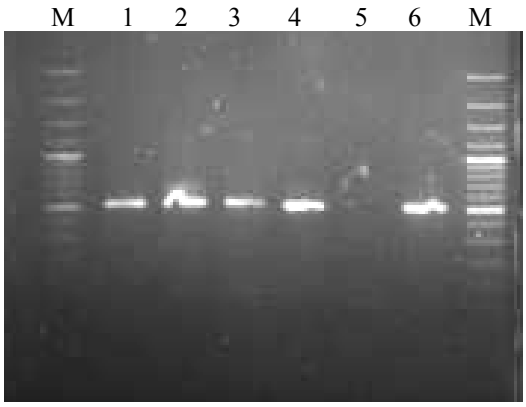

Fig. 2. Agarose gel electrophoretic analysis of RT–PCR products obtained from test–plants inoculated with CMV *Gaillardia* isolate. Lanes M, DNA fragment size standard, fragment sizes: 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp. Lane 1, *Cucumis sativus*; 2, *Chenopodium quinoa*; 3, *Nicotiana rustica*; 4, *N. glutinosa*; 5, healthy *N. rustica* plant (negative control); 6, CMV from *D. cultorum* (positive control). CMV specific product, 540 bp.

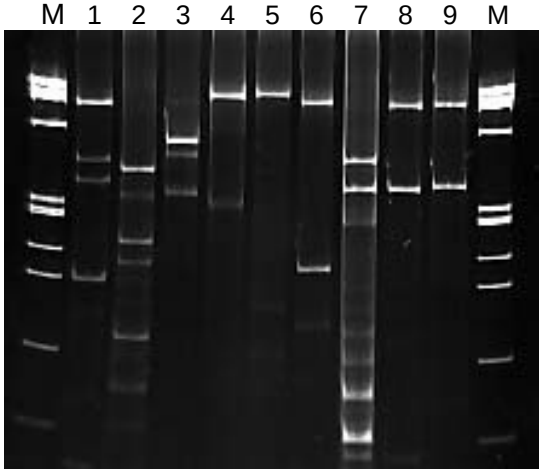

Fig. 3. RFLP analysis of 16S rRNA gene sequence amplified in nested PCR primed by primer pair R16F2n/R16R2 from gaillardia yellows phytoplasma strain GaiY. Lanes: 1, *Alu*I; 2, *Mse*I; 3, *Kpn*I; 4, *Hha*I; 5, *Hpa*II; 6, *Hae*III; 7, *Rsa*I; 8, *Hinf*I; 9, *Taq*I; M, Marker, *Phi*X174 DNA/*Bsu*RI (*Hae*III) digest size standard with fragment sizes of 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118 and 72 bp.

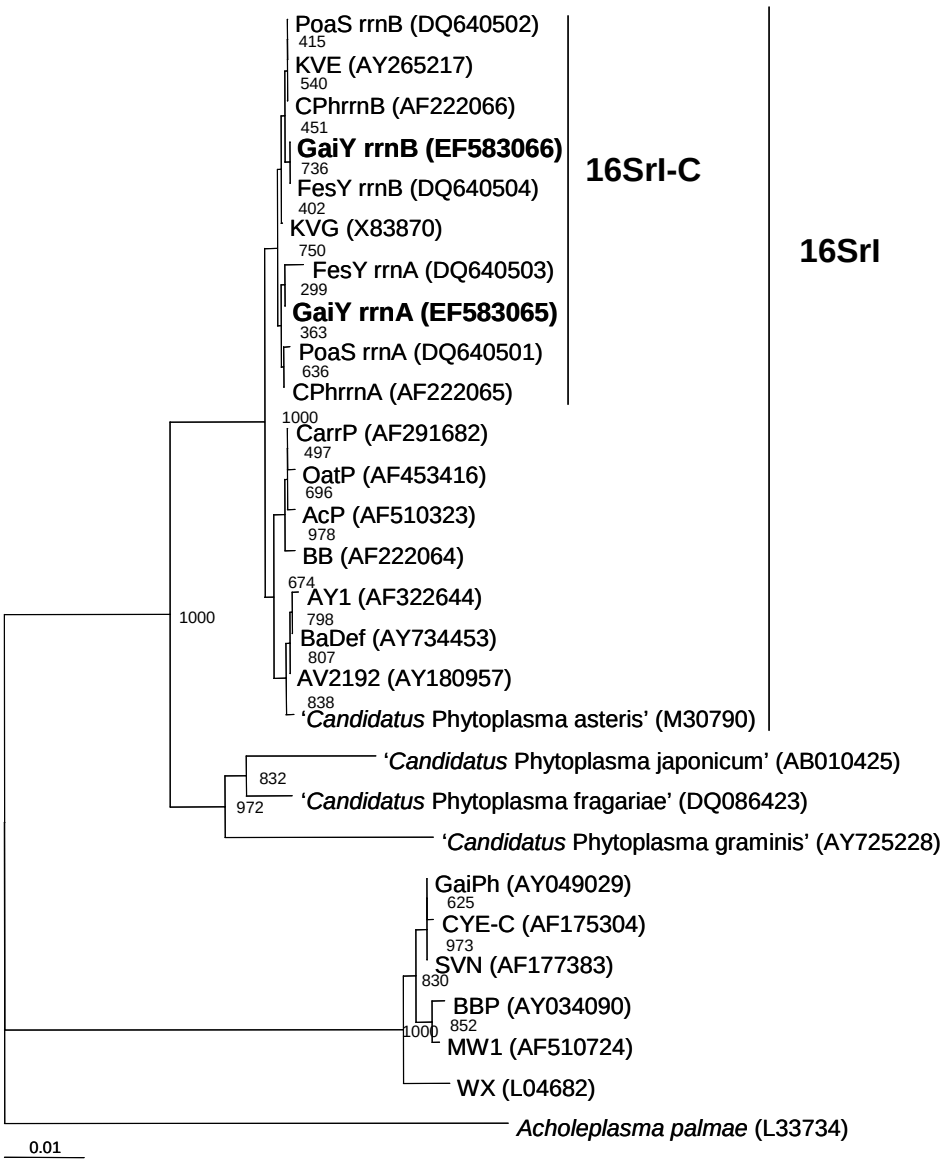

Fig. 4. A genetic distance-based on phylogenetic tree constructed by the Neighbor-Joining method of 16S rDNAs from 23 phytoplasma strains, including phytoplasma subgroup 16SrI–C strains, and *Acholeplasma palmae*, employing *A. palmae* as the outgroup. GenBank accession numbers of 16S rDNA sequences are in parentheses. Group 16SrI and subgroup 16SrI–C strains are indicated. Sequences from strain GaiY determined in the present work are in bold type.

On the basis of particle morphology data, symptom expression on host plants and inoculated test–plants, positive reaction in serological test, RT–PCR results, and in accordance with published descriptions of CMV (Francki et al., 1979; Brunt et al., 1996), the virus isolated from *Gaillardia* was identified as CMV.

CMV is distributed worldwide and has an extremely wide host range, infecting more than 1000 species in 85 families. The virus is transmissible by mechanical inoculation and is transmitted in non–persistent manner by more than 60 species of aphids. It systemically infects plant hosts and thus can be spread through vegetative propagation of plant parts, and it is transmitted through true seeds of 19 plant species (Francki et al., 1979; Brunt et al., 1996; Dijkstra, Khan, 2006; Aramburu et al., 2007).

Molecular detection and classification of phytoplasma. Nucleic acid for use as a template in PCR was extracted from *G. aristata* plants bearing symptoms of general yellowing and stunting of plants, virescence and phyllody of flowers. Amplification of phytoplasmal 16S rRNA gene sequences in PCRs from diseased plants yielded phytoplasma characteristic 1.8 kbp and 1.2 kbp PCR products indicating that the diseased plants were infected by phytoplasma (data not shown; Lee et al., 1998). The phytoplasma was named *Gaillardia* yellows (GaiY) phytoplasma. The 1.2 kbp 16S rDNA products were subjected to RFLP analysis involving single enzyme digestions with different restriction endonucleases. The RFLP patterns revealed that the *Gaillardia* plants were infected by a phytoplasma belonging to group 16SrI (aster yellows phytoplasma group), subgroup 16SrI–C (clover phyllody (CPh) phytoplasma subgroup) (Fig. 3).

Phytoplasmas of subgroup 16SrI–C have previously been detected in association with clover phyllody disease on *Trifolium repens* in Lithuania (Staniulis et al., 2000), France and Germany and on *T. sativum* in Canada (Marcone et al., 2000); with green petal of *Fragaria x ananassa* (strawberry) in Canada; with yellows disease of *Vitis vinifera* (grapevine) in Germany; and with witches' broom disease of *Olea europaea* (olive) in Italy (Lee et al., 2004). Strains of this phytoplasma subgroup have also been detected in gramineous plants, *Poa pratensis* and *Festuca arundinacea*, in Lithuania (Valiūnas, 2003, Valiūnas et al. in press), as well as in virescence–diseased *Anemone coronaria* and phyllody–diseased *Ranunculus* in Italy (Lee et al., 2004). Results from the present study add *Gaillardia* to the list of plants harboring a subgroup 16SrI–C phytoplasma.

16S rDNA sequence similarity and putative restriction site analysis. Amplified fragments (1.2 kb in size) of two heterogeneous rRNA operons (rrn) from GaiY phytoplasma were cloned and sequenced. The sequence data were deposited in the GenBank database under accession nos. EF583065 (GaiY rrnA) and EF583066 (GaiY rrnB). The putative restriction site maps of 16S rDNAs from both GaiY rrn operons were expectedly in agreement with the results from the enzymatic RFLP analysis (data not shown).

Alignments of nucleotide sequences (three clones from each amplicon were sequenced) revealed that the 1.2 kb GaiY rrnA sequence differed from the corresponding sequence of CPh rrnA (reference strain of 16SrI-C phytoplasma subgroup) by 2 positions and shared 99.5% sequence similarity. The rrnB sequences from GaiY and CPh phytoplasmas differed by 1 base and shared 99.9% sequence similarity. The alignment of rrnA and rrnB of GaiY phytoplasma revealed that they differ in 3 base positions.

Phylogenetic analysis. A phylogenetic tree of phytoplasma subgroup 16SrI–C strains and other phytoplasma strains was constructed on the basis of 16S r RNA gene sequences to visualize relationships of the GaiY phytoplasma with other strains (Fig. 4). The branching order of the tree confirmed that GaiY phytoplasma was related to CPh phytoplasma classified in phytoplasma subgroup 16SrI–C. All 16S rDNA sequences derived from 16SrI–C phytoplasma subgroup formed a distinct branch in the group 16SrI clade, underscoring their distinctness from other strains in the group.

CONCLUSIONS

1. *Gaillardia* viral disease expressed by plant stunting, flower breaking and malformation of petals is associated with *Cucumber mosaic cucumovirus* identified by the methods of test–plant inoculations, electron microscopy, the double–diffusion serological test, and RT–PCR.

2. *Gaillardia* yellows disease is associated with a phytoplasma (strain GaiY) belonging to phytoplasma group 16SrI (aster yellows phytoplasma group), subgroup 16SrI–C (clover phyllody, CPh, phytoplasma subgroup).

3. 16S rDNA sequence analysis confirmed that GaiY phytoplasma is closely related to CPh phytoplasma and is appropriately classified in phytoplasma subgroup 16SrI–C.

4. In Lithuania, *Gaillardia* can be naturally infected by a broad biodiversity of phytoplasma species, belonging to subgroups 16SrIII–B, 16SrI–A and 16SrI–C, as well as by *Cucumber mosaic cucumovirus*.

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