Multilocus typing for characterization of ‘Candidatus Phytoplasma asteris’-related strains in several ornamental species in Italy

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Abstract

Different ornamental plants showing symptoms indicating phytoplasma presence were collected between 1993 and 2016 in various floricultural areas in north and south of Italy, including Sicily. These samples were determined to be infected by '*Candidatus* Phytoplasma asteris'-related strains, and after PCR/RFLP identification based on the 16Sr RNA\gene were assigned to the 16SrI-B subgroup. These infected samples were employed for phytoplasma strain differentiation on *tuf*, *groel*, *rp* and *amp* genes. Phytoplasma strains were from hydrangea, primula, *Centaurium erythraea,* petunia and gerbera samples showing flower virescence; from gladiolus samples both *in vivo* and in micropropagation showing the "germs fins" symptomatology, from statice with stunting and lack of flower production, from ranunculus and carnation with virescence and malformation of flowers. All the genes were amplified in nested PCR except the *amp* gene. For the *tuf* gene all samples resulted amplified, and *Tru1*I RFLP analyses confirmed identical profiles with those of 16SrI group phytoplasmas. However, for the other genes only samples from ranunculus, gladiolus *in vivo*, statice and hydrangea were amplified. For these genes the phytoplasmas were identical to each other and to reference strains belonging to 16SrI-B subgroups; RFLP analyses with *Tru1*I and *Alu*I further indicated placement in the rpI-B and GroELI-III groups. Considering that these samples have been collected in different Italian regions during 23 years, the relevant conservation in the studied genotypes can perhaps be linked to the presence of common leafhopper vectors, not always identified nor detected in the cultivation areas where the diseased plants were collected. It is important to highlight that '*Ca*. P. asteris' is the prevalent phytoplasma reported in flower cultivations worldwide, and its lack of genetic polymorphisms may also indicate a globalized trading of the pathogen together with its propagation material.

Keywords: PCR/RFLP analyses, *16S rRNA* gene, *rp* gene, *GroEl* gene, *tuf* gene

INTRODUCTION

Phytoplasmas are bacteria present worldwide in several plant species (Bertaccini et al., 2014), but they are most common and recognized as pathogens in ornamental plants since they dramatically reduce their value as both plants and cut flowers. Symptoms include virescence and phyllody that severely modify the colour, the shape and the behaviour of infected plants. Among the different phytoplasmas those affiliated with 16SrI-B ('*Candidatus*

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Phytoplasma asteris' commonly known also as aster yellows phytoplasma) (Lee et al., 2004) are most reported in ornamental plants. Therefore, in a period of 23 years (1993-2016) a study was undertaken in a number of diverse species reported to be infected by this phytoplasma in diverse geographic areas of Italy.

The purpose of this study was to verify the possible presence of genetic variability in the 16SrI-B phytoplasmas in Italy by using a multilocus typing approach on genes other than the *16S rRNA*, that is employed for general phytoplasma classification. This characterization could be helpful in epidemiologic studies aimed to reduce the impact of phytoplasma diseases and to verify their transmission pathways in Italy.

MATERIALS AND METHODS

Samples

Samples from different ornamental plant species showing symptoms indicating phytoplasma presence were collected between 1993 and 2016 in various floricultural areas in north and south Italy. In particular, the 23 phytoplasma strains employed were from hydrangea (*Hydrangea macrophylla* L.) (5), *Primula* spp. (3), *Centaurium erythraea* (2), *Petunia* spp. (2) and gerbera (*Gerbera jamesonii* L.) (1) samples showing flower virescence; from *Gladiolus* spp. samples both *in vivo* and in micropropagation (2) showing the "germs fins" symptomatology; from statice (*Limonium sinuatum* L.) (2) with stunting and lack of flower production collected in a greenhouse located in Sicily (Southern Italy) (Figure 1); from *Ranunculus asiaticus* L. (2) and carnation (*Dianthus barbatus* L.) (4) with virescence and flower malformation.

Figure 1. *L. sinuatum* plants in greenhouse showing symptoms of lack of flower, stunting and malformation due to the presence of aster yellows phytoplasmas (16SrI-B).

Phytoplasma detection and ribosomal subgroup identification

Nucleic acid extraction from frozen or fresh samples used a chloroform/phenol extraction (Prince et al., 1993) followed by nested-PCR analyses with previously reported procedures (Duduk et al., 2013). In particular, for samples in which the phytoplasma detection is reported here for the first time (Table 2), direct PCR assays used 20 ng of template DNA with primers R16F2n/R2 (Gundersen and Lee, 1996) followed by primers R16(I)F1/R1 (Lee et al., 1994) in nested PCR assays, using amplicons diluted 1:30 with sterile distilled water as template was used. A tube with reaction mixture devoid of DNA template was included as negative control in each PCR reaction. PCR amplifications were performed following reported parameters (Schaff et al., 1992) and products were electrophoresed in 1.0% agarose gel; the gel was stained with ethidium bromide and visualised under UV light. RFLP analyses with *Tru1*I and *Hha*I (Fermentas, Vilnius, Lithuania) restriction enzymes following the manufacturer's instructions were then carried out on positive samples; the RFLP products were separated by electrophoresis in 6.7% polyacrylamide gels, and visualised with an UV transilluminator.

Multilocus RFLP characterization

Amplification of the samples with direct or nested PCR yielded positive results after amplification of the *16S rRNA* gene was carried on *tuf* gene amplifying primers (Makarova et al. 2012) and on *groel* and *rp* genes in nested PCR with primer combinations reported in Table 1. The amplification of *amp* gene was achieved in direct PCR with primers listed in Table 1. Amplicons of the expected length amplified from samples and reference strains NJ-AY (New Jersey aster yellows, 16SrI-A), AY-J (aster yellows from France, 16SrI-B) and AY-27 (aster yellows from Canada, 16SrI-B) from the micropropagated phytoplasma collection (Bertaccini, 2015) were obtained using the amplification conditions reported in the respective literature (Table 1). They were then examined by RFLP analyses as reported above with selected informative enzymes and restriction profiles were compared with those available in the literature.

Table 1. Primers and primer combination employed for the multilocus analyses of phytoplasma molecular diversity.

		Direct PCR	
Gene	Primer	Sequence 5'-3'	Literature
rp	rpF1	GGACATAAGTTAGGTGAATTT	Lim and Sears, 1992
	rpR1	ACGATATTTAGTTCTTTTTGG	
amp	Amp-N1	AAGAATTCCATATGCAAAATCAAAAAACTCA	Kakizawa et al., 2004
	Amp-C1	AAGAGCTCGAGTTATTTATTGTTTTTGTTTTTTTTAAC	
groEL	AYgroelF	GGCAAAGAAGCAAGAAAAG	Mitrović et al., 2011
	AYgroelR	TTTAAGGGTTGTAAAAGTTG	
		Nested PCR	
rp	rp(I)F1A	TTTTCCCCTACACGTACTTA	Lee et al., 2004
	rp(I)R1A	GTTCTTTTTGGCATTAACAT	
groEL	AYgroesF	ATCAGAAAAAGAAAAATCCT	Mitrović et al., 2011a
	AYampR	GCAACAGCAGCAAATAAAAC	

RESULTS AND DISCUSSION

All samples in this study were determined to be infected by aster yellows strains that after PCR/RFLP analyses on *16S rRNA* gene were assigned to 16SrI-B subgroup (Table 2). All

23 samples also were positive after amplification of *tuf* gene, and yielded identical RFLP results for this gene (data not shown). However, only some of the tested species were positive for amplification of the other genes (Table 2). In particular, samples of statice, gladiolus, ranunculus and hydrangea were amplified with *amp*, *rp* and *groEl* gene primers, whereas primula samples were only amplified with *groEl* gene primers. In contrast, samples of *C. erythraea* and *D. barbatus* were only amplified with primers for the *rp* gene. Results from primers for the other genes do not appear related to the year of collection (storage time) nor to the diversity of phytoplasmas in the other amplified genes. RFLP analyses show that no differences were present among the detected strains allowing to group all the studied aster yellows strains into the same multilocus type cluster (Figure 2). Overall the obtained profiles demonstrate high genetic RFLP homogeneity among all the strains studied. RFLP results were indistinguishable among all samples in this study and the reference strain AY-27 in periwinkle originally collected in field infected samples in Canada.

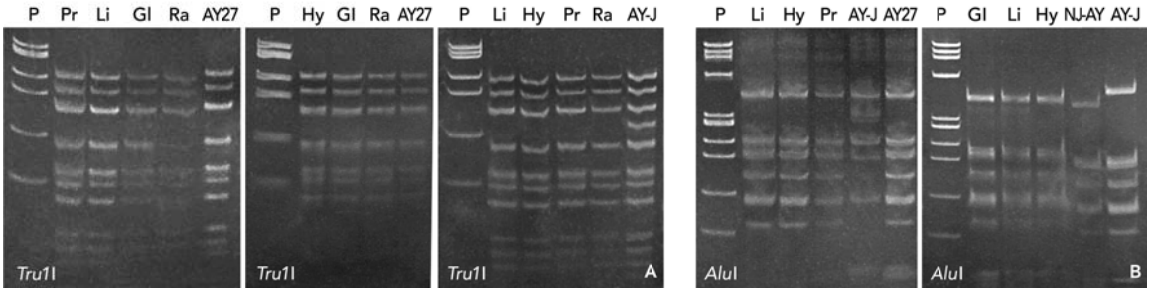

Figure 2. RFLP analyses on 6.7% polyacrylamide gels of *groel* gene amplicons obtained after nested-PCR on some of the samples analyzed. The restriction enzymes used are indicated at the bottom. Acronyms of samples: Pr, *Primula* spp.; Li, *L. sinuatum*; Gl, *Gladiolus* spp.; Ra, *Ranunculus* spp., Hy, *Hydrangea* spp. Reference strains AY27, aster yellows from lettuce from Canada; AY-J, aster yellows from France; and NJ-AY, New Jersey aster yellows. In A marker P, phiX174 *Hae*III-digested with fragment sizes, from top to bottom, of 310, 281, 271, 234, 194, 118 and 72 bp in B marker P, marker phiX174 *Hae*III-digested with fragment sizes, from top to bottom, of 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp.

However, the RFLP characterizations carried out on the *amp* gene by *Tru1*I allowed to determine that the profiles of all samples collected in Italy were identical to each other, but were different from all the reference strains used. Considering that the strains positive on this gene (statice, hydrangea, gladiolus and ranunculus) were collected in different regions of Italy i.e Sicily, Campania, Tuscany and Liguria, respectively, and over more than 20 years, some epidemiologic considerations are suggested. The phytoplasma membrane proteins have important roles in the attachment to the host cell, and amp coded proteins were suggested as candidates for involvement in host-phytoplasma interactions; considering their high expression especially in aster yellows phytoplasmas (Kakizawa *et al.*, 2004) they may also determine insect specificity (Suzuki *et al.*, 2006). Phytoplasmas' amp localized in the microfilaments of the insect's intestine in aster yellows (strain OY) was shown in literature to form a complex with insect proteins actin and myosin. In addition, the formation of amp–microfilament complexes was correlated with the phytoplasma-transmission capability of leafhoppers, suggesting that the interaction plays a major role in determining the transmissibility of phytoplasmas (Suzuki *et al.*, 2006). The finding of no variability in this gene in the Italian samples indicates that there was very little phytoplasma

interaction with the environment including insect vectors. Considering the limited information available on insect vectors of phytoplasmas infecting ornamental plants in Italy and also worldwide, there is only a recent report of presence of aster yellows in *Laodelfax striatellus* detected on aster yellows infected hydrangeas (Bertaccini et al., 2015), it is very likely that the vegetative propagation of ornamental material could play an important role in maintaining the genetic homogeneity detected in the studied samples.

Table 2. Results of PCR/RFLP characterization on different genes for the ornamental plant samples studied and reference phytoplasma strains.

Plant species tested	**Year sampling**	***16S rRNA* subgroup***	***tuf* gene****	***rp* gene§**	***amp* gene°**	***groEL* gene°°**	**Literature**
Limonium sinuatum	2015	I-B	AY	B	Ita	III	This work
Hydrangea macrophylla	2014	I-B	AY	B	Ita	III	This work
Primula spp.	1998	I-B	AY	Nd	Nd	III	This work
Centaurium erythraea	1998	I-B	AY	B	Nd	Nd	Contaldo et al., 2010
Petunia spp.	1998	I-B	AY	Nd	Nd	Nd	This work
Dianthus barbatus	1996	I-B	AY	B	Nd	Nd	This work
Gerbera jamesonii	1996	I-B	AY	Nd	Nd	Nd	Bertaccini e Bellardi, 1998
Gladiolus spp.	1993	I-B	AY	Nd	Ita	III	Bertaccini et al., 1992
Ranunculus asiaticus	1993	I-B	AY	B	Ita	III	Bertaccini et al. 1993
NJ-AY	From collection	I-A	AY	A	Usa	I	Lee et al., 1998
AY-J	From collection	I-B	AY	Nt	Fr	IV	Mitrović et al., 2011
AY-27	From collection	I-B	AY	Nt	Can	III	This work

*, ribosomal subgroups are reported by Lee et al. (1998); **, AY, tuf gene profile referable to aster yellows; §, identical letter correspond to identical RFLP profile, °*, Ita=Italy; Usa=United States of America; Fr=France; Can=Canada;°°, roman numbers correspond to different profiles as reported in Mitrović et al., 2011. Nd, not determined.

CONCLUSIONS

The results of this research shows that there is a tremendous need for further study of epidemiology of phytoplasma diseases in ornamental plants, especially considering the continuous increase of new symptomatic species (Davino et al., 2007; Paltrinieri et al., 2015). The 16SrI-B phytoplasmas identified in several ornamental plants in Italy were also detected in *Laodelfax striatellus* on hydrangea with virescence (Bertaccini et al., 2015), but no further information is available on insect vectors of these phytoplasmas in the different

environments in which the infected samples were collected. It is also possible to hypothesize that given the genetic homogeneity of detected strains over two decades, the main mode of transmission is the vegetative propagation of asymptomatic infected materials. However, it is also possible that in some cases seed transmission could have a role in the epidemiology of these diseases as reported for other phytoplasma associated diseases (Calari et al., 2011, Satta et al., 2016). More investigations are needed and some are in progress (Contaldo et al., 2012; 2016) to increase the epidemiologic knowledge about these diseases and propose adequate management measures towards reducing the risk of severe losses due to epidemic outbreaks.

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