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A new grapevine yellows phytoplasma from the Buckland Valley of Victoria, Australia

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Summary

A new phytoplasma detected in grapevines with grapevine yellows disease from the Buckland Valley of Victoria, Australia was characterized. Buckland Valley grapevine yellows phytoplasma (BVGYp) could not be amplified by PCR using primers specific for the stolbur (STOL, 16SrXII) group of phytoplasmas indicating that it was unlikely to be a STOL group phytoplasma. BVGYp was amplified by PCR using primers specific for both the aster yellows (AY, 16Sr I) and STOL phytoplasma groups, indicating that it may be more closely related to the AY group phytoplasmas. Sequence analysis of 16SrRNA gene sequences showed that BVGYp clustered with AY and STOL groups of phytoplasmas. Sequence similarities were determined by pairwise comparisons of the 16S rDNA sequence of BVGYp to AY and STOL group phytoplasmas and BVGYp was more closely related to the AY group phytoplasmas. Although the data indicate BVGYp may form a new AY subgroup, the similarity coefficients between BVGYp and phytoplasmas from the AY, STOL and Mexican periwinkle virescence groups, derived from putative RFLP patterns, were less than 90 %, so BVGYp may actually form a new phytoplasma group.

K e y w o r d s : grapevine yellows, phytoplasma, Australia.

Introduction

Phytoplasmas have been associated with yellows diseases of grapevines in many viticultural regions worldwide. Molecular techniques have shown that there are several distinct grapevine yellows phytoplasmas from different regions that represent various phylogenetic groups including the aster yellows (AY, 16Sr I), faba bean phyllody (FBP, 16SrII), Western X (16Sr III), Elm yellows (EY, 16Sr V) and Stolbur (STOL, 16Sr XII) phytoplasma groups (Bianco *et al.* 1993; Daire *et al.* 1993; Prince *et al.* 1993; Chen *et al.* 1994; Daire *et al.* 1994; Davis and Prince 1994; Arzone *et al.* 1995; Maixner *et al.* 1995; Padovan *et al.* 1996; Davis *et al.* 1997, 1998; Lee *et al.* 1998; Gibb *et al.* 1999).

Three phytoplasmas, differentiated by sequence analysis and restriction enzyme analysis of the 16S rRNA gene and the 16S-23S spacer region, have been detected in Australian grapevine yellows affected grapevines (Constable *et al.* 1998; Gibb *et al.* 1999). These phytoplasmas include the Australian grapevine yellows phytoplasma (AGYp), of the STOL group of phytoplasmas, and the Australian tomato big bud phytoplasma (TBBp), of the FBP group of phytoplasmas. The third, uncharacterized, phytoplasma was detected in Chardonnay grapevines with the grapevine yellows disease (GYd) from one vineyard in the Buckland Valley of north eastern Victoria, Australia (Gibb *et al.* 1999). Restriction fragment length polymorphism (RFLP) analysis of the DNA amplified by PCR from the Buckland Valley grapevine yellows phytoplasma (BVGYp) and AGYp indicated that the two phytoplasmas were closely related but distinguishable and that BVGYp may be a variant of AGYp (Gibb *et al.* 1999).

The aim of this study was to further characterize BVGYp using polymerase chain reaction (PCR) techniques and DNA sequencing of the 16S rRNA gene and the 16S rRNA/23S rRNA spacer region.

Material and Methods

S o u r c e o f p h y t o p l a s m a s : Samples from 9 grapevines with GYd and three symptomless grapevines were collected from three blocks of Chardonnay in a vineyard in the Buckland Valley of Victoria. Samples from grapevines that tested positive for AGYp or TBBp were collected from the Sunraysia district of northwestern Victoria.

E x t r a c t i o n o f D N A f r o m g r a p e v i n e : Leaf veins, petioles and scrapings of vascular tissue from stems were used for DNA extractions from shoots. DNA was extracted from each sample using a DNeasy plant DNA extraction kit (Qiagen, Inc. Mississauga, ON, USA) according to the method of Green *et al.* (1999).

P r i m e r s f o r a m p l i f i c a t i o n o f p h y t o p l a s m a D N A i n g r a p e v i n e : A nested PCR procedure was used with the first primer pair fP1 (Deng and Hiruki 1991) and rP7 (Schneider *et al*. 1995) and the nested primer pair R16F2n (Gundersen and Lee 1996) and m23SR (Padovan *et al.* 1995) for universal detection of phytoplasmas in grapevines. These primers are specific for a region of the 16S rRNA and 23S rRNA genes in all known phytoplasmas. The primer pair ftufAY and rtufAY (Schneider *et al.* 1997) was used to amplify the gene coding for the elongation factor Tu (*tuf*). This pair is specific to members of the AY and STOL phytoplasma groups, and was also used for detection of phytoplasmas. The primer pair fstol and rstol (Maixner

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et al. 1995) was used in nested PCR to amplify a region of the 16S rRNA and 16S-23S spacer region, specifically from the STOL group of phytoplasmas.

Polymerase Chain Reaction (PCR): For PCR, each reaction contained 0.2 mM of each dNTP, 0.625 µM of each primer, 1.5mM $MgCl_2$, 1xDNA polymerase buffer supplied with the enzyme and 2.5 U thermostable *Taq* DNA polymerase (Gibco/BRL, Life Technologies, Rockville MD, USA). For the first round or single PCR, 0.5 µl of undiluted or 1/10 diluted nucleic acid sample was added to the PCR mixture. For the nested PCR, 0.5 µl of the first round PCR reaction mixture was added to the PCR mixture containing the second primer pair. The total reaction volume was 20 µl in an MJM PTC-100 thermocycler (Geneworks, Adelaide, SA, Australia). For the first round of the nested PCR test with primer pair P1/P7, a hot start at 94 °C for 1 min was followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1.5 min. PCR test conditions were the same for the second round of nested PCR with the generic primer pairs R16F2n/m23SR. The annealing temperature was 58 °C for the second round of nested PCR using the primer pair fstol/rstol. Single PCR test conditions with primer pair ftufAY/rtufAY were also the same except that the annealing temperature was 52 °C. Total nucleic acids, extracted from asymptomatic grapevines, were subjected to the PCR as a negative control and water controls were included, in which no plant nucleic acid was added to the PCR mix.

After PCR amplification, 5 µl from each sample was subjected to electrophoresis in a 1 % agarose gel using 0.5xTBE (0.045M Tris-borate, 1mM EDTA, pH 8.0) running buffer. Products in gels were stained with ethidium bromide and visualised by UV transillumination. DNA markers were pUC19/*Hpa*II and SPP-1 Phage DNA/*Eco* RI markers (GeneWorks).

Restriction fragment length polymorphism (RFLP) analysis: The endonuclease *Hpa*II distinguishes BVGYp from AGYp and TBBp (GIBB *et al.* 1999) and was used to confirm the presence of BVGYp in the grapevine samples from the Buckland Valley that were positive by nested PCR of the 16SrRNA gene and the 16S-23S spacer region. More information about the differences between BVGYp and AGYp was obtained by RFLP analysis using the endonucleases *Alu*I, *Hpa*II and *Rsa*I to digest the *tuf* gene PCR product. 5 µl of the nested PCR product or *tuf* gene product were digested using the restriction endonucleases in the buffer supplied by the manufacturer (New England Biolabs, Beverly, MA, USA). Digestions were incubated overnight at 37 °C and the fragments were separated by electrophoresis in an 8 % acrylamide gel or 1.2 % agaose gel in 0.5xTBE buffer and visualized by staining with ethidium bromide and photographed on a UV transilluminator.

PCR amplification of DNA for sequencing: DNA was amplified for sequencing from BVGYp by nested PCR with the first round primer pair fP1/rP7 and the nested primer pair p25f and m23SR as described above. The primer p25f (5'-AGG ATT AAC GCT GGC G-3') was designed so that a nested PCR product could be amplified that included more of the 16S rRNA gene at the 5' end of the gene. After PCR amplification the PCR products were run on a 1 % agarose gel in 0.5xTBE and gel purified using an UltraClean™ Gelspin DNA purification kit (Mo Bio Laboratories, Inc., Solana Beach CA, USA) according to the manufacturer's instructions.

Sequencing directly from PCR products: PCR products of the 16SrRNA gene from the new phytoplasma were sequenced using the T7 Sequenase PCR Sequencing Kit (Amersham Bioseciences, Uppsala, Sweden) according to the manufacturer's directions. Two isolates of BVGYp were sequenced, and sequences of both strands of DNA from each isolate were determined at least twice. The primers used for sequencing are listed in Tab. 1.

Sequencing cloned PCR products: When regions of the PCR products could not be sequenced directly, these regions of DNA were amplified by PCR, gel-purified as described above and cloned. The primer p593r (5'-CCT ACG CAC CCT TTA CGC C-3') was designed and used with the primer p25f to amplify a 568 bp region of phytoplasma DNA near the 5' end of the 16S rRNA gene.

Table 1

PCR primers used for sequencing the BVGYp 16S rRNA gene and 16S-23S spacer region sequences

Primer name (direction)	Primer sequence	Reference
R16F2n (forward)	5'-GAA ACG ACT GCT AAG ACT GG-3'	GUNDERSEN *et al.* 1996
fU5 (forward)	5'-CGG CAA TGG AGG AAA CT-3'	LORENZ *et al.* 1995
ng (forward)	5'-AGG CGG CTT GCT GGG TCT T-3'	LIEFTING *et al.* 1996
fU3 (forward)	5'-TGT TAC AAA GAG TAG CTG AA-3'	LORENZ *et al.* 1995
r8 (reverse)	5'-CTC GTT GCG GGA CTT AAC CC-3'	LIEFTING *et al.* 1996
rU3 (reverse)	5'-TTC AGC TAC TCT TTG TAA CA-3'	LORENZ *et al.* 1995
rP3 (reverse) - reverse of fP3	5'-AAG GAG GTG ATC CAT CC-3'	SCHNEIDER *et al.* 1995
m23SR (reverse)	5'-TAG TGC CAA GGC ATC CAC TGT G-3'	PADOVAN *et al.* 1995
M13f (forward)	5'-GTT TTC CCA GTC ACG ACG TTG TA-3'	
M13r (reverse)	5'-TTG TGA GCG GAT AAC AAT TTC-3'	

The primer pair fP3 and m23SR was used to amplify a region of phytoplasma DNA, approximately 350 bp, which incorporated the 16S-23S spacer region. PCR was done as described above except that the annealing temperature for the p25f/p593r primers was 49 ºC. The amplified products were cloned using the pGEM®-T Easy Vector System I (Promega, Madison, WI, USA) according to the manufacturers' instructions and transformed into competent *Escherichia coli* TG1 cells (HANAHAN 1983).

Cloned PCR products of the 16SrRNA gene and the 16S-23S spacer region from the new phytoplasma were sequenced using the T7 Sequenase Sequencing Kit (Amersham Pharmacia Biotech) according to the manufacturers' directions. Two isolates of the new phytoplasma were sequenced in two directions and sequences were determined at least twice. The primers used were those provided with the sequencing kit.

The reaction mixtures from direct sequencing of PCR products or sequencing of cloned DNA were denatured and run on denaturing polyacrylamide gels (6 % (w/v) acrylamide, 0.3 % (w/v) bisacrylamide, 7 M urea) in 1xTBE at constant power for 3-5 h. Prior to loading the reaction mixtures the gels were pre-electrophoresed at 50 V until the gel temperature was approximately 50 °C.

Sequence analysis of the 16S rRNA gene and the 16S-23S spacer region: Pairwise comparisons of the 16S rRNA gene and 16S-23S spacer region sequences of the new phyto-plasma were each compared to several other phytoplasmas using Gap (gap weight 5.0 and length weight 0.3, Genetics Computer Group Inc., Madison, Wisconsin, USA), to calculate percentage sequence similarity. The 16S rRNA gene sequence of the new phytoplasma was aligned with other phytoplasma 16S rRNA gene sequences representing the major phylogenetic groups using EclustalW (THOMPSON *et al.* 1994). The aligned sequences were analysed by the program DNAdist (FELSENSTEIN 1989), which generates a matrix of the pairwise evolutionary distances between aligned sequences using the Kimura model of nucleotide substitution. The program Neighbour (FELSENSTEIN 1989) was used to calculate a phylogenetic tree from the distance matrix using the neighbour joining method. The tree was displayed using the TreeView program (PAGE 1996). The phytoplasmas used for the sequence analyses of the 16S rRNA gene sequence and for the 16S-23S spacer region are listed in Tabs 2 and 3, respectively.

Theoretical restriction fragment length polymorphism (RFLP) patterns of 16S rDNA sequence from BVGYp, AAYp, ACLRp, BBp, CPhp, PaWBp, SAYp, MPVp, STOLp and AGYp between the primer pair R16F2n and R16R2 (LEE *et al.* 1993) were predicted using the program Mapsort (Genetics Computer Group Inc.) for 17 restriction endonucleases. The

Table 2

A list of the phytoplasma strains, origins and accession numbers for the 16S rRNA gene

Abbreviation*)	Name	16Sr group	Origin	Accession number
AAYp	American aster yellows	16SrIB	USA	X68373
ACLRp	Apricot chlorotic leafroll	16Sr IF	Spain	X68338
AGYp	Australian grapevine yellows	16Sr XIIB	Australia	X95706
AshYp	Ash yellows	16Sr VIIA	USA	X68339
ATp	Apple proliferation	16Sr XA	Germany	X68375
BBp	Tomato big bud	16Sr IA	USA	L33760
BGWLp	Bermuda grass white leaf	16Sr XIV	Italy	Y16388
CPp	Clover proliferation	16Sr VIA	Canada	L33761
CPhp	Clover phyllody	16Sr IC	Canada	AF222065
EYp	Elm yellows	16Sr VA	USA	L33763
HibWBp	Hibiscus witches' broom	16Sr XV	Brasil	AF147708
LfWBp	Loofah witches' broom	16Sr VIII	Taiwan	L33764
LYp	Coconut lethal yellows	16Sr IVA	USA	U18747
MPVp	Mexican periwinkle virescence	16Sr XIII	Mexico	AF248960
PaWBp	Paulownia witches' broom	16Sr ID	Taiwan	AF279271
PnWBp	Peanut witches broom	16Sr IIA	Taiwan	L33765
PPWBp	Pigeon pea witches' broom	16Sr IXA	USA	U18763
PYLp	Phormium yellow leaf	16Sr XIIB	New Zealand	U43570
RYDp	Rice yellow dwarf	16Sr XIA	Japan	D12581
SAYp	Western aster yellows	16Sr IB	USA	M86340
STOLp	Stolbur	16Sr XIIA	Serbia	X76427
TBBp	Tomato big bud	16Sr IIB	Australia	Y08173
VKp	German grapevine yellows	16Sr XIIA	Germany	X76428
WXp	Western X-disease	16Sr IIIA	USA	L04682

*) Throughout the text, tables and figures "p" after the phytoplasma strain abbreviation refers to phytoplasma.

Table 3

A list of the phytoplasma strains, origins and accession numbers for the 16S-23S spacer region

Abbreviation	Name	16Sr group	Origin	Accession number
AGYp	Australian grapevine yellows	16Sr XIIB	Australia	X95706
BBp	Tomato big bud	16Sr IA	USA	AF222064
CPhp	Clover phyllody	16Sr IC	Canada	AF222065
FPWBp	Florida periwinkle witches' broom		USA	AF025426
MPVp	Mexican periwinkle virescence	16Sr XIIIA	Mexico	AF025428
SAYp	Western aster yellows	16Sr IA	USA	M86340
STOLp	Stolbur	16Sr XIIA	France	AF035361
VKp	German grapevine yellows	16Sr XIIA	Germany	AF035362

endonucleases were *Alu*I, *Bam*HI, *Bfa*I, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mse*I, *Rsa*I, *Sau*3A I, *Ssp*I, *Taq*I and *Tha*I. The predicted 16S rDNA RFLP patterns of BVGYp were compared to those of the other phytoplasmas and the similarity coefficient (F) was determined using the method of NEI and LI (1979).

EclustalW, DNAdist, Neighbour, Gap and Mapsort were accessed through BioManager by the Australian National Genomic Information Service, Sydney, Australia.

Results

Detection of BVGYp by nested PCR and RFLP: The phytoplasma 16S rDNA gene and 16S-23S spacer region were amplified from all 9 GYd-affected grapevine samples by nested PCR using the primer pairs fP1 and rP7 followed by R16F2n and m23SR (data not shown). The restriction profiles generated by digestion of the PCR products with *Hpa*II were the same as the profile described previously for BVGYp (GIBB *et al.* 1999, data not shown). No amplification products were observed from symptomless grapevines.

Detection of BVGYp using group specific PCR primers and RFLP: BVGYp was not amplified in PCR tests in any of the 9 samples from the Buckland Valley using the primer pair fstol/rstol (data not shown). BVGYp was amplified in PCR tests in all 9 samples using the fTufAy/rTufAy primers and the product was of the expected size (approx.1000 bp; Fig. 1). RFLP analysis using the endonucleases *Alu*I, *Hpa*II and *Rsa*I of the *tuf* gene showed that BVGYp was distinguishable from AGYp (Fig. 2).

Sequence analysis of the 16S rRNA gene and spacer region: A pileup tree guide was determined from the sequence analysis of the 16S rRNA gene of BVGYp (accession number AY083605) and showed BVGYp clustering with the AY and STOL phytoplasma group (Fig. 3). Gap analyses indicated that the greatest sequence similarity was between BVGYp and CPhp (97.1 %, Tab. 4). Sequence analysis of the 16S-23S spacer region indicated that BVGYp had greatest sequence similarity to AGYp (89.0 %, Tab. 5).

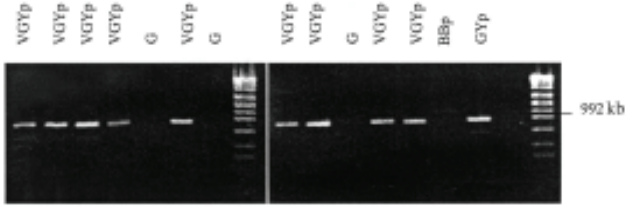

Fig. 1: PCR amplification of the *tuf* gene using the primer pair ftufAY/rtufAY. The PCR product was approximately 1000 bp. BVGYp = Buckland Valley grapevine yellows phytoplasma, HG = symptomless grapevine, TBBp = tomato big bud phytoplasma, AGYp = Australian grapevine yellows phytoplasma, C = water control and M = DNA marker (SPP-1 Phage DNA/*Eco* RI).

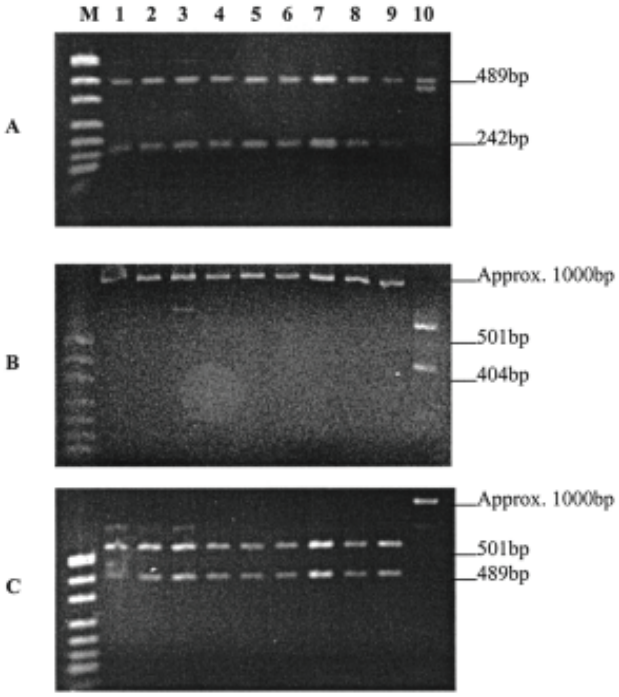

Fig. 2: RFLP analysis of the *tuf* gene product amplified by the primer pair fTufAy/rTufAy using the endonucleases *Alu*I (**A**), *Hpa*II (**B**) and *Rsa*I (**C**). Lanes 1-9 = BVGYp, lane 10 = AGYp and M = pUC19/*Hpa*II markers.

When BVGYp was compared to other phytoplasmas the similarity coefficient (F) derived from the comparison of predicted RFLP patterns of 16S rDNA sequences was 83 % with

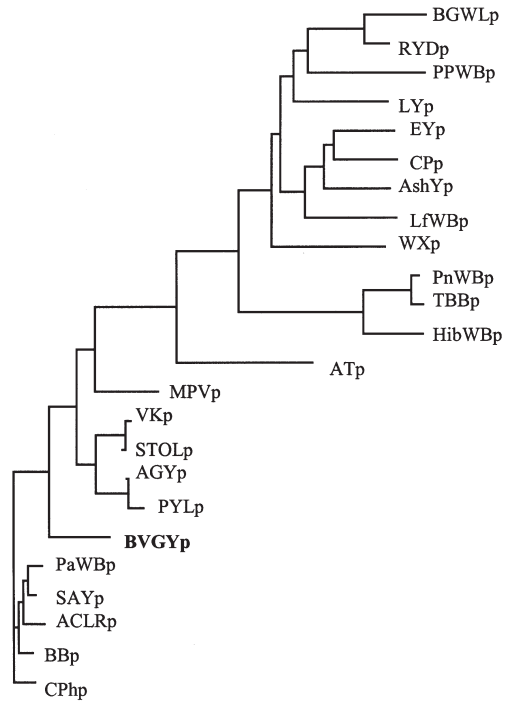

Fig. 3: A phylogenetic tree constructed from the pairwise analysis of an EClustalW alignment of selected phytoplasma 16S rDNA sequences. Abbreviations are given in Tab. 2.

CPhp, 80 % with BBp and SAYp, 79 % with AAYp, 77 % with STOLp, 76 % with ACLRp and PaWBp, 69 % with AGYp and 65 % with MPVp.

Phytoplasma signature sequence and unique 16S rRNA sequences: The sequence 5'-GUUGC-3', which is unique to members of AY group phytoplasmas (GUNDERSEN *et al.* 1994) and MPVp, was located at positions 1000-1004 of the BVGYp 16S rRNA sequence. In addition, the 16S rRNA gene of BVGYp contained sequences that had a unique arrangement of nucleotides (Tab. 6). Each of these sequences differed between 1-5 nucleotides with AY and STOL group phytoplasmas.

Discussion

In this study the relationship between BVGYp and other phytoplasma strains was analysed using group specific PCR tests, RFLP and sequence analysis. Results showed that BVGYp was most closely related to AY group phytoplasmas.

BVGYp DNA was not amplified by PCR using the primer pairs fstol/rstol, which amplify stolbur group phytoplasmas (GIBB *et al.* 1999). This indicated that the new phytoplasma was unlikely to be a member of the STOL group of phytoplasmas. However, PCR amplification with the fTufAy/rTufAy

Table 4

Sequence similarity (%) between the 16S rRNA genes of BVGYp and other phytoplasmas

	CPhp	SAYp	BBp	PaWBp	ACLRp	AGYp	VKp	STOLp	AAYp	MPVp
BVGYp	97.1	96.9	96.7	96.7	96.5	96.3	96.1	96.1	95.8	96.0
CPhp		99.3	99.3	99.0	98.8	96.8	96.5	96.6	98.1	96.1
SAYp			99.2	99.4	99.0	96.5	96.4	96.4	98.7	96.3
BBp				98.9	98.8	96.5	96.1	96.2	98.2	95.9
PaWBp					99.0	96.3	96.4	96.5	99.2	96.1
ACLRp						96.2	96.0	96.2	98.8	95.9
AGYp							97.5	97.8	95.5	96.1
VKp								99.5	95.3	96.5
STOLp									95.4	96.5
AAYp										95.0

Table 5

Sequence similarity (%) between the 16S-23S spacer region of BVGYp and other phytoplasmas

	AGYp	STOLp	VKp	FPWBp	MPVp	SAYp	BBp	CPhp
BVGYp	89.0	88.6	88.6	88.6	87.2	81.4	80.6	76.8
AGYp		94.9	94.9	94.0	93.0	92.3	92.3	91.0
STOLp			99.5	95.8	93.5	92.5	92.1	88.8
VKp				95.8	93.5	92.5	92.1	88.8
FPWBp					98.2	92.5	92.1	88.8
MPVp						90.7	90.2	86.9
SAYp							97.3	95.0
BBp								93.9

Table 6

The presence of sequences in the 16S rRNA gene of the Buckland Valley grapevine yellows phytoplasma that had a unique arrangement of nucleotides

Sequence	Position
5'-CGCGTAACGAATCTGCCTCTAAG – 3'	86-108
5'-AGATAAGAAGGCATCTTCTTATTT-3'	150-173
5'-AGAGAGGAG-3'	201-209
5'-TAGAGTAAAAG-3'	233-243
5'-AAAAGTGGTGGAAAAACCATTCT-3'	429-451
5'-GCTAAGTA-3'	559-566
5'-ACGTTGGGTTAAACC-3'	801-815
5'-AAGCTTTAGAAACAAAGTG-3'	971-989
5'-AATTGCCAGCACGTTATGGT-3'	1084-1103
5'-TGAAACGTAAGTTCTTGGCAA-3'	1222-1242
5'-TTGCAAGAAGAGGGAGCC-3'	1413-1430

primers, which only amplifies AY and STOL group phytoplasmas (SCHNEIDER *et al.* 1997), did amplify a product of the expected size (1000 bp) from grapevine samples infected with BVGYp. This indicated that BVGYp was closely related to phytoplasmas of the AY group. RFLP analysis of the *tuf* gene provided further evidence the BVGYp was different to AGYp.

The AY group of phytoplasmas is the largest and most genetically diverse of all the phytoplasma groups. More than 7 subgroups have been described within the AY group based on comparative analyses of RFLP patterns and near full length sequences of the 16S rRNA gene and ribosomal protein genes (LEE *et al.* 1998; SEEMÜLLER *et al.* 1998). AY group phytoplasmas from both the 16SrIA (DAVIS *et al*. 1998) and 16SrIB (ALMA *et al.* 1996) subgroups have been detected in grapevines from the USA and Italy, respectively. Based on 16SrDNA sequence BVGYp was distinguishable from all AY group phytoplasmas and other phytoplasmas used for comparison in this study.

Sequence analysis of 16SrRNA gene sequences showed that BVGYp was closely related to members of the AY and STOL group phytoplasmas. Gap analyses for sequence similarity of the 16SrRNA gene showed that BVGYp had greatest sequence similarity with CPhp of the 16SrIC subgroup (97.1 %). When revising the classification scheme for phytoplasmas, LEE *et al.* (1998) noted that 16S rRNA sequence similarities were between 95-98 % between two subgroups of phytoplasmas, within a given 16Sr group. BVGYp could be considered an AY group phytoplasma since BVGYp contains greater sequence similarity to AY phytoplasmas (95.8-97.1 %) compared to STOL phytoplasmas (96.1-96.3 %). The BVGYp 16S rRNA also contains the sequence 5'-GUUGC-3', which is unique to AY group phytoplasmas (GUNDERSEN *et al.* 1994) and MPVp. Several regions of unique DNA sequence exist within the BVGYp 16S rRNA gene indicating that BVGYp may represent a new subgroup of the AY group phytoplasmas. Sequence analysis of the 16S-23S spacer region indicated a greater variability in this region compared to other sequences of the same region from different phytoplasmas and the greatest sequence similarity observed was with AGYp (89 %). This result also supports the hypothesis that BVGYp may represent a distinct subgroup of phytoplasmas.

The similarity coefficient of phytoplasmas, based on predicted restriction sites, also confirmed that BVGYp was distinct from all other phytoplasmas used in this study. The greatest similarity coefficient was between BVGYp and CPhp (83 %). Previous research has suggested that the similarity coefficient of RFLP patterns between two distinct groups of phytoplasmas is equal to or less than 90 % (LEE *et al.* 1998). This raises the possibility that BVGYp represents a new phytoplasma group rather than an AY subgroup.

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