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A new wilt disease of banana plants associated with phytoplasmas in Papua New Guinea (PNG)

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Abstract Nested PCR indicated a possible causal relationship between presence of phytoplasmas and unusual wilt symptoms in cooking banana plants in PNG. Sequence analysis showed that phytoplasmas from diseased banana plants in four Provinces were unique, but most closely related to a phytoplasma associated with a new lethal disease of coconuts in PNG's Madang Province, related to phytoplasmas in the 16SrIV group.

Keywords Phytoplasma · *Musa* sp. · Wilt disease · Papua New Guinea

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Since 1989, quarantine plant pathologists from PNG and Australia have regularly jointly surveyed PNG's border regions. These surveys (conducted in recent years by PNG's National Agricultural Quarantine and Inspection Authority (NAQIA) and Australia's Department of Agriculture, Fisheries and Forestry (DAFF)) have demonstrated freedom from some of the world's most devastating banana diseases. Investigating wilt—like problems of banana has always been a high priority and this is done by destructive sampling of any plants showing yellowing and leaf death followed by examination of internal psudostem and other tissues for characteristic symptoms of fusarium or bacterial wilt. Many cooking banana plants (ABB genome) obviously yellowing or dying (Fig. 1), have been investigated in this way and the symptoms seen were clearly not those of fusarium or bacterial wilt. However, no other clear explanation for decline could be found in these plants. Inside these pseudostems, discontinuous streaking was present, appearing as small regions of brown or black vascular tissues, usually with wet, necrotic pockets (Figs. 2, 3). In 2008, a sample of such material tested PCR positive for phytoplasma in the laboratory of PNG's Oil Palm Research Association (Carmel Pilotti OPRA, personal communication).

In response, NAQIA/DAFF conducted disease surveys of banana plants in Madang Province (MaP), Morobe Province (MoP), Milne Bay Province (MBP) and East Sepik Province (ESP) in 2009, and in Western Province (WP) and North Solomons Province (NSP) in 2010. Vascular samples were taken from the lower pseudostems of 16 yellowing and dying banana plants showing internal symptoms as described above (suspect phytoplasmas) and two associated

Fig. 1 External symptoms (leaf yellowing and leaf death) associated with BWAP isolates RID5519 in East Sepik Province (*left*) and RID5837 in Western Province (*centre*), and with unidentified phytoplasma isolate RID5861 in North Solomons Province (*right*)

suckers as well as from banana plants not showing such internal symptoms (controls). Some controls were symptomless and some were showing yellowing or leaf death. However, some problems were experienced with returning vascular material in good condition to Australia, especially from locations where refrigeration was not available. Only control DNA extracts from PNG that were known, from using 16S rDNA internal control primers, to be of excellent PCR integrity were included. Therefore, seven samples with no internal symptoms from PNG and five additional symptomless plants sourced from Australia served as controls. All details on test plants, tissue sample preparation and treatment, DNA extraction and PCR are provided in Table 1.

All 16 symptomatic banana plants plus the two associated suckers tested phytoplasma positive (Table 1). The seven control samples, collected from PNG with no internal symptoms, plus the five symptomless Australian controls, tested negative (Table 1). This suggests that phytoplasmas are consistently association with banana wilt disease, rather than just being opportunistic phloem inhabitants, sometimes present in diseased plants.

The 16S rRNA gene, 16S-23S spacer region and a part of the 23S rRNA gene and the ribosomal protein (rp) S19 (rps19), ribosomal protein L22 (rpl22), and ribosomal protein S3 (rps3) genes of the phytoplasmas from samples RID5365 and RID5376 were amplified using P1/P7 and the rpL2F3/rp(I)R1A primer pair (Martini et al. 2007), respectively. Platinum® *Taq* DNA Polymerase (Invitrogen, USA) was used according to the manufacturer's instructions except that the total reaction volume was 25 µl. Amplicons of expected size were obtained from both samples and were purified and cloned using the pGEM-T Easy Vector system according to the manufacturer's protocol (Promega, USA). Transformants were screened and selected using standard protocols. Three clones of each gene region were sequenced using primers SP6 and T7, with an ABI PRISM® BIGDYE™ Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, USA). Sequences of 16S rRNA gene, 16S-23S spacer region and a part of the 23S rRNA gene and the rp gene region of both samples were identical and have been deposited in Genbank (JN872863 and JN872864 Banana wilt associated phytoplasma: BWAP).

Fig. 2 Internal symptoms (discontinuous necrotic vascular streaks and pockets of rot and discolouration) associated with BWAP isolate RID5546 at Taman, Morobe Province, PNG

Fig. 3 Internal symptoms (discontinuous necrotic vascular streaks and adjacent necrosis) associated with unidentified phytoplasma isolate RID5545 at Taman, Morobe Province, PNG

Table 1 Banana plants sampled in 2009 and 2010, and phytoplasma test results obtained

Collection #	Location[A]	Genome: sub group/ (local name)	Symptoms[B]	PCR test result[C]	Phytoplasma identity[D]
RID 5365	Furan, MaP*	ABB: (Daru)	Death and yellowing of leaves, DVS and necrotic pockets	+	BWAP
RID 5376	Dugumur, MaP*	ABB: (Daru)	Death and yellowing of leaves, DVS and necrotic pockets	+	BWAP
RID 5586	Tobenham, MaP*	ABB: Kalapua	Death and yellowing of leaves, DVS and necrotic pockets, underdeveloped fruits	+	16SrII group
RID 5587	Tobenham, MaP*	ABB: Kalapua	None (sucker of RID 5586 plant)	+	Undetermined phytoplasma
RID 5602	Furan, MaP*	ABB (Daru)	Yellowing of leaves, minor DVS and necrotic pockets	+	Undetermined phytoplasma
RID 5519	Kjabraka No.1, ESP	ABB: (Daru)	Death and yellowing of leaves, DVS and necrotic pockets	+	BWAP
RID 5545	Taman, MoP	ABB: Kalapua	Death and yellowing of leaves, DVS and necrotic pockets	+	Undetermined phytoplasma
RID 5546	Taman, MoP	ABB: Kalapua	Death and yellowing of leaves, DVS and necrotic pockets	+	BWAP
RID 5561	Gabensis, MoP	ABB: Kalapua	Death and yellowing of leaves, minor DVS and necrotic pockets	+	Undetermined phytoplasma
RID 5565	Sangun, MoP	ABB: Kalapua	Death and yellowing of leaves, DVS and necrotic pockets	+	Undetermined phytoplasma
RID 5566	Sangun, MoP	ABB: Kalapua	Death and yellowing of leaves, very minor DVS and necrotic pockets (sucker of RID 5565 plant)	+	Undetermined phytoplasma
RID 5569	Bilibam, MoP	ABB: Kalapua	Death and yellowing of leaves, basal necrosis.	+	16SrVIII group
RID 5837	Debepari, WP	ABB: Kalapua	Death of leaves, DVS and necrotic pockets	+	BWAP
RID 5838	Debepari, WP	ABB: Kalapua	Yellowing of leaves, DVS and necrotic pockets	+	Undetermined phytoplasma
RID 5822	Wipim, WP	ABB (Goya)	Slight DVS and necrotic pockets, underdeveloped fruits	+	Undetermined phytoplasma
RID 5861	Buka, NSP	ABB: Kalapua	Yellowing of leaves, DVS	+	Undetermined phytoplasma
RID 5989	Sanakoba, NSP	ABB: Kalapua	Strong yellowing of leaves, DVS	+	Undetermined phytoplasma
RID 5990	Sanakoba, NSP	ABB: Kalapua	Strong yellowing of leaves, DVS	+	Undetermined phytoplasma
RID 5615	Daduai, MBP	ABB (Daru)	Death of leaves only[E]	–	
RID 5824	Wipim, WP	ABB (Goya)	Yellowing of leaves only[E]	–	
RID 5867	Buka, NSP	ABB: Kalapua	Yellowing of leaves only[E]	–	
RID 5941	Bana, NSP	ABB: Kalapua	None	–	
RID 5954	Dererevati, NSP	ABB: Kalapua	None	–	
RID 5985	Tabub, NSP	ABB: Kalapua	None	–	
RID 5986	Tabub, NSP	ABB: Kalapua	Yellowing of leaves only[E]	–	
RID 5854	Australia[F]	ABB: Kalapua (Rana)	None	–	
RID 5855	Australia[F]	ABB: Kalapua (Dwarf Kalapua)	None	–	
RID 5856	Australia[F]	ABB: Kalapua a (Kalapua No. 2)	None	–	
RID 5857	Australia[F]	ABB: Kalapua (Benganai)	None	–	
RID 5858	Australia[F]	ABB: Kalapua (Giant kalapua)	None	–	

[A] ESP: East Sepik Province, MaP: Madang Province, MBP: Milne Bay Province, MoP: Morobe Province, NSP: North Solomons Province (autonomous region of Bougainville), WP: Western Province. *: indicates banana plants cut down in an active epidemic of phytoplasma disease in coconut trees.

[B] DVS: Discontinuous vascular streaking

[C] Nested PCR test result: phytoplasmas detected (+) or phytoplasmas negative (−)

[D] BWAP: Banana wilt associated phytoplasma, based on cloning and sequencing of the 16S rRNA gene, the 16S-23S spacer region and part of the 23S rRNA gene and the ribosomal protein (rp) S19 (rps19), ribosomal protein L22 (rpl22), and ribosomal protein S3 (rps3) genes. Unidentified phytoplasma: Cloning and sequence analysis not conducted.

[E] Death and/or yellowing possibly attributable to other causes such as root problems.

[F] Banana plants originally obtained from PNG, now grown at the Queensland Department of Agriculture Fisheries and Forestry's germplasm collection at the South Johnstone Research Station.

Each sample consisted of approximately 0.5–1 g vascular tissues excised from immediately above and below obvious pockets of necrosis, when present. The material was chopped finely and rapidly desiccated in the field over anhydrous calcium chloride. Samples were stored at 4°C until fully desiccated, and at −20°C thereafter. All PNG samples were returned to Australia under biological material import permit and were treated with gamma irradiation at 25 kGy. DNA was extracted using Qiagen DNeasy plant minikits following the manufacturer's instructions with the following modifications. Quantities of initial plant material and corresponding volumes of buffers AP1 and AP2 were increased by 150 % (i.e., 50 mg dry weight instead of 20 mg). The AP1 buffer incubation period, at 65 C, was increased from 10 minutes to 30 minutes. An additional clean up step using buffer AW was conducted. Initial diagnosis by nested PCR using the P1/P7 primer pair for first round and the R16F2n/m23SR primer pair for second round was conducted as described by Constable et al. (2003). The PCR integrity of all DNA extracts was verified using the PCR primers rP2/fD1 (Weisburg et al. 1991).

Phylogenetic analyses of the 16S rRNA gene using 'Cocos nucifera' lethal yellowing phytoplasma1 (GQ227853) and 44 formally recognised phytoplasma species (Wei et al. 2007; http://plantpathology.ba.ars.usda.gov/pclass/pclass_phytoplasmaclassification_system2.html, Fig. 4) and the rp gene regions using 75 phytoplasma species or strains (Fig. 5) were conducted using MEGA4 (Tamura et al. 2007). These analyses showed that the phytoplasma from samples RID5365 and RID5376 clusters most closely with phytoplasmas associated with lethal yellows diseases of coconut in PNG and other countries but do form a distinct lineage from all other phytoplasma groups

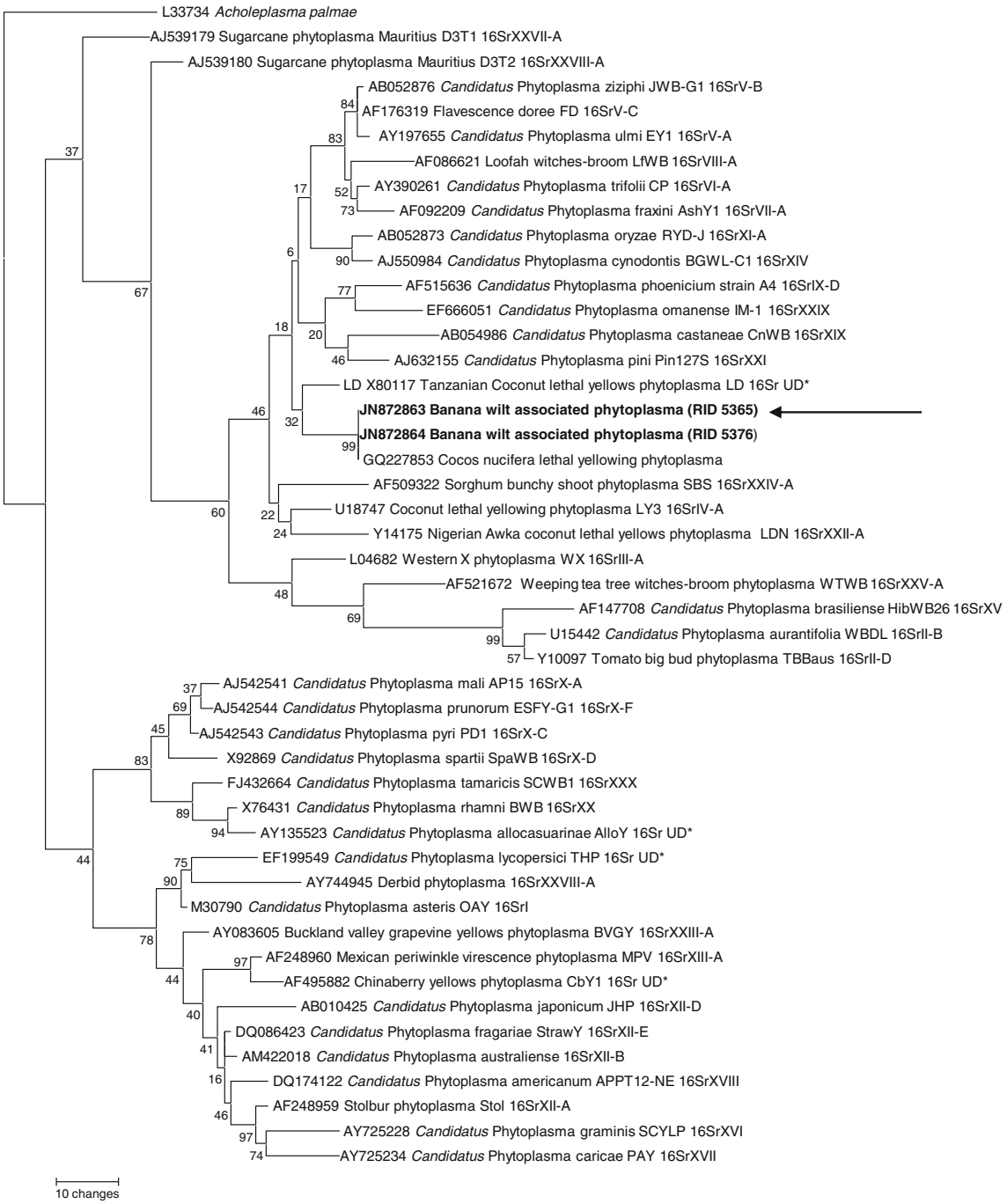
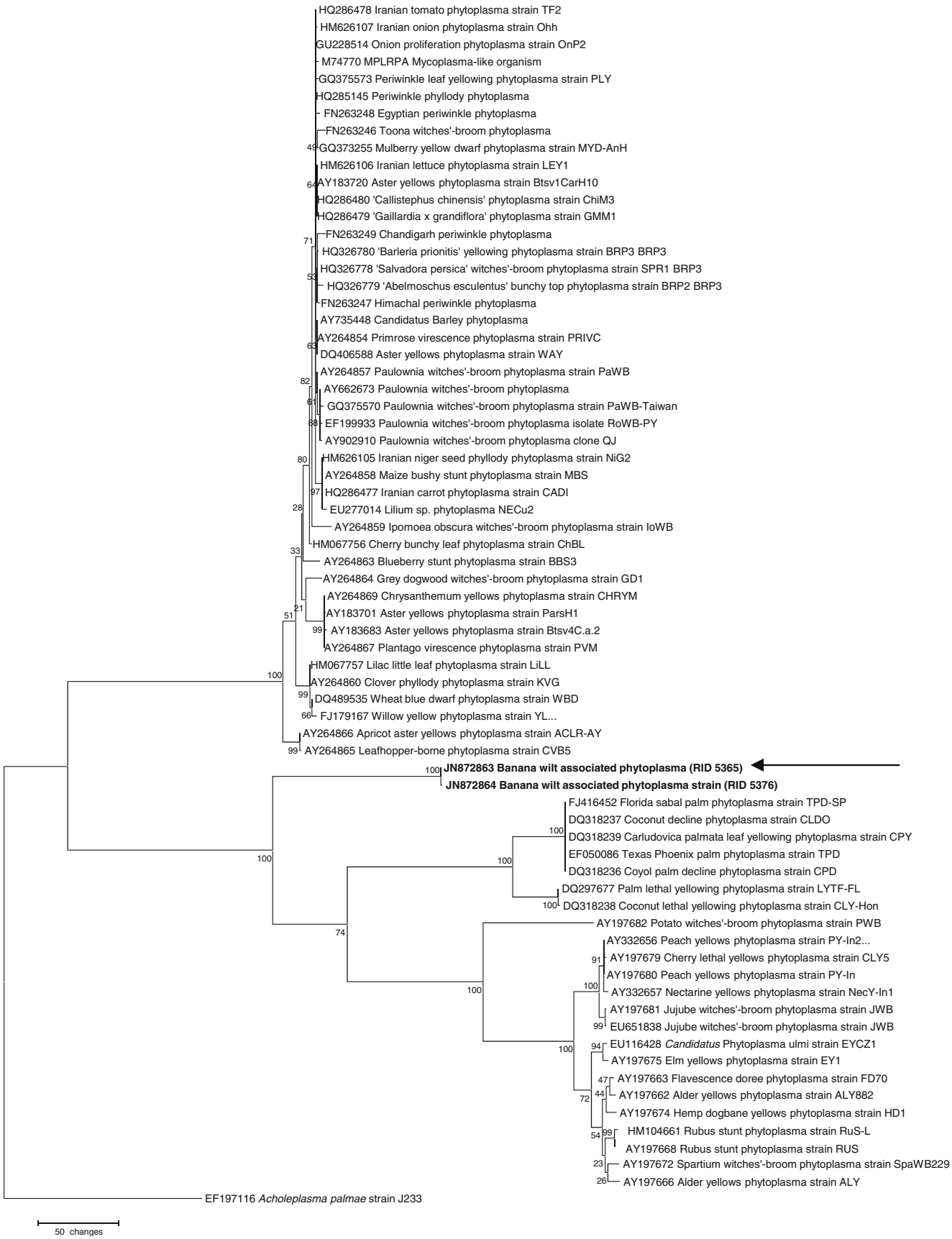


Fig. 4 A phylogenetic tree representing the evolutionary relationships of 47 phytoplasma species based on the 16 S ribosomal protein gene using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. *16Sr UD indicates that the 16Sr group of the phytoplasma has not been determined. The tree is drawn to scale, with branch lengths calculated using the average pathway method (Nei and Kumar 2000) and are in the units of the number of changes over the whole sequence


HQ286478 Iranian tomato phytoplasma strain TF2
HM626107 Iranian onion phytoplasma strain Ohh
GU228514 Onion proliferation phytoplasma strain OnP2
M74770 MPLRPA Mycoplasma-like organism
GQ375573 Periwinkle leaf yellowing phytoplasma strain PLY
HQ285145 Periwinkle phyllody phytoplasma
FN263248 Egyptian periwinkle phytoplasma
FN263246 Toona witches'-broom phytoplasma
GQ373255 Mulberry yellow dwarf phytoplasma strain MYD-AnH
HM626106 Iranian lettuce phytoplasma strain LEY1
AY183720 Aster yellows phytoplasma strain Btsv1CarH10
HQ286480 'Callistephus chinensis' phytoplasma strain ChiM3
HQ286479 'Gaillardia x grandiflora' phytoplasma strain GMM1
FN263249 Chandigarh periwinkle phytoplasma
HQ326780 'Barleria prionitis' yellowing phytoplasma strain BRP3 BRP3
HQ326778 'Salvadora persica' witches'-broom phytoplasma strain SPR1 BRP3
HQ326779 'Abelmoschus esculentus' bunchy top phytoplasma strain BRP2 BRP3
FN263247 Himachal periwinkle phytoplasma
AY735448 Candidatus Barley phytoplasma
AY264854 Primrose virescence phytoplasma strain PRIVC
DQ406588 Aster yellows phytoplasma strain WAY
AY264857 Paulownia witches'-broom phytoplasma strain PaWB
AY662673 Paulownia witches'-broom phytoplasma
GQ375570 Paulownia witches'-broom phytoplasma strain PaWB-Taiwan
EF199933 Paulownia witches'-broom phytoplasma isolate RoWB-PY
AY902910 Paulownia witches'-broom phytoplasma clone QJ
HM626105 Iranian niger seed phyllody phytoplasma strain NiG2
AY264858 Maize bushy stunt phytoplasma strain MBS
HQ286477 Iranian carrot phytoplasma strain CADI
EU277014 Lilium sp. phytoplasma NECu2
AY264859 Ipomoea obscura witches'-broom phytoplasma strain IoWB
HM067756 Cherry bunchy leaf phytoplasma strain ChBL
AY264863 Blueberry stunt phytoplasma strain BBS3
AY264864 Grey dogwood witches'-broom phytoplasma strain GD1
AY264869 Chrysanthemum yellows phytoplasma strain CHRYM
AY183701 Aster yellows phytoplasma strain ParsH1
AY183683 Aster yellows phytoplasma strain Btsv4C.a.2
AY264867 Plantago virescence phytoplasma strain PVM
HM067757 Lilac little leaf phytoplasma strain LiLL
AY264860 Clover phyllody phytoplasma strain KVG
DQ489535 Wheat blue dwarf phytoplasma strain WBD
FJ179167 Willow yellow phytoplasma strain YL...
AY264866 Apricot aster yellows phytoplasma strain ACLR-AY
AY264865 Leafhopper-borne phytoplasma strain CVB5
JN872863 Banana wilt associated phytoplasma (RID 5365)
JN872864 Banana wilt associated phytoplasma strain (RID 5376)
FJ416452 Florida sabal palm phytoplasma strain TPD-SP
DQ318237 Coconut decline phytoplasma strain CLDO
DQ318239 Carludovica palmata leaf yellowing phytoplasma strain CPY
EF050086 Texas Phoenix palm phytoplasma strain TPD
DQ318236 Coyol palm decline phytoplasma strain CPD
DQ297677 Palm lethal yellowing phytoplasma strain LYTF-FL
DQ318238 Coconut lethal yellowing phytoplasma strain CLY-Hon
AY197682 Potato witches'-broom phytoplasma strain PWB
AY332656 Peach yellows phytoplasma strain PY-In2...
AY197679 Cherry lethal yellows phytoplasma strain CLY5
AY197680 Peach yellows phytoplasma strain PY-In
AY332657 Nectarine yellows phytoplasma strain NecY-In1
AY197681 Jujube witches'-broom phytoplasma strain JWB
EU651838 Jujube witches'-broom phytoplasma strain JWB
EU116428 Candidatus Phytoplasma ulmi strain EYCZ1
AY197675 Elm yellows phytoplasma strain EY1
AY197663 Flavescence doree phytoplasma strain FD70
AY197662 Alder yellows phytoplasma strain ALY882
AY197674 Hemp dogbane yellows phytoplasma strain HD1
HM104661 Rubus stunt phytoplasma strain RuS-L
AY197668 Rubus stunt phytoplasma strain RUS
AY197672 Spartium witches'-broom phytoplasma strain SpaWB229
AY197666 Alder yellows phytoplasma strain ALY
EF197116 Acholeplasma palmae strain J233
50 changes

◀ **Fig. 5** A phylogenetic tree representing the evolutionary relationships of 70 phytoplasma species based on the ribosomal protein S19 (rps19), ribosomal protein L22 (rpl22), and ribosomal protein S3 (rps3) genes using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths calculated using the average pathway method (Nei and Kumar 2000) and are in the units of the number of changes over the whole sequence

(Figs. 4 and 5). Sequence similarity matrices were generated for the 16S rRNA gene and the rp gene regions using BioEdit (Hall 1999). Based on the 16S rRNA gene the banana phytoplasma is identical to the 'Cocos nucifera' lethal yellowing phytoplasma1 (GQ227853) recently reported from coconut trees in PNG (Kelly et al. 2011). 16S rRNA gene sequence similarities ranged from 90–95.4 % and rp gene region similarities ranged from 70–75.2 % for the remaining phytoplasma species analysed, suggesting the BWAP is a unique species.

To the author's knowledge, this may be the first description of a completely new wilt disease of banana. The only other records of phytoplasma detection in banana plants are two phytoplasmas in the 16SrI (Aster yellows group) found in association with disease symptoms similar to banana bunchy top disease. One is from China (Li et al. 1999) and one from India (Singh et al. 2009).

Almost all PNG phytoplasma records so far are from herbaceous dicotyledonous hosts (Davis and Ruabete 2010). An important exception being the recent find of a phytoplasma related to the 16SrIV (coconut lethal yellows) group associated with severe disease in coconut palms in MaP (Kelly et al. 2011). RID5365 and RID5376 isolates were obtained from active coconut phytoplasma disease outbreaks in MaP (Table 1), suggesting that banana should be investigated as a possible alternative host in PNG's new coconut disease epidemic. Little is known of what species from other plant families may act as inoculum reservoirs in coconut phytoplasma disease epidemics. Recently, three herbaceous weeds were identified as alternative hosts of phytoplasmas in the 16SrIV group in Jamaica (Brown and McLaughlin 2011), and a fourth was found to be a host of a related coconut disease associated phytoplasma in Ghana (Danyo 2011). However, based on PCR detection, cloning and sequencing as described above, the BWAP was also found in banana plants from WP, MoP and ESP (Table 1, Fig. 6),

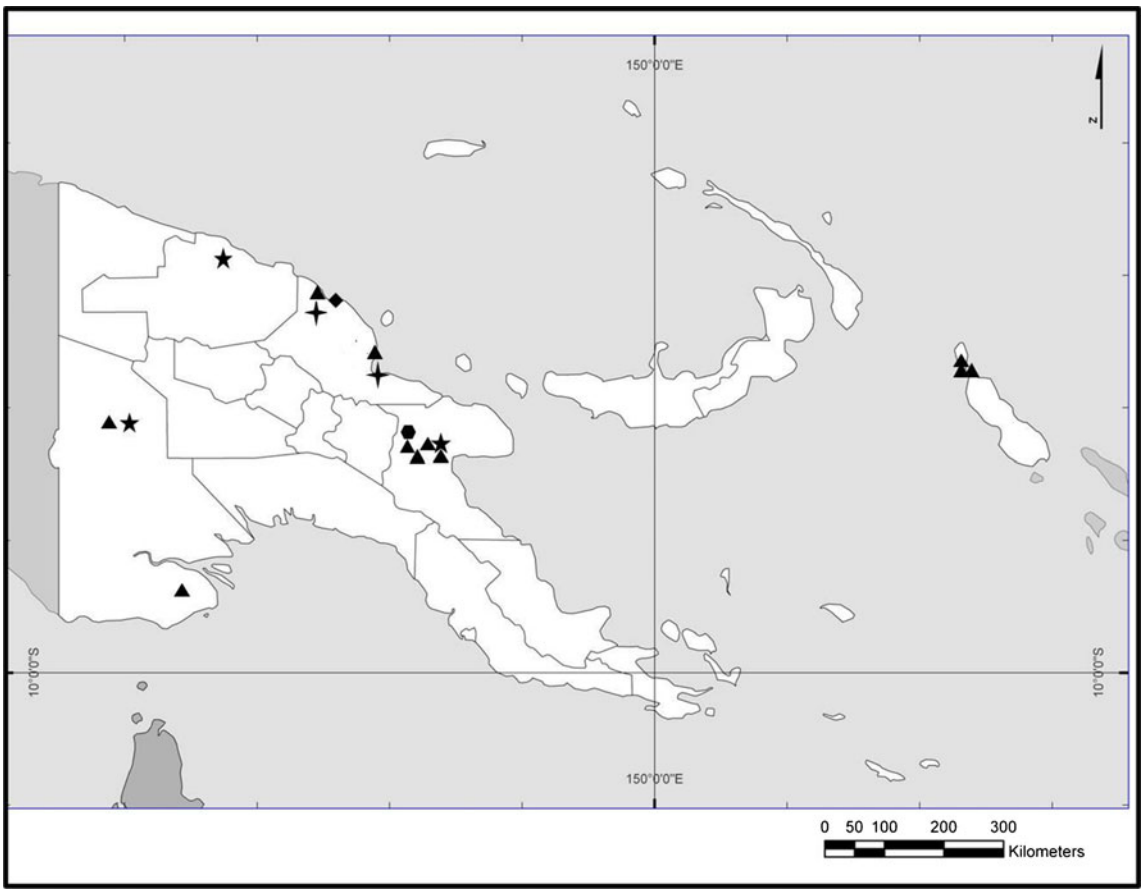


Fig. 6 Map of PNG, indicating the approximate location of banana plants from which the following phytoplasmas were obtained. BWAP, in a location where active coconut disease epidemics were in progress (*black four pointed star*); BWAP, in a location where no coconut disease problems were apparent (*black star*); as yet unidentified phytoplasmas (*black up-pointing triangle*); and phytoplasmas most closely related to phytoplasmas in the 16SrII (*black diamond suit*) and 16SrVIII (*black circle*) groups

where coconuts were abundant and showing no signs of phytoplasma—like disease. This apparent lack of transfer between host species might be explained by so far unidentified differences within the BWAP, or because of differences in the feeding behaviour of insect vectors present. Further work on the phytoplasmas involved is clearly needed. A 16SrII group phytoplasma was detected in one banana plant showing the disease symptoms described above in MaP and a 16SrVIII group phytoplasma was detected in another banana plant not showing discontinuous vascular streaking in MoP (Table 1, Fig. 6). The latter could be a slightly different disease relationship, or alternatively a chance 'infection', which should be expected to occasionally occur in perennial crops like banana. This is the first record of a 16SrVIII phytoplasma from PNG.

Further investigations into the phytoplasma disease status of monocotyledonous crops and weeds, plus studies to determine what are the insect vectors in these areas of PNG are essential. This information would underpin sustainable disease management strategies for banana and possibly also coconut growers of PNG.

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