'Candidatus Phytoplasma wodyetiae', a new taxon associated with yellow decline disease of foxtail palm (Wodyetia bifurcata) in Malaysia

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Abstract

Landscape-grown foxtail palm (Wodyetia bifurcata A. K. Irvine) trees displaying symptoms of severe foliar chlorosis, stunting, general decline and mortality reminiscent of coconut yellow decline disease were observed in Bangi, Malaysia, during 2012. DNA samples from foliage tissues of 15 symptomatic palms were analysed by employing a nested PCR assay primed by phytoplasma universal ribosomal RNA operon primer pairs, P1/P7 followed by R16F2n/R2. The assay yielded amplicons of a single band of 1.25 kb from DNA samples of 11 symptomatic palms. Results from cloning and sequence analysis of the PCR-amplified 16S rRNA gene segments revealed that, in three palms, three mutually distinct phytoplasmas comprising strains related to ‘Candidatus Phytoplasma asteris’ and ‘Candidatus Phytoplasma cynodontis’, as well as a novel phytoplasma, were present as triple infections. The 16S rRNA gene sequence derived from the novel phytoplasma shared less than 96 % nucleotide sequence identity with that of each previously described species of the provisional genus ‘Ca. Phytoplasma’, justifying its recognition as the reference strain of a new taxon, “Candidatus Phytoplasma wodyetiae”. Virtual RFLP profiles of the R16F2n/R2 portion of the 16S rRNA gene and the pattern similarity coefficient value (0.74) supported the delineation of ‘Ca. Phytoplasma wodyetiae’ as the sole representative subgroup A member of a new phytoplasma ribosomal group, 16SrXXXVI.

Diverse plant species in the family Areaceae, commonly known as palms, have enormous appeal due to their high economic and aesthetic values. In the vast tropical and subtropical areas around the globe, palms provide unparalleled elegance and architectural stature in landscapes; their fruits, seeds, fronds (leaves) and stems are rich sources of food, biofuels, cosmetic products and timber [1]. However, the health of palms and the palm industry are vulnerable to destructive diseases attributed to infection by phytoplasmas [2], a group of cell-wall-less bacteria that parasitize host plant vascular sieve elements and are transmitted by phloem-feeding insects [3]. Possessing a unique genome architecture [4, 5], phytoplasma chromosomes are rich in phage-derived genomic islands [6] but lack many metabolic pathway genes that are present in most free-living bacteria [7, 8]. Absence of genes encoding phosphoenolpyruvate-dependent sugar phosphotransferase system, of genes critical for maintaining intracellular redox homeostasis and of genes essential for cell membrane genesis, among others, may have led to an inability of phytoplasmas to survive outside their host cells in nature [9, 10]. As discussed previously [11], over the last four decades, numerous efforts have been devoted to establish phytoplasma axenic culture; while several claims of success have appeared in the literature [12–19], none has been verified independently. Consequently, phytoplasmas are still accommodated in a provisional genus, ‘Candidatus Phytoplasma’ [20]. Thus far, 41 species of the provisional genus ‘Ca. Phytoplasma’ have been described formally [21, 22]. An additional nine potentially novel phytoplasma taxa have been suggested or cited incidentally but remain to be described formally [20, 23].
Phytoplasmal diseases in palms can be lethal. At least 38 palm species have been proven susceptible to phytoplasmal infections, and the number continues to grow [24, 25]. Foxtail palm (Wodyetia bifurcata), a once endangered species native to northeastern Queensland, Australia, has become a widely cultivated landscape ornamental because of its spectacular foliage displays and its exceptional adaptability to diverse soil conditions [26]. This palm species was previously considered to be resistant to phytoplasmal diseases. Such assumption was called into question in the late 1990s when symptoms of crown necrosis, general stunting and leaf yellowing were observed among foxtail palms growing in Howard Springs, NT, Australia, even though no phytoplasma was actually detected in any of the affected palms by use of a PCR assay at that time [27]. Only recently were phytoplasmal yellow decline and lethal yellowing diseases of foxtail palms positively diagnosed in Malaysia [28] and Antigua, West Indies [29], respectively. Recently, foxtail palms affected by a yellow decline disease in the town of Bangi, Selangor State, western Malaysia, were found to have mixed phytoplasmal infections. Such mixed infections were comprised two previously characterized phytoplasmas [28] as well as an unknown phytoplasma. The novel phytoplasma became the focus of our current investigation, culminating in its recognition as a representative of a novel taxon.

Prior to the present communication, diverse phytoplasmal diseases in palms have been attributed to seven mutually distinct species of the provisional genus ‘Ca. Phytoplasma’, namely ‘Ca. Phytoplasma palmae’ [30, 31], ‘Ca. Phytoplasma cynodontis’ [32, 33], ‘Ca. Phytoplasma cocostanzanii’ [20], ‘Ca. Phytoplasma oryzae’ [34], ‘Ca. Phytoplasma malaysianum’ [35], ‘Ca. Phytoplasma palmicolae’ [36] and ‘Ca. Phytoplasma asteris’ [37, 38]. Among them, ‘Ca. Phytoplasma palmae’ and ‘Ca. Phytoplasma cocostanzanii’ are incidentally cited species [20]. The new phytoplasma that we identified in the present study represents yet another new palm-affecting taxon, ‘Ca. Phytoplasma wodyetiae’.

TRIPLE PHYTOPLASMAL INFECTIONS IN FOXTAIL PALMS DISPLAYING YELLOW DECLINE SYMPTOMS IN BANGI, MALAYSIA

In the spring of 2012, foxtail palms grown in the town of Bangi, Selangor State, Malaysia, exhibited symptoms resembling those of coconut yellow decline (CYD) disease [39]. The symptoms included stunting, leaf chlorosis, crown necrosis and general decline (Fig. 1); the disease was designated foxtail palm yellow decline (FPYD) [28]. Foliar tissue samples were collected from 15 symptomatic palms from which total DNA was extracted as described previously [33]. Sample DNAs were analysed using a nested PCR assay employing the phytoplasmal 16S rRNA gene universal primer pairs P1/P7 [40] and R16F2n/R16R2 [41]. The PCR parameters used were the same as those described previously [33]. Resulting amplicons (1.25 kb) were cloned as described previously [33] and sequenced independently by two DNA sequencing service providers (Bioneer and Medigene SDN BHD) to achieve at least four times coverage per base position. The consensus sequence of each phytoplasma strain was deposited in the GenBank nucleotide database. Results showed that 11 of the 15 symptomatic foxtail palm trees were phytoplasma-positive, and that three palms each simultaneously harboured three mutually distinct phytoplasmas. Among the three co-infecting phytoplasmas, two were identified as closely related to the phytoplasma strains previously reported in FPYD-diseased foxtail palms in Serdang, Selangor State, Malaysia [28], i.e. a strain related to ‘Ca. Phytoplasma asteris’ (FPYD Bangi-1, GenBank accession KC844878) and a strain related to ‘Ca. Phytoplasma cynodontis’ (FPYD Bangi-3, GenBank accession KT070559). The third phytoplasma was novel; it did not match any previously known strain (Table 1). While the complex etiology of the FPYD disease in Bangi remains under investigation, the present communication focuses upon characterization of the novel phytoplasma.

**FPYD BANGI-2 REPRESENTS A NEW AND DISTINCT 16SR RFLP GROUP**

Phytoplasmas are often classified into groups and subgroups based on RFLP analysis of a 1.25 kb PCR-amplified 16S rRNA gene segment, termed the F2nR2 fragment, using a defined set of 17 restriction enzymes [42]. While this classification scheme continues to be embraced by the phytoplasma research community, the RFLP analysis method has advanced to include DNA sequence-based computer simulation [23, 43]. Thus far, 35 ribosomal (16Sr) groups have been delineated [44, 45], although the delineations of the two most recent groups [45] are incomplete and need to be amended [46]. Among the previously established groups, six contain phytoplasma strains that were associated with palm diseases; these groups are 16SrIV, 16SXI, 16SrXIII, 16SrXIV, 16SrXXII and 16SrXXXII [47].

![Fig. 1. Symptoms of foxtail palm yellow decline disease. (a) General yellowing of the fronds; (b) gradual necrosis and collapse starting from the oldest fronds and spreading to the younger ones.](image-url)
Table 1. Phytoplasma strains used in this study for delineation of 'Ca. Phytoplasma wodyetiae'

This table is adapted from Nejat et al. [35], with newer taxa updated based on Davis et al. [54, 55, 21]; Quaglino et al. [56]; Win et al. [57]; Harrison et al. [36], Šafářová et al. [51] and Fernández et al. [22].

<table>
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<tr>
<th>Strain name</th>
<th>GenBank accession no</th>
<th>16Sr group classification</th>
<th>Percentage identity with FPYD Bangi-2</th>
<th>Reference</th>
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<td>16SrXXXVI</td>
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<td>U18747</td>
<td>16SrV</td>
<td>93.5</td>
<td>[20]</td>
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The virtual RFLP profile of the 16S rDNA F2nR2 fragment from the phytoplasma strain FPYD Bangi-2 (Fig. 2) had a similarity coefficient of 0.74 or less with the profile of each previously recognized 16Sr group based on analyses using iPhyClassifier [48]. RFLP patterns generated by restriction enzymes BfaI, Hinfl or RsaI were each sufficient to distinguish FPYD Bangi-2 phytoplasma from strains composing all other 16Sr groups (Fig. S1, available with the online Supplementary Material). According to the guidelines of the phytoplasma 16Sr classification scheme, a new group can be proposed if two criteria are met [46]. The first criterion specifies that the collective RFLP pattern of a given phytoplasma strain must have a similarity coefficient value lower than 0.85 when compared with the RFLP patterns of all previously recognized groups; this criterion is met by strain FPYD Bangi-2, as noted above. The second criterion is met if the new group hosts at least one species of the provisional genus ‘Ca. Phytoplasma”; this criterion is also met by strain FPYD Bangi-2 according to the analyses presented below.

Therefore, we establish a new 16Sr group, designated group 16SrXXXVI, the foxtail yellow decline phytoplasma group, with FPYD Bangi-2 phytoplasma representing the first known member of this group and subgroup A (16SrXXXVI-A).

**FPYD BANGI-2 REPRESENTS A NOVEL TAXON OF THE PROVISIONAL GENUS ‘CANDIDATUS PHYTOPLASMA’**

A detailed examination of the 16S rRNA gene of the novel strain FPYD Bangi-2 revealed that it contained a sequence block, 1275'-CAAGACCATGATGTGTAGCTGGACT-3'1515, that matches the signature sequence (5'-CAAGAYBATKA TGTKTAGCGGDC3') characteristic of the provisional genus ‘Candidatus Phytoplasma’ [20]. A phylogenetic analysis of the 16S rRNA gene sequence of the FPYD Bangi-2 phytoplasma together with the reference sequences of all previously established taxa of the provisional genus ‘Ca. Phytoplasma’ was performed, and a phylogenetic tree was reconstructed using the maximum-likelihood method based on the Tamura–Nei model [49] in MEGA 6 [50]. The topology of the resulting phylogenetic tree (Fig. 3) demonstrated that, within the phytoplasma clade, strain FPYD Bangi-2 shares a common ancestor with the recently established taxon ‘Ca. Phytoplasma cirsii’ [51], as well as with several other phytoplasma taxa implicated in palm lethal yellowing and palm yellow decline diseases, including ‘Ca. Phytoplasma oryzae’, ‘Ca. Phytoplasma cynodontis’, ‘Ca. Phytoplasma cocostanzaniae’ and ‘Ca. Phytoplasma palmicola’.

The FPYD Bangi-2 16S rRNA gene sequence shared at most, 96% similarity with that of each previously recognized species of the provisional genus ‘Ca. Phytoplasma’ (Table 1), a similarity that is lower than the threshold value (97.5%) that posits a species boundary [20]. Such a result signals that the FPYD Bangi-2 phytoplasma should be considered as a new taxon within the provisional genus ‘Ca. Phytoplasma’. To identify unique sequence blocks in the 16S rRNA gene that distinguish the FPYD Bangi-2 phytoplasma from all previously established species of the provisional genus ‘Ca. Phytoplasma’, a multiple sequence alignment was constructed using the clustalw option of the lasergene MegAlign program (DNASTAR). Comparisons of the aligned sequences revealed
Fig. 3. Phylogenetic relationships among three foxtail palm yellow decline (FPYD) phytoplasmas and previously described ‘Ca. Phytoplasma’ taxa. The evolutionary history was inferred from analysis of 16S rRNA gene sequences using the maximum-likelihood method implemented in MEGA 6 [50]. The tree with the highest log likelihood (–7534.8857) is shown. The taxa included in the analysis are reference strains of 41 formally described species of the provisional genus ‘Ca. Phytoplasma’, four incidentally cited species (marked with an asterisk *), six potentially novel but yet to be described species (marked with two asterisks **) and three FPYD strains identified from diseased foxtail palms in Bangi, Malaysia, in the present study. The taxa that have been implicated in palm diseases are in bold type. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. Numbers at the nodes of the branches are bootstrap values; bootstrap values of less than 50 were not shown.

five regions that are unique to the FPYD Bangi-2 phytoplasma (see below). These distinguishing regions serve as ‘signature sequences’ of the phytoplasma.

Results from the foregoing analyses, together with identification of a natural host (foxtail palm) and geographical occurrence (Malaysia), justify recognition of the phytoplasma strain FPYD Bangi-2 as representative of a novel taxon. We propose that the phytoplasma be designated the reference strain of a distinct species of the provisional genus ‘Ca. Phytoplasma’, ‘Candidatus Phytoplasma wodyetiae’.

DESCRIPTION OF ‘CANDIDATUS PHYTOPLASMA WODYETIAE’

‘Candidatus Phytoplasma wodyetiae’ (wo.dy.e’i.ae. N.L. gen. n. Wodyetiae of Wodyetia the scientific name of foxtail palm; epithet referring to the plant host).

Reference strain, FPYD Bangi-2\(^3\), is associated with foxtail palm trees exhibiting yellow decline symptoms.

\[
[(\text{Mollicutes}) \text{ NC; NA; O, wall-less; NAS (GenBank accession number KC844879)}; \text{ oligonucleotide sequences of unique regions of the 16S rRNA gene are:} \\
\begin{align*}
&\text{115}^5\text{-}\text{AAGCCTACCAAGACCA-3}^3_{134} \\
&\text{595}^5\text{-}\text{CTTACCGAC-3}^3_{602} \\
&\text{855}^5\text{-}\text{GTAGAACACAGTG-3}^3_{866} \\
&\text{1050}^5\text{-}\text{TTTGCAACCT-3}^3_{1058} \text{and} \\
&\text{1189}^5\text{-}\text{AATCACGAAT-3}^3_{1198} \\
\end{align*}
\]

P (Wodyetia bifurcata, phloem); M]. Naderali et al., this study.

Recognition of the new taxon, ‘Ca. Phytoplasma wodyetiae’, and establishment of the new foxtail yellow decline phytoplasma 16Sr group, further highlight the extent of the genetic diversity among phytoplasmas affecting palms. Apparently, diverse phytoplasma strains and strain combinations may induce indistinguishable symptoms in palms, including foxtail palms, echoing the hypothesis of ‘a common ancestral origin of phytoplasma pathogenicity genes and a limited repertoire of plant responses to phytoplasma pathogen signals’ [52]. It emphasizes the need for using molecular or other advanced discriminatory techniques to precisely identify etiological agents associated with a disease, especially when dealing with diseases that occur in areas where multiple phytoplasmas may be present [53].

References

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Conflicts of interest
The authors declare that there is no conflict of interest, and no humans or animals were subjects in this work.


