

Review

Palm yellows phytoplasmas and their genetic classification

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Palm yellows phytoplasmas have been a subject of debate because of two recent outbreaks. Firstly, a lethal yellowing-type phytoplasma disease was recorded on a number of palm species of mainly the genus *Phoenix* in Florida in 2008. Shortly afterwards, *Sabal palmetto* which has never been threatened by a phytoplasma before, was suddenly attacked by a phytoplasma strain similar to the one that attacked *Phoenix* in 2008. Both these recent outbreaks have made phytoplasmatologists realize the need to characterize palm phytoplasma strains in order to rapidly determine the phytoplasma palm yellows in future disease outbreaks. Various workers have made attempts to genetically characterize palm phytoplasmas but a lot of crucial knowledge is still lacking. This review focuses on the little progress that has been made to characterize palm phytoplasmas and we also recommend further steps to provide more tools for characterization.

Key words: *Cocos nucifera*, palm yellows phytoplasmas, *Sabal palmetto*, *Phoenix*.

INTRODUCTION

A widespread yellows disease called aster yellows was first reported in 1902 (Kunkel, 1926). During that period, yellows diseases were generally thought to be caused by viruses. To put an end to this myth, Japanese scientists discovered mycoplasma-like organisms (MLOs) on ultrathin sections of phloem in plants affected by yellows diseases (Doi et al., 1967). Since then the term mycoplasma-like organisms was used to refer to the causal agents of yellows diseases. It was not until 1994 that the name phytoplasma was adopted by the Phytoplasma Working Team of the 10th Congress of International Organization of Mycoplasmatology to refer to MLOs. Single cross sections of phytoplasmas showed them to be rounded, pleiomorphic with a diameter between 200 and 800 μm , but repeated ultrastructural observations showed that they are filamentous (Haggis and Sinha, 1978; Waters and Osborne, 1978; Lee and Davis, 1983; 1992). Phytoplasmas cause several hundred diseases of various plant hosts (McCoy et al., 1989). Among the known plant hosts are palms in which various symptoms ranging from premature leaf fall in coconut, decline and yellowing are

common. Palm yellows caused by phytoplasmas were observed in the Cayman Islands in the 1830s and were later discovered in other parts of the Caribbean. The focus of this review is on palm yellows phytoplasmas, the little progress made to genetically characterize them as well as suggestions to genetically characterize palm yellows phytoplasmas by copying methods that have been successful in other groups of phytoplasmas.

TAXONOMY AND GENERAL CHARACTERISTICS OF PHYTOPLASMAS

Phytoplasmas belong to the super kingdom Prokaryota, the kingdom Monera, the domain Bacteria, the phylum Firmicutes, the class Mollicutes and the *Candidatus* genus Phytoplasma. Based on analysis of the sequence of the 16S rRNA, gene phytoplasmas share between 88 and 99% similarity among themselves and between 87 and 88.5% similarity with Achleplasmas, their closest relatives (Seemüller et al., 1998; Gundersen et al., 1994).

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Phytoplasmas are mollicutes that inhabit phloem sieve tubes of their plant hosts. For a long time, phytoplasmas remained unculturable until Contaldo et al. (2012) grew phytoplasmas in axenic culture independently of their hosts. For transmission, they depend on phloem-feeding insect vectors of the order Hemiptera (Kirkpatrick, 1992). These insects harbor and spread them to different plants in a plant-insect-phytoplasma relationship known as the matrimonial triangle. Phytoplasmas are associated with over 1000 plant diseases (Seemüller et al., 1998; Lee et al., 2000), and typical symptoms on infected plants include virescence (development of green flowers and the loss of flower pigmentation), phyllody (development of leafy appearance on flowers), sterility of flowers, witches'-brooms, abnormal internode elongation, stunting, discoloration of the foliage, leaf distortion, malformation of stem ends and plant decline. However, some plant species are tolerant to phytoplasmas and therefore show mild or no symptoms. Phytoplasmas have small genomes ranging between 530 and 1200 kb and a GC content between 23 and 29 mol%, two rRNA operons, few tRNAs and limited metabolic activity (Razin, 1985; Bové, 1997; Marcone et al., 1999; Oshima et al., 2004; Bai et al., 2006). Phylogenetic analysis based on analysis of the 16S rRNA gene showed that phytoplasmas form a distinct clade within the class *Mollicutes* (Schneider et al., 1995; Gundersen et al., 1994; Lim and Sears, 1992). Since phytoplasmas are unculturable, their characterization is limited to mainly molecular-based methods. Due to this limited characterization, they have been assigned the provisional genus status "Candidatus Phytoplasma (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group, 2004; Murray and Stackenbrandt, 1995).

A comprehensive classification of phytoplasmas is based on combined analyses of restriction fragment length polymorphism of 16S rRNA and ribosomal protein gene sequences (Lee et al., 1998). According to this classification scheme, phytoplasmas were designated into 14 16Sr groups and 32 subgroups. Phytoplasmas causing lethal yellowing (LY) and lethal yellowing-type diseases on palms were classified as group 16SrIV.

GROUP 16SrIV PHYTOPLASMAS

Group 16SrIV phytoplasmas cause lethal yellowing and lethal yellowing-like diseases of palms and have been known for more than hundred years. Reports of mortality of palms due to yellowing symptoms were made in the Cayman Islands (Maramorosch, 1978; Ollagnier and Weststeijn, 1961; Nutman and Roberts, 1955; Martyn, 1945; Johnston, 1912), Cuba (Johnston, 1912; DeLa Torre, 1906), and Jamaica (Martyn, 1945; Fawcett, 1891). After these first records, palm lethal yellows were also reported in the Dominican Republic (Schieber and Hichez-Frias, 1970; Ciferri and Ciccarone, 1949), Haiti (Leach, 1946), Bahamas

(Leach, 1946) and Florida in the USA (Martinez and Roberts, 1967). Other outbreaks were also reported in the Yucatán Peninsula (Cardeña et al., 1991; McCoy et al., 1983), Belize (Escamilla et al., 1994), West and East Africa (Epko and Ojomo, 1992; Schuiling and Mpumani, 1990; Dollet et al., 1977; Dabek et al., 1976; Bull, 1955). By 1997, lethal diseases of palms had reached the Pacific coast of the Americas (Harrison et al., 2002). Most lethal disease reports were made on *C. nucifera* which has been devastated in various epide-mics. In Florida and Jamaica, lethal yellowing epidemics killed most of the Jamaican Tall variety from the 1970s until the 1990s. This occurred concurrently with spread of the disease to neighbouring regions including the Pacific coast of the Americas (Harrison et al., 2002) and in the late 1990s most disease activity was along the Atlantic coasts of Belize and Honduras (Harrison and Oropeza, 1997; Ashburner et al., 1996). In West Africa, epidemics of lethal yellowing-like diseases devastated palms mainly in Nigeria, Cameroon, Togo and Ghana (Mpunami et al., 1999; Dery et al., 1997). In some communities these epidemics caused severe economic losses.

In Canary Island date (*Phoenix canariensis*) palms, a lethal yellowing-type phytoplasma was detected in Corpus Christi, Texas in 2001 (Harrison et al., 2002). Symptoms on *P. canariensis* resembled those on *P. canariensis* and *P. dactylifera* during an epidemic in the Brownsville and Rio Grande Valley in southern Texas during the late 1970s (McCoy et al., 1980). Later records of lethal yellowing-type diseases on palms were made on silver date (*P. sylvestris*), Canary Island date, edible date (*P. dactylifera*), Queen (*Syagrus romanzoffiana*) and Mexican fan (*Washington robusta*) palms in west central Florida (Harrison et al., 2008). A lethal decline of cabbage palms (*Sabal palmetto*) was also recorded in West Central Florida in 2008. Initial DNA-based characterization found a phytoplasma which is identical to the strain previously affecting *P. canariensis* in Texas (Harrison and Elliott, 2009). In all its hosts, (*Phoenix*, *Syagrus*, *Washingtonia* and *Sabal*) this phytoplasma is called Texas Phoenix palm decline (TPPD) phytoplasma, a 16SrIV-D phytoplasma (Harrison and Elliott, 2009). Although sequence analysis of the 16S rRNA gene is the primary parameter for classifying phytoplasmas, others genes have been useful in phytoplasma taxonomy and differentiation. Few additional genes have been used for further classification of palm yellows phytoplasmas. This manuscript reviews genes that have been used for genetic classification of palm yellows phytoplasmas and we further recommend other genes that may also be tested.

RIBOSOMAL RNA GENES

Ribosomal RNA genes are an essential component of the protein synthesis apparatus and are therefore universally distributed in all organisms. They are conserved and

have sufficient variation to allow distinction between taxa (Woose, 1987). In eukaryotic genomes, copies of rRNA coding genes may reach several thousands. In prokaryotes, the rRNA gene copy number is less, averaging three or four in a single genome (Fogel et al., 1999). Schneider and Seemüller (1994), analyzed 28 phytoplasmas and found two copies of rRNA operons in phytoplasma and their work has been confirmed through the sequencing of the genomes of aster yellows witches' broom phytoplasma, *Candidatus* Phytoplasma australiense, *Ca.* Phytoplasma mali, and onion yellows phytoplasmas (Kube et al., 2008; Tran-Nguyen et al., 2008; Bai et al., 2006; Oshima et al., 2004). The exception is that of Western X-disease which has one copy of the rRNA operon (Kirkpatrick et al., 1987). Phytoplasmas also contain a tRNA in the spacer between the 16S and the 23S rRNA genes (Razin et al., 1998; Smart et al., 1996). Analysis of 16S rRNA gene is by far the most commonly used classification scheme for phytoplasmas. Signature sequences of the 16S rRNA gene have been used to distinguish phytoplasmas from other prokaryotes. Work has also been done to classify phytoplasmas into 14 major groups and 32 sub-groups based on analysis of sequence of the 16S rRNA gene and restriction fragment length polymorphisms combining 16S rRNA and ribosomal protein genes (Lee et al., 1998). Although analysis of the 16S rRNA gene is the primary parameter for classification of phytoplasmas, the 23S rRNA gene which is almost twice the size of the 16S rRNA gene has potential to provide additional information and variation for differentiating strains.

The 23S rRNA gene is useful in differentiating phytoplasma groups with potential for finer differentiation among the 16Sr groups (Guo et al., 2000). However, the 23S rRNA gene was found to be equally or even more conserved than the 16S rRNA gene in the Western X-disease phytoplasma group (Guo et al., 2000). The 23S rRNA gene sequence was used successfully to regroup the 16SrIX pigeon pea witches'-broom and the 16SrXI napier grass stunt phytoplasmas (Hodgetts et al., 2008). In a study in which the resolving power of the 23S rRNA gene was compared with that of the 16SrRNA, and the 16S-23S IGS, the 23S rRNA gene was found to have the same resolution with these other genomic regions but it however confirmed grouping based on them (Ntushelo et al., 2012). However, the usefulness of the 23S rRNA needs to be investigated in a sample bigger than the one used in the study by Ntushelo et al. (2012). Similarly, the 16S-23S rRNA gene spacer region which is more variable than the 16S rRNA gene was proved useful in classifying subspecies of the gram-positive bacterium *Clavibacter michiganensis* (Li and DeBoer, 1995).

The spacer region between the 16S rRNA and 23S rRNA genes

Length and nucleotide polymorphisms of the spacer

region between the 16S and the 23S rRNA genes offer variation for classification of phytoplasmas. The 16S-23S rRNA gene spacer region has fewer evolutionary constraints and can provide an alternative parameter to phytoplasma classification. This spacer region has a highly conserved tRNA^{le} flanked by variable regions. The presence of both conserved and variable regions makes it useful for detection and differentiation of closely related phytoplasma strains. Analysis of the sequence of the 16S-23S rRNA gene spacer region was used to classify the Argentinean strawberry phyllody phytoplasma (Fernández et al., 2013) and 26 phytoplasma strains from 12 host plants which included palm trees (Smart et al., 1996).

nusA Gene

The *nusA* gene, a transcription termination factor gene, like the 16S rRNA gene, is ubiquitous and conserved among bacteria (Borukhov et al., 2005). In a previous study, the *nusA* gene was found useful in classifying phytoplasmas, with the branching order of a phylogenetic tree inferred from the *nusA* gene sequence similar to the branching order inferred from the 16S rRNA gene sequence for the same phytoplasma isolates (Shao et al., 2006). The consistency of the *nusA* gene in resolving phytoplasma strains was also demonstrated by correlations between *nusA* phylogenetic trees with trees inferred from the sequences of ribosomal protein genes (Lee et al., 2004), *tuf* gene sequences (Marcone et al., 2000; Schneider et al., 1997), as well as *gcp* gene sequences (Davis et al., 2003). This demonstration of *nusA* as a pertinent taxonomic tool argues for using *nusA* to differentiate between the TPPD and the LY phytoplasmas as it is yet to be fully exploited in characterizing palm yellows phytoplasmas. Among the challenges limiting its use is the difficulty in amplifying it from infected palm tissue with low phytoplasma titre.

hf/B Gene

The *hf/B* gene is possibly associated with strain virulence (Lithgow et al., 2004; Beier et al., 1997). The *hf/B* gene whose sequence polymorphisms were useful in differentiating strains of *Candidatus* Phytoplasma mali (Schneider and Seemüller, 2009) is also useful in differentiating between the TPPD phytoplasma from the coconut LY phytoplasma. On restriction fragment lengths profiles of this gene using a few restriction enzymes, there are distinct band differences that show differences between the TPPD phytoplasma and the LY phytoplasma (unpublished data).

gcp Gene

The protein encoded by the *gcp* gene, O-galactosidase

endopeptidase, is possibly a host adaptation and virulence factor and is a member of the M22 peptidase family (Rawlings and Barret, 1995). Phylogenetic branching of phytoplasmas from other bacteria was shown by analysis of the *gcp* gene sequence and was similar to the pattern shown by the 16S *rRNA* gene (Gundersen et al., 1994). Differentiating the TPPD phytoplasma strain from the coconut lethal yellowing strain, phylogenetic analysis based on the sequence of the *gcp* gene is positively correlated with differentiation based on the spacer between the 16S rRNA gene and the 23S rRNA gene (unpublished data).

secA Gene

Although the 16S *rRNA* gene has been crucial in grouping phytoplasmas associated with palms, within group resolution of the 16SrIV phytoplasmas has been made possible by, among other genes, the *secA* gene. Using sequence analysis of the *secA* gene, coconut lethal yellowing phytoplasmas have been differentiated into three distinct groups that are correlated with geographic origin. Based on analysis of the sequence of the *secA* gene combined with analyses of the sequences of the ribosomal operon, 16SrIV phytoplasmas were split into 16SrIV-A phytoplasmas which represented the lethal yellowing group in the Americas and 16SrIV-B group which represented the lethal yellowing group in Tanzania as well as Cape St Paul wilt in Ghana (16SrIV-C) (Hodgetts et al., 2008).

groEL Gene

Because phytoplasma characterization based on the 16S *rRNA* gene does not always provide a clear distinction between phytoplasmas within a 16Sr group, genes that can provide a finer differentiation are always explored and tested on various phytoplasmas. The *groEL* gene is a genes that can be utilized for finer subgroup differentiation among 16SrIV group phytoplasmas. Using the *groEL* gene for subgroup differentiation of 16SrV phytoplasmas collected from the oil palm, group differentiation could be of epidemiological relevance. Restriction fragment length polymorphisms of the *groEL* gene digested with *TruI* showed two groups of the 16SrV group collected from the oil palm and digestion of this gene with the *AluI* showed eight groups. Furthermore, grouping of 16SrIV phytoplasmas that cause onion phyllody and virescence using sequence of the *groEL* gene digested with the same enzymes also segregated these phytoplasmas into two groups for each enzyme. The same result was achieved with another phytoplasma that causes carrot proliferation and grindelia virescence (Mitrović et al., 2011). The *groEL* gene was also used to develop a SybrGreen system which accurately detects

the coconut lethal yellowing phytoplasma (Myrie et al., 2011). This successful use of this gene warrants its further utilization in characterizing palm yellows phytoplasmas further.

Other genes that may be used to classify palm yellows phytoplasmas

Ribosomal protein (rp) genes

Ribosomal protein (*rp*) genes were used to revise the phytoplasma classification scheme according to Lee et al. (1998). Other studies in which *rp* genes were used for finer differentiation of phytoplasmas include the classification of phytoplasmas isolated from diseased strawberry (Jomantiene et al., 1998). In a phylogenetic study by Martini et al. (2007), classification based on *rp* genes could be correlated with classification based on analysis using the 16S rRNA gene. Because of these and other previous successes in using this gene in phytoplasma classification, it is worth considering using ribosomal protein genes for assessing diversity among palm yellows phytoplasmas.

secY Gene

The *secY* gene was used for finer differentiation into 10 lineages of the aster yellows phytoplasmas. This subgroup differentiation was positively correlated with differentiation of the aster yellows phytoplasmas based on *rp* gene sequences (Lee et al., 2006). Together with other non-ribosomal genetic loci *aceF*, *pnp*, and *imp* genes, the *secY* gene was used to determine genetic variability among isolates of *Ca. Phytoplasma prunorum*, *Ca. P. mali*, and *Ca. P. pyri* (Danet et al., 2007). Danet et al., (2011) also showed the use of multi-genetic loci in phytoplasma classification, a strategy that can also be copied in classifying 16Sr IV phytoplasmas.

Immunodominant membrane protein (imp) Gene

Apart from the study of Danet et al., (2007) mentioned above, the *imp* gene was also used to assess genetic diversity among strains of *Ca. Phytoplasma mali* (Seemüller et al., 2010). Combined with the *aceF* gene, the *imp* gene was also used to determine genetic diversity among European fruit tree phytoplasmas (Danet et al., 2007). An attempt should be made to use this gene to classify palm yellows phytoplasmas.

tuf Gene

Profiles of restriction fragment length polymorphisms of

the *tuf* gene were used to delineate subgroups of aster yellows-group phytoplasmas. This *tuf*-gene based classification was consistent with classification using rDNA sequences (Marccone et al., 2000). Similar work was done by Lee et al. (2004) to resolve the diversity among aster yellows phytoplasmas. The *tuf* gene was also used to differentiate a broad range of phytoplasmas (Schneider et al., 1997).

CONCLUSION

Genes that have been used to classify phytoplasmas belonging to other 16Sr groups can be used to classify phytoplasmas within the 16SrIV. A high chance of potential success with these genes is based on the high genetic similarity among phytoplasmas.

Techniques that are used to study single genes of phytoplasma usually involve the PCR amplification of the single gene and analysis of its sequence. These techniques are laborious and time-consuming. High-throughput, fast and efficient methods of genome characterization are rapidly gaining popularity in many applications and can be utilized in the genetic characterization of phytoplasmas. Once adopted, these new techniques will increase the speed at which palm yellows phytoplasmas are genetically characterized and phytoplasma genetic characterization may no longer be based on genes but significantly on genomes.

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