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BACTERIAL AND PHYTOPLASMA DISEASES

First report on the association of '*Candidatus* Phytoplasma asteris' with lethal wilt disease of coconut (*Cocos nucifera* L.) in India

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Abstract
Lethal wilt disease of coconut, reported as a disease of unknown etiology as early as 2007, was for the first time found consistently in association with a phytoplasma in diseased coconut trees in Thanjavur, Thiruvarur and Pudukkottai districts in Tamil Nadu on the southeastern coast of India. Nested PCR with phytoplasma-specific universal primers and in silico restriction analysis of 16S rDNA sequences revealed the association of '*Candidatus* Phytoplasma asteris' (16SrI-B) group phytoplasma, which was confirmed by sequence analysis of translation elongation factor (*tuf*) and ribosomal protein (*rp*) genes. Because this disease kills affected palms within 5 months, recommendations are given to arrest the spread of the pathogen to other coconut-growing regions in the country.

Keywords Palm phytoplasma · 16SrI-B · Aster yellows · Monocot · Tamil Nadu

Introduction

Worldwide, coconut (*Cocos nucifera* L.; Arecaceae), a native of humid tropics, is grown in 94 countries, providing employment and livelihood options to 64 million farm families (Nampoothiri et al. 2018). In India, one of the leading producers of coconut in the global market, cultivation is mainly confined to the southern states: Kerala, Karnataka, Tamil Nadu, and Andhra Pradesh. Diseases occupy a prime position among the major production constraints in the coconut sector across peninsular India. Bhaskaran et al. (2007) reported sporadic occurrence of a lethal disease of unknown etiology in coconut plantations in Thanjavur district of Tamil Nadu that was symptomatically different from the commonly prevalent basal stem rot disease caused by species of the fungus *Ganoderma*. Preliminary etiological investigations based on fungal cultures isolated from the infected tissues also failed to yield confirmatory results. The geographic spread and incidence of the new disease in question has gradually increased over the years in Thanjavur, Thiruvarur, and Pudukottai districts of Tamil Nadu, with an average incidence level of 1.4% in 2018 (Thangeswari et al. 2018). The symptoms manifested by the diseased palms were found to be analogous to those induced by phytoplasmal infection. The randomly distributed, discrete foci of diseased trees in the affected plantations and the symptomatology, suggested the involvement of a vector-transmitted phytopathogenic mollicute. Hence, we focused our attention on detecting a phytoplasma to establish the etiology of this lethal disease.

Materials and methods

Symptomatology and distribution of the disease

Ccoconut plantations in Thanjavur, Thiruvarur, and Pudukottai districts of Tamil Nadu, India where the lethal disease has been endemic were surveyed to assess the spread and distribution of the disease. Geocoordinates of the diseased plots and affected palms were recorded using a global positioning system (GPS). The base map of the study area

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was prepared using the Survey of India (SOI) map and the administrative map from the Revenue Division. Ground truth data were collected from the diseased plots. Spatial information on the disease incidence was mapped using ArcGIS version 10 (ESRI; Redlands, CA, USA). Ten diseased palms were identified in the early stage of the disease, and progression of symptoms was documented systematically to reconfirm symptom expression and for further collection of samples.

Collection of samples, detection and molecular characterization of pathogen

Samples were collected during 2017–2018 from Thanjavur and Thiruvarur districts in Tamil Nadu, India. From among 60 symptomatic palms selected, we collected spear leaves from all 60 and inflorescences from 56 palms. Spear leaves and inflorescences were also collected from seven asymptomatic coconut palms. Total DNA was extracted from collected samples by the CTAB method (Lodhi et al. 1994). Because symptoms of this lethal disease typically resemble those caused by phytoplasma, a nested PCR with universal primer pairs P1/P6 (Deng and Hiruki 1991) and R16F2n/R2 (Gundersen and Lee 1996) was used to detect any pathogen from symptomatic samples. Bermuda grass white leaf phytoplasma (‘*Ca.* P. cynodontis’) served as a positive control. The thermal conditions of the primary PCR were initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 63 °C for 1 min and extension at 72 °C for 2 min; final extension at 72 °C for 10 min. The P1/P6 primary PCR product was then diluted to 1:10 with sterile distilled water and 2 µl used as the DNA template in the nested PCR with primer pair R16F2n/R2. An annealing temperature of 56 °C was maintained for R16F2n/R2.

Ribosomal protein (*rp*) genes were also amplified using primer pairs rpFI/rpRI (Lim and Sears 1992) nested with rp(I-B)F1/rp(I-B)R2 (Lee et al. 2003). Primer pairs fTuf1/rTuf1, nested with fTufAy/rTufAy (Schneider et al. 1997), were used to amplify the translation elongation factor (*tuf*) gene. All PCR amplifications were carried out in Flexigene thermal cycler (Techne, Duxford, Cambridge, UK). The amplicons were resolved in 1.0% agarose gels and documented using Gel Doc (Bio-Rad Laboratories, Hercules, CA, USA). PCR products amplified with P1/P6, rp(I-B)F1/rp(I-B)R2, and fTufAy/rTufAy primers were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced at M/s AgriGenome Labs Pvt Ltd. (Kochi, Kerala, India).

Pathogen identification and phylogenetic analysis

In silico RFLP analysis of 16S rDNA sequences with restriction endonucleases *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I and virtual gel plotting were carried out using the *i*PhyClassifier online tool (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) as described by Zhao et al. (2009). The NCBI database was searched using BLAST (Altschul et al. 1990) for sequences homologous to the 16S rDNA, *rp* and *tuf* gene sequences under study. For constructing phylogenetic trees, selected sequences were aligned using CLUSTAL W (Thompson et al. 1994), and the tree was assembled using MEGA 6.0 software (Tamura et al. 2013) by the neighbour joining method with 1000 replications for bootstrap analysis. *Acholeplasma oculi* strain 19L (GenBank accession NR_025960) was used as the outgroup for the phylogenetic tree of 16S rDNA. Phylogenetic trees of *tuf* and *rp* genes were constructed using respective sequences from ‘*Ca.* P. australiense’ (GenBank accession AM422018) as the outgroup.

Results

Symptomatology

The primary visible symptom of the disease is shedding of nuts of all stages within 3–5 days (Fig. 1a), followed by inflorescence necrosis and shedding of male flowers (Fig. 1b, c). As the inflorescence necrosis progresses, yellowing starts from the outer whorls of leaves and expands to the inner whorls (Fig. 1d). The chlorotic leaves then turn brown and necrotic, with the dried leaves hanging intact on the crown (skirting of leaves around the trunk) for a few days before detachment from the trunk (Fig. 1e). In the advanced stage of the disease, necrosis, and rotting are seen on spear leaves and growing points (Fig. 1f). Eventually, the entire crown dies, leaving a bare trunk. Affected palms die within 3–5 months after the initial nut fall. The symptoms of this emerging disease completely differ from all other diseases reported so far on coconut palms from India. Because the disease is lethal and affected palms show wilting symptoms, the disease is hereby referred to as lethal wilt disease (LWD).

Distribution of the disease

From the disease incidence map developed for Thanjavur, Thiruvarur, and Pudukottai districts of Tamil Nadu (Fig. 2), LWD had spread near the coastal region in these three districts, but in randomly distributed foci of infection. In all regions surveyed, the symptoms first manifested in December after the cessation of the northeast monsoon and extended up to June with peak incidence during the summer months (February to May). The seasonality of the disease occurrence was attributed to the abundance of insect

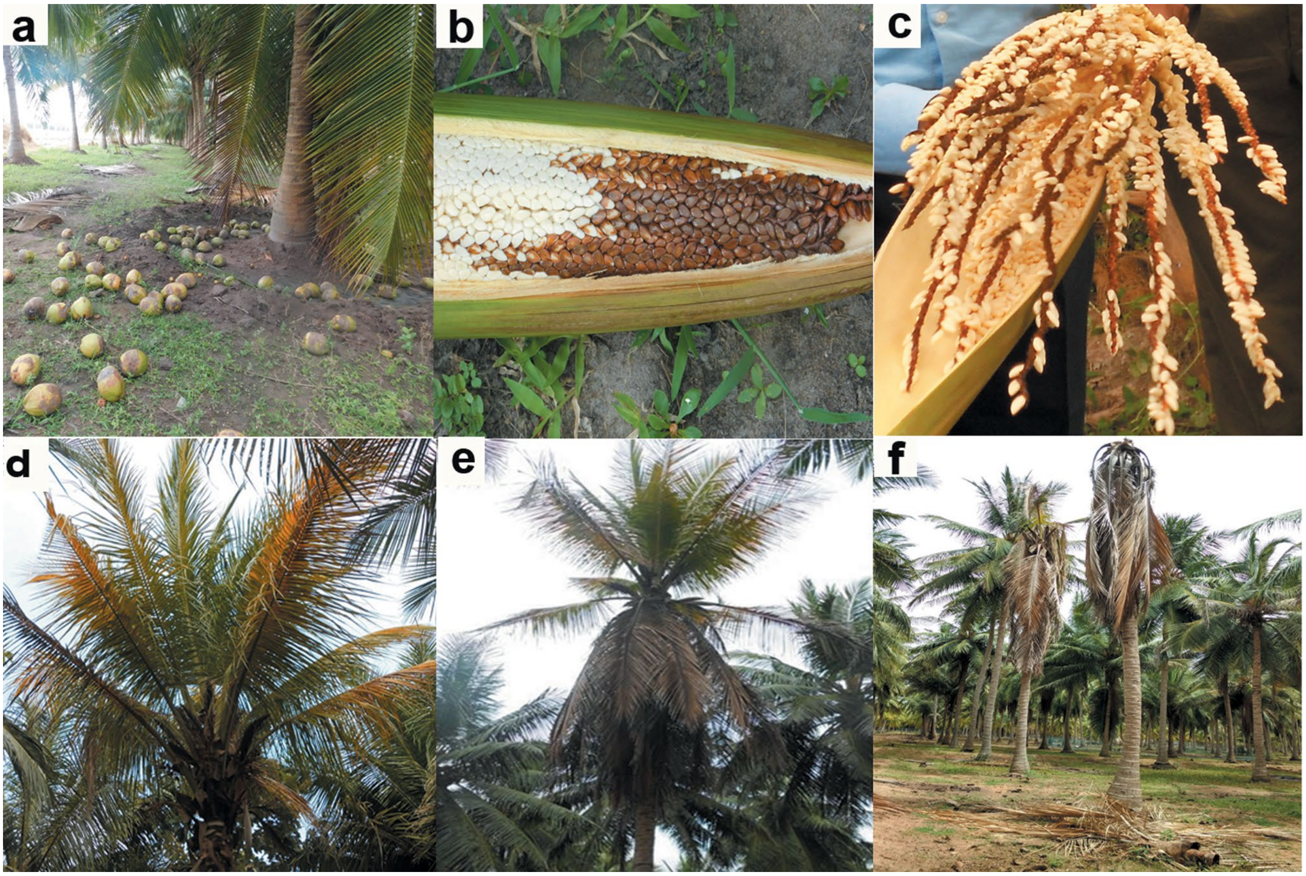

Fig. 1 Symptoms of lethal wilt disease (LWD). **a** Nut fall. **b** Inflorescence necrosis. **c** Shedding of male flowers. **d** Leaf yellowing. **e** Bronzing, drying and drooping of leaves. **f** Death

vector(s) and enhanced inter-plant mobility of vector(s) during the favourable summer season. This highly random scattering of foci is indicative of the involvement of an insect vector typical for a phytoplasma. An intensive search for a vector during disease outbreak period has to be systematically instituted.

Identification and characterization of the pathogen

In the direct PCR with primers P1/P6, 34 palms had an amplicon of approximately 1.4 kb, and in the nested PCR with primers R16F2n/R16R2, 59 had an amplicon of around 1.2 kb, confirming the association of phytoplasma with the disease. Of the 60 spear leaf samples examined, 49 (81.7%) tested positive in nested PCR using primers P1/P6-R16F2n/R16R2. The four palms with advanced disease from which only spear leaves were collected also tested positive in the nested PCR. For inflorescence samples, which had a higher detection rate than in spear leaves, 55 of the 56 (98.2%) were positive. None of the primer pairs amplified the target in any of the samples from asymptomatic palms. The consensus sequences of the amplicons obtained using primers P1/P6 were deposited in NCBI (GenBank: KY814724, MK617534). BLAST analysis of the 16S rDNA sequence of the LWD phytoplasma showed 99.9% similarity with *Brassica oleracea* phyllody phytoplasma and *Pisum sativum* yellows phytoplasma (GenBank: MK440287, MK440284). Analysis with *i*PhyClassifier revealed that 16S rDNA sequence of coconut lethal wilt phytoplasma shares more than 99% similarity with that of the '*Candidatus* Phytoplasma asteris' reference strain (GenBank: M30790). Also, the virtual RFLP pattern derived from 16S rDNA fragment of LWD phytoplasma was identical (similarity coefficient 1.00) to the reference pattern of 16Sr group I, subgroup B (GenBank: AP006628). Based on the results obtained from 16S rDNA sequence analysis and virtual RFLP pattern generated by *i*PhyClassifier, the phytoplasma causing LWD is identified as '*Ca.* P. asteris'-related strain belonging to subgroup 16SrI-B.

In the phylogenetic tree constructed using the 16S rDNA sequences, the LWD phytoplasma clustered with the 16SrI group phytoplasmas ('*Ca.* P. asteris') (Fig. 3). The root (wilt) disease phytoplasma ('*Ca.* P. oryzae') belonging to 16SrXI-B group (GenBank: FJ94816, JX273772) already reported

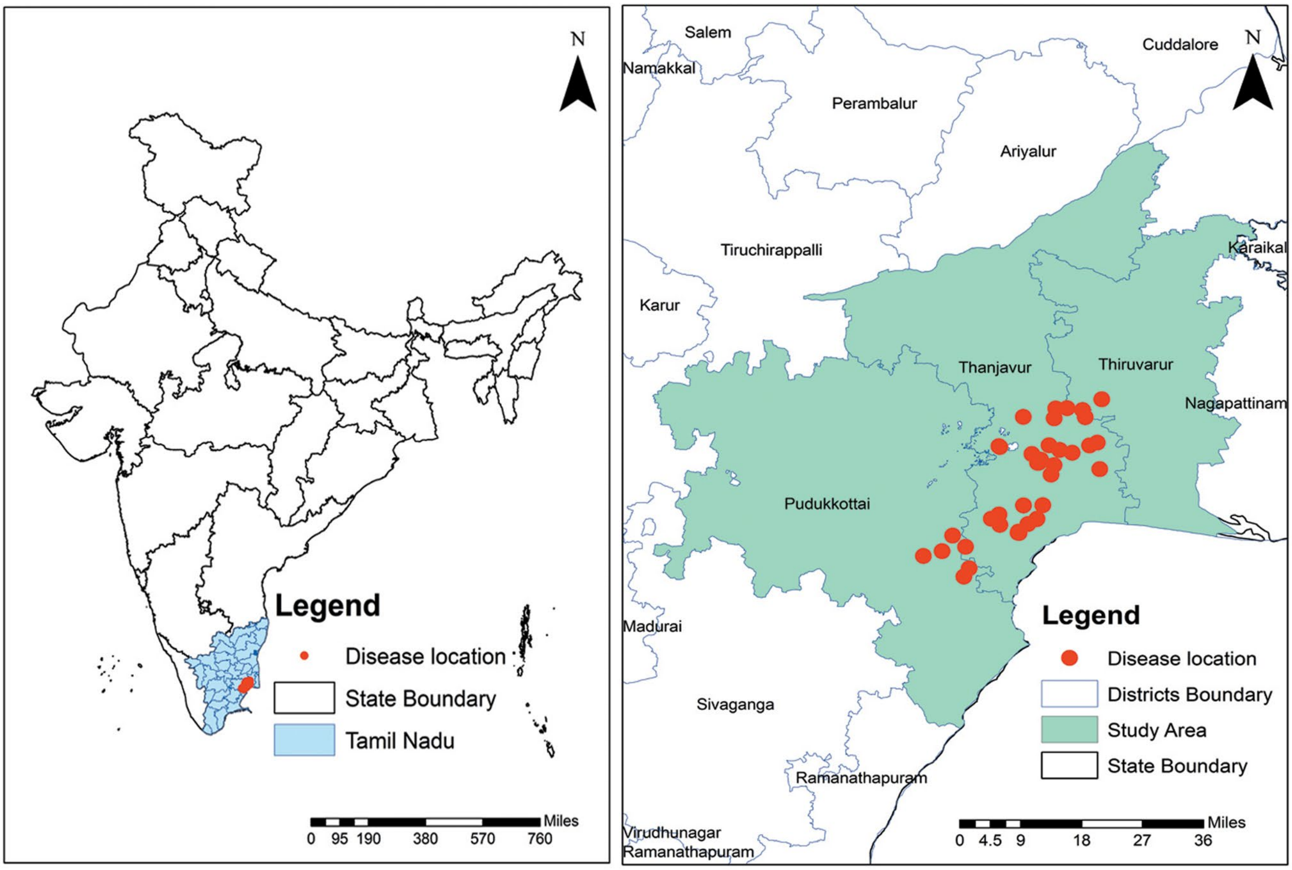

Fig. 2 Distribution of lethal wilt disease (LWD) in Tamil Nadu, India

from coconut in India formed a separate cluster with the Weligama wilt phytoplasma reported from Sri Lanka.

The identity of the pathogen was further confirmed by the molecular characterization of translation elongation factor (*tuf*) and ribosomal protein (*rp*) genes. The symptomatic palms yielded the expected amplicon of approximately 900 bp for the translation elongation factor using specific primer pairs in the nested PCR. The partial sequences for *tuf* gene of LWD phytoplasmas (GenBank: MK614890, MT108188) shared more than 99% identity with aster yellows group (AY) phytoplasma (GenBank: MG744259, MH061368, KP219000). In the phylogenetic tree constructed using *tuf* sequences of AY group phytoplasmas, the LWD phytoplasma formed a subclade in *tufI*-B subgroup (Fig. 4).

Nested PCR with primer pairs specific to the ribosomal protein yielded amplicons of approximately 800 bp. The amplified fragments (GenBank: MK614889, MT090178) contained partial sequences of ribosomal protein L22 (*rpl22*) and ribosomal protein S3 (*rps3*) of LWD phytoplasma. The *rpl22* sequence shared 100% identity with that of AY group phytoplasma (GenBank: CP035949, MH061366, KY689740). The partial sequences of *rps*3 shared more than 99% similarity with that of Goldenrain phytoplasma isolate GRP (GenBank: FJ705831). Phylogenetic analysis using partial sequences of ribosomal protein revealed the delineation of LWD phytoplasma isolates into a separate cluster along with the *rpI*-L subgroup phytoplasma isolates, viz., Maize bushy stunt phytoplasma MBS and the Iranian isolates affecting carrot (CADI), niger seed (NiG2) and onion (SOZ1) (Fig. 5).

Discussion

The results of the present study established the association of phytoplasma with the lethal disease of coconut in India. The symptoms of LWD were similar to the already reported lethal yellowing (LY) and lethal yellowing-type phytoplasmal diseases of coconut palm. In the present study, the detection rate of LWD phytoplasma was higher in inflorescence samples than spear leaves, perhaps due to the high titre of phytoplasma in the inflorescences of LWD-affected palms; an uneven distribution of phytoplasma in palms due to movement of the phytoplasma from source to sink tissues has been reported (Oropeza et al. 2011; Parthasarathy 1974;

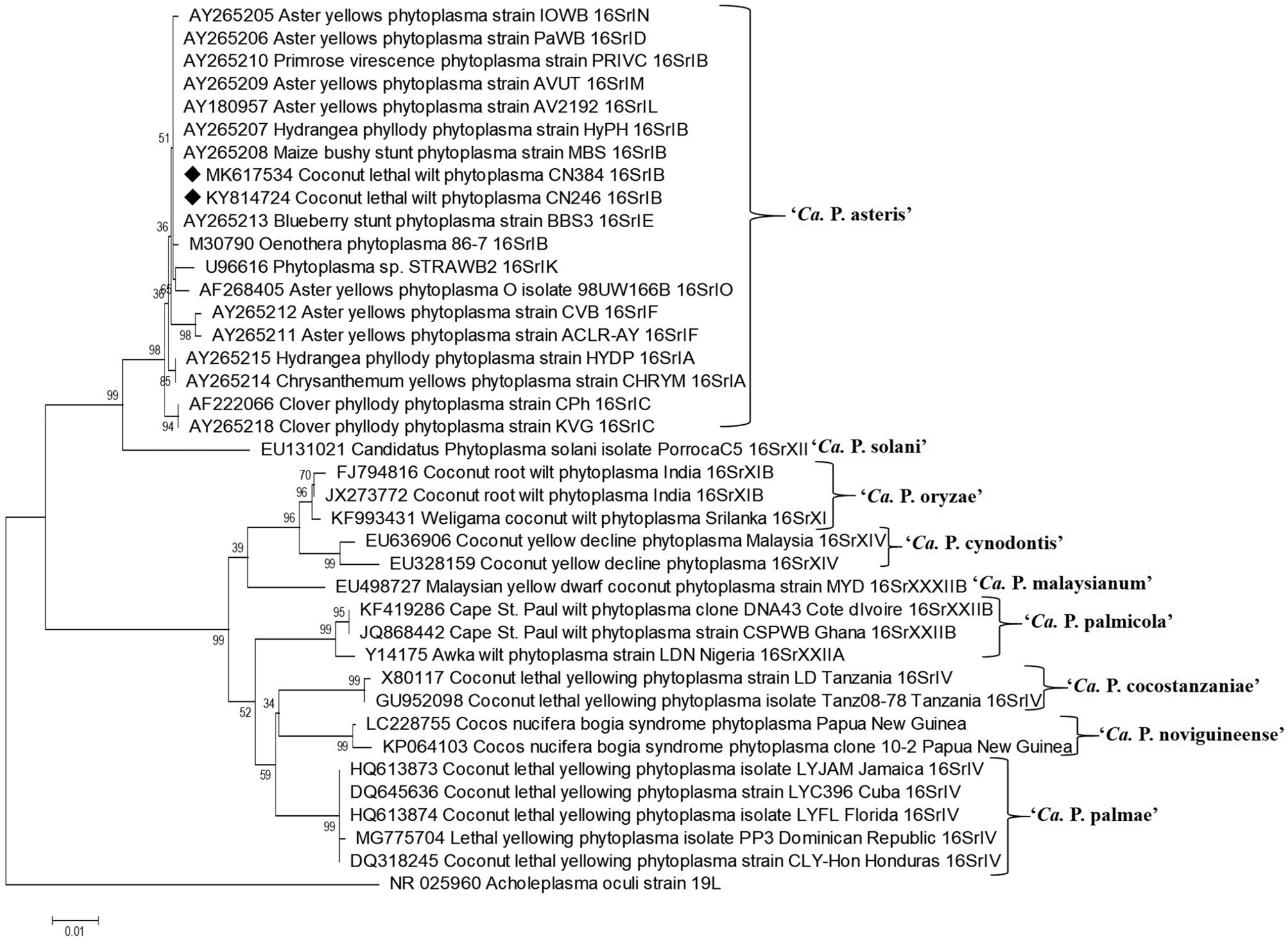

Fig. 3 Phylogenetic tree constructed using 16S rDNA sequences of aster yellows group and various other groups of phytoplasmas that infect coconut

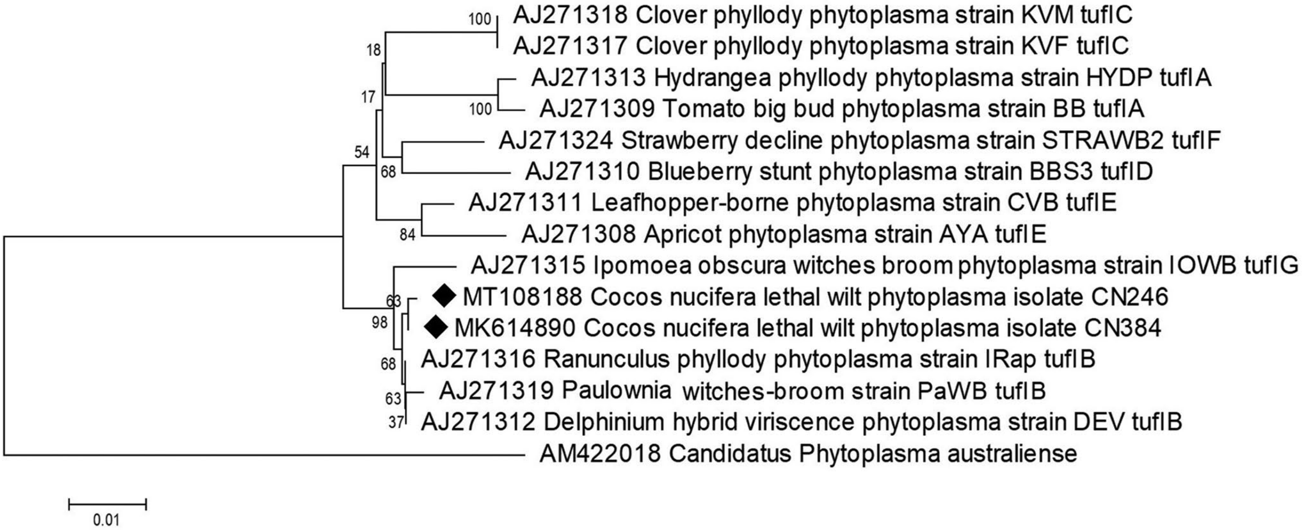

Fig. 4 Phylogenetic tree constructed by the neighbour joining method using *tuf* gene sequences of '*Ca*. P. asteris' isolates

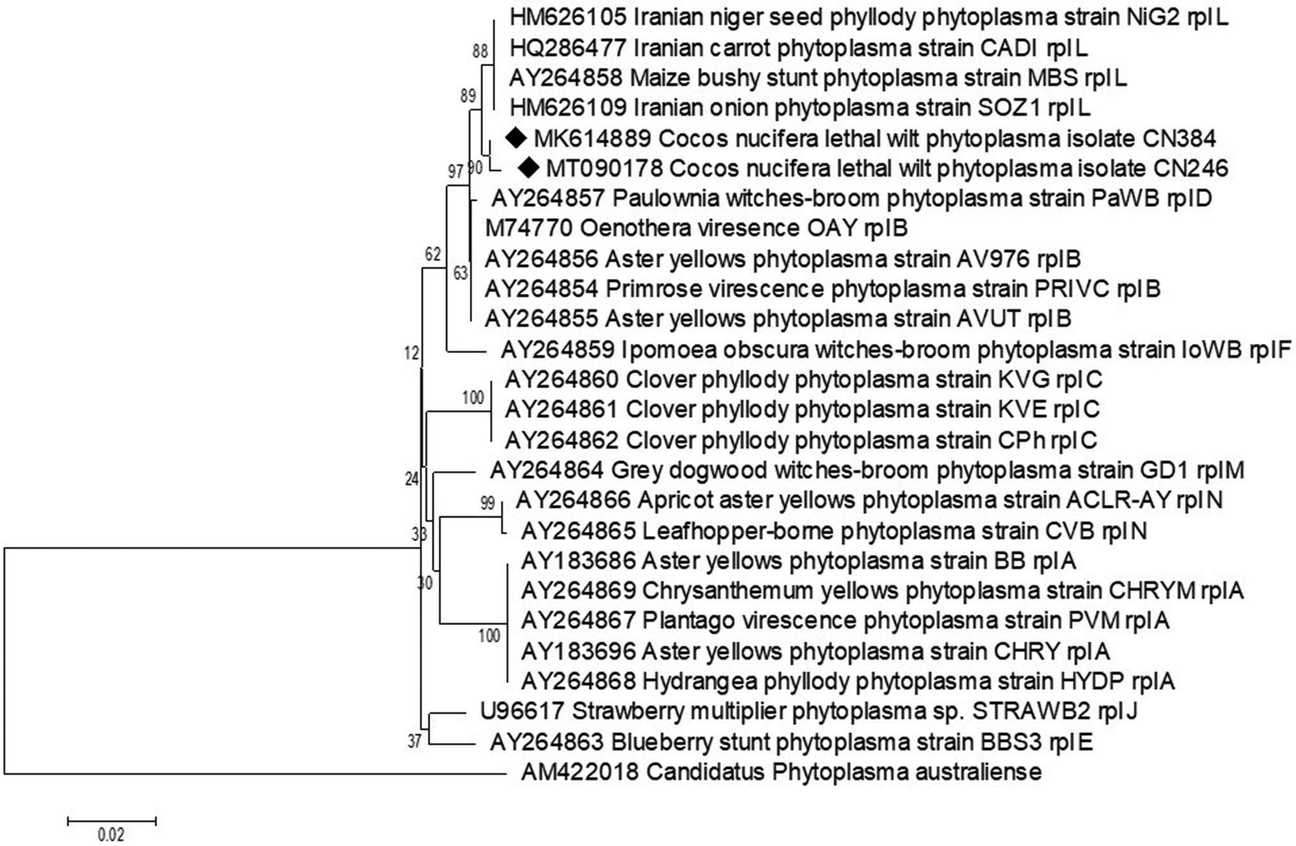

Fig. 5 Phylogeny based on the ribosomal protein gene (*rp*) from the lethal wilt disease (LWD) phytoplasma

Thomas 1979). Cordova et al. (2014) reported the highest concentration of phytoplasma in trunk of LY-affected coconut palms followed by primary root apex, inflorescence, spear leaf, flag leaf, and mature leaf in the decreasing order.

Worldwide coconut palm is affected with phytoplasmas belonging to diverse groups, viz., '*Ca.* P. palmae' (groups 16SrIV-A, -B, -E), '*Ca.* P. cocostanzaniae' (16SrIV-C), '*Ca.* P. palmicola' (16SrXXII-A, -B), '*Ca.* P. oryzae' (16SrXI), '*Ca.* P. cynodontis' (16SrXIV), '*Ca.* P. malaysianum' (16SrXXXII-B), and '*Ca.* P. noviguineense' with varied expression of symptoms as well under each group (Arocha-Rosete et al. 2014; Bertaccini et al. 2014; Harrison et al. 2014; Nejat et al. 2013; Perera et al. 2012; Wijesekhara et al. 2013). Root (wilt) disease (RWD) and tatipaka are the two phytoplasma diseases of coconut reported from India (Solomon et al. 1983; Solomon and Geetha 2004). The phytoplasma associated with RWD belongs to group 16SrXI (Manimekalai et al. 2010) and differs from the LWD phytoplasma taxonomically and symptomatically. Flaccidity, yellowing, and marginal necrosis of leaflets are typical symptoms of RWD, so the symptoms of LWD are quite distinct from RWD. RWD is a nonlethal, debilitating disease, whereas LWD kills the palm within 5 months. The results of the present study confirmed that the phytoplasma was consistently associated with LWD-affected palms. To our knowledge, this is the first report on the identification and characterization of the phytopathogenic mollicute associated with lethal wilt disease of coconut palm in India.

'*Ca.* P. asteris' (16SrI) has been reported to be associated with several crops including palms (Alvarez et al. 2014; Lee et al. 2004; Mehdi et al. 2012). It is one of the most diverse group of phytoplasmas with a very broad host range. Recently, Kwadjo et al. (2018) reported a mixed/sole infection with 16SrI on four palms affected by Côte d'Ivoire lethal yellowing (CILY). The phytoplasma associated with LWD has been identified as '*Ca.* P. asteris' based on multigene sequencing and virtual RFLP of 16S rDNA. This forms the first report of the association of subgroup 16SrI-B phytoplasma ('*Ca.* P. asteris') with lethal wilt disease of coconut in the world.

Among the 10 16Sr phytoplasma groups reported so far from India, 16SrI is the most prevalent and affects around 64 plant species (Rao et al. 2017b). The 16SrI-B isolates of '*Ca.* P. asteris' in India that were reported to be associated with sandalwood spike (Khan et al. 2008), black pepper yellows (Adkar-Purushothama et al. 2009), oil palm stunting (Mehdi et al. 2012), sesamum phyllody (un Nabi et al. 2015), and maize leaf redness (Rao et al.

2017a) diseases share more than 99% identity with the 16S rDNA sequences of coconut LWD isolates. The *rpl-L* and *tufI-B* subgrouping of Coorg black pepper yellows phytoplasma (CBPYp) that is associated with yellows disease of black pepper in southern India (Adkar-Purushothama et al. 2011) were found to be similar to that of LWD phytoplasma, warranting in-depth studies to establish evolutionary relationships and host range of the pathogen. Despite the associated pathogen belonging to a very common 16Sr group, the disease is spreading very slowly; hence, coconut is presumed to be a dead-end host. The possibility of cross transmission will be further examined because several plant species that are reported as hosts of '*Ca*. P asteris' viz., sesamum (Singh et al. 2018; un Nabi et al. 2015), maize (Bedendo et al. 2000; Rao et al. 2017a), eucalyptus (Salehi et al. 2016), and rice (Valarmathi et al. 2013) are prevalent in LWD-affected tracts. The vector(s) involved in LWD transmission and factors determining the rate of spread have to be elucidated for evolving strategies to prevent a sudden upsurge in disease incidence in ensuing years. We could not clearly associate any insect vectors with the disease, but intensive investigations are ongoing to identify the vectors. Recently, *Nedotepa curta* Dmitriev (Hemiptera: Cicadellidae: Typhlocybinae) was suspected as a potential vector of the Côte d'Ivoire lethal yellowing phytoplasma in coconut palms under sole or in mixed infection with a '*Ca*. P. asteris'-related strain, but its phytoplasma-vectoring ability could not be ascertained (Kwadjo et al. 2018). However, this species or its close relative has not been observed in the Indian subcontinent so far. The insect vectors of coconut lethal yellowing continue to be elusive; long-term studies of more than 50 years in the Caribbean, Central America and the southern United States have implicated some species but were not validated through experimental transmission (Wilson 2019).

The consistent association of phytoplasma in LWD palms warrants a thorough look for vectors involved in disease transmission in the LWD-endemic regions in Tamil Nadu and the development of an emergency preparedness module to advise the farming community in the region. Mapping of the diseased palms provided preliminary information on the distribution of the disease. Uprooting infected palms is the first step in a disease eradication strategy. Breeding tolerant coconut varieties is a viable option for pluralistic strategies and integrated farming systems to decrease the risk to farmers in the long run. The endemic nature of the disease, confined within the eastern coast of Tamil Nadu for more than a decade highlights the need for a very close scrutiny of potential insect vectors. Diseases caused by aster yellows group phytoplasma are reported from different plant species across varied agro-ecological zones and it indicates a need to focus on insect vectors that are likely to greatly impact disease epidemics and management options.

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Data availability The nucleotide sequence data reported are available in the GenBank database under accessions KY814724, MK617534, MK614890, MT108188, MK614889 and MT090178.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/ or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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