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Characterization of Aster Yellows Phytoplasma Affecting *Cycas revoluta* in Egypt

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ABSTRACT

In 2015, Pale yellowing symptoms were observed on *Cycas revoluta* (family: Cycadaceae) leaves rachis and lamina grown as ornamental plant in gardens of the Alexandria Governorate, Egypt. Since the symptoms were suggestive the phytoplasma presence, samples were tested by PCR amplification of 16S rRNA gene nucleotide sequences using phytoplasma universal primers P1/P7 in direct, and in nested R16F2n/R16R2 PCR. RFLP-PCR product of the nested primer with *Mse*I restriction enzyme and phylogenetic analyses of 16S rRNA gene nucleotide sequences suggested that the phytoplasma of Aster yellows designated in 16SrI group. Various 16Sr groups of phytoplasmas, e.g. 16SrI, 16SrII, 16SrX, 16SrXII, and 16SrXVII, are recognized to be associated in abnormalities of ornamental plants in many regions.However, this study reports for the first time the incidence of 16SrI phytoplasma in association with aster yellows disease in *Cycas revoluta* grown in Egypt.

INTRODUCTION

Cycads are an ancient group of gymnosperms, and in fact they are increasingly grown as ornamentals in many countries In India 2012, '*Candidatus* Phytoplasma aurantifolia ' related to group16SrII has been noted closely associated with *Cycas* and *Zamia* (Kumar *et al*., 2012). Cycads phytoplasms, including the groups of 16SrII or 16SrI, have already been addressed with epidemiological effects that may be critical for incidence disease control in worldwide (Kumar *et al.* 2012; Omar and Alsohiam, 2016).

Previous records indicate that ornamental plants in India, Saudi Arabia, USA and Egypt are particularly susceptible to infection with phytoplasma. '*Candidatus* Phytoplasma aurantifolia' found in *Albizia* and periwinkle related to 16S rII-B group . Meanwhile, *Hibiscus* and *Catharanthus* plants were affaired by papaya yellow crinkle belonging to subgroup 16S rII-D (Omar and Alsohiam, 2016; Behiry and Bertaccini 2017),in addtion, there were leaves defects have been noted with '*Candidatus* Phytoplasma pini' belong to group 16S rV and '*Candidatus* Phytoplasma phoenicium' related to group16Sr IX-E (Schneider *et al*., 2005; Davis *et al*., 2010). Moreover, *Araucaria heterophylla* little leaf and stunting have been linked with '*Candidatus* Phytoplasma tripoli' (group 16SrVI) (Gupta *et al*., 2010). Considering the disease noted in different regions, collections were conducted from symptomless and infected cycas plants in the gardens of Alexandria Governorate in 2015 to further lessen the chances of phytoplasma incidence, ancestry and disease correlation.

MATERIALS AND METHODS

The CTAB method was used to extract the genomic DNA from leaves middle vein of four infected and five asymptomatic *C. revoluta* plants as previously described by Saghai-Maroof *et al.* (1984). PCR reactions were conducted using universal primer pairs amplifying 16S rRNA gene, in direct PCR reactions (R16mF2/R16mR1) or P1/P7 (Gundersen and Lee, 1996; Deng and Hiruki, 1991). The employed direct primer pairs , quickly followed by nested PCR assay using R16F2n/R16R2 primers under reported conditions (Gundersen and Lee, 1996). PCR reactions were conducted using sense and antisense primer pair R16F2n/ R16R2, then the nested primer pairs R16(I)F1/R1 or 16R758f/16S1232r (=M1/M2) were used to amplify the desired fragment (Lee *et al*., 1994; Gibb *et al*., 1995). A negative control, sterile distilled water (SDW) and phytoplasmas DNA positive controls provided from Prof. Bertaccini, Bologna University,Italy (Bertaccini, 2014) e.g. Paulownia withes'-broom from Taiwan PaWB (AY265206, 16SrI-D); Ash yellows Ash Y from New York (X68339, 16SrVII-A) were used in each assay.

A 25 µl PCR reaction tube accomplished by add 12.5 µl master mix (iNtron, Korea), 0.5 µl of each primer (Macrogen inc., Korea), 1 µl of total DNA (10–50 ng) and finally high molecular grade water up to vol. 25 µl. A 10 µl of the amplified products were subject to run in 1% agarose gel, 0.5 X TAE Buffer, stained with ethidium bromide, washed with dioinized water and finally visualized on transilluminator. On the other hand, Restriction fragment length polymorphism (RFLP) performed on the positive amplicons amplified by primer pairs M1/M2 or R16(I)F1/R1, were subjected to digestion with the restriction enzyme (*Mse*I) as described by manufacturer (BioLabs, MA, New England). RFLP digestions were run into 6.7% acrylamide gels,electrophoresed, stained and visualized as mentioned above. Other amplicon products amplified by R16F2n/ R16R2 primer pair were purified and sequenced by Macrogen, Inc., Korea. Phylogenetic relationships were analyzed using the 16S rRNA phytoplasmas nucleotide sequences cited from *C. revoluta* (*Cycas* yellowing phytoplasma), and 46 other 16S rDNA reference phytoplasmas nucleotide sequences obtained from GenBank using MEGA 6 maximum parsimony method. *Acholeplasma laidlawaii* bacteria were used to root the phylogenetic tree.

RESULTS AND DISCUSSION

In multiple gardens or orchards of Alexandria Governorate, Egypt, *Cycas* plants showing pale yellowing symptoms suggestive of a phytoplasma disease. More reliability and sensitive results were obtained by the sequence of amplification primers (R16F2n/R16R2 -direct PCR) and (R16(I)F1/ R1 or M1/M2 -nested PCR) than the other primer amplification priming sequence (P1/P7 direct) then

(R16F2n/R16R2 as nested) which, were less worthwhile and influential priming (partially data not shown). All the symptoms-bearing plants resulted positive to presence of aster yellows-related phytoplasmas. RFLP of strains amplified with M1/M2 allow identifying phytoplasma group as 16SrI as reported by Bertaccini, (2007) Fig. 1.

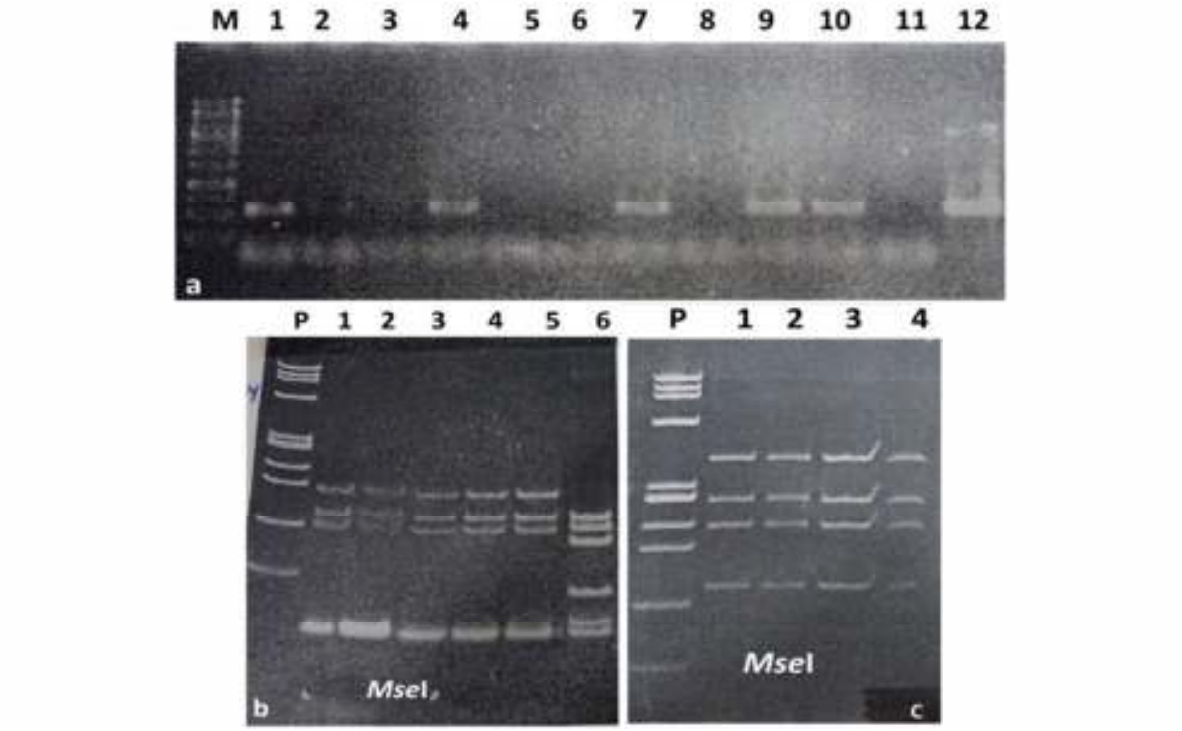

Fig.1. Agarose 1% gel with results of double nested PCR amplification using a) the primers M1/M2 with Cycas samples; 1, 2,3,4,5,6,7,8,9, 10; Paulownia withes'-broom PaWB (16SrI-D),11 ; SDW, 12; Ash yellows AshY (16SrVII-A); M, band sizes of 1 kb DNA ladder from top to bottom in base pairs (bp) 10,000; 8,000; 6,000; 5,000; 4,000; 3,000; 2,500; 2,000; 1,500; 1,000; 750; 500 and 250., in b) Restriction Fragment Length Polymorphism (RFLP) pattern of 6.7% acrylamide gel dedicated from M1/M2 primer pair products digested with *Mse*I restriction enzyme listed at the bottom of the figure from *Cycas* yellows phytoplasma (1, 2 , 3 and 4) ; 5, Paulownia withes'-broom PaWB (16SrI-D); 6, Ash yellows AshY (16SrVII-A), in c) RFLP patterns of R16(I)F1/ R1 primer pair products digested with *Mse*I from *Cycas* yellows phytoplasma (1, 2 , 3 and 4) P, marker phiX174 *Hae*III the bands base pairs from top to bottom 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72

The nucleotide sequences of 4 amplicons obtained from R16F2n/R16R2 fragments were deposited in GenBank under accession numbers MH985240-MH985243 (1050 bp). The NCBI-BLAST analyses revealed that the homology of the16S rRNA nucleotide sequences of *Cycas* yellowing phytoplasmas were about 99% in samilarity with the other aster yellow phytoplasmas belonging to group 16SrI and have a distinct subgroup by 93% identity with chrysanthemum yellows phytoplasma CHRYM AY265214 16SrI-A. It could be concluded that a new subgroup might be found as reported by Wei *et al*. (2007), A completely new subgroup becomes affirmed when a phytoplasma strain has a F value equal to or less than 0.97 compared to all existing truly representative strains of a given group. The *Cycas* yellow phytoplasmas have F values varied from 0.91 to 0.93 in regards to the phytoplasmas belonging to one of 16SrI subgroups, possibly leading us to suggest that our phytoplasma defines a totally new subgroup. Phylogenetic bootstrap tree verfied the nucleotide sequence similarity analysis since the four *Cycas* yellowing phytoplasmas nucleotide sequences clustered together in different phylogenetic branch of 16SrI phytoplasmas (Fig. 2).

This may the first notation of *Cycas revoluta* to be affected by the group of 16SrI aster yellows phytoplasmas, the most widespread phytoplasma group in the world. In this study we suggest a new subgroup presence of phytoplasma enclosed in 16SrI group according to the sequence analysis .However, we could not confirm that suppose because we need more multigenic studies and condensed RFLP characterization to confirm that is a new subgroup or not meanwhile, there are phytoplasmas has been reported previously inserted in related 16SrI subgroup in Italy (Chrysanthemum yellows, CHRYM 16SrI-A) and in Canada (Clover phyllody, CPh ,16SrI-C) (Lee *et al.* 1998; Bertaccini, 2014) in Chrysanthemum and Clover respectively. Finally, in brief, more work is needed to improve if this phytoplasma could induce the symptoms as noticed in the gardens or there is an association with another disease or not.

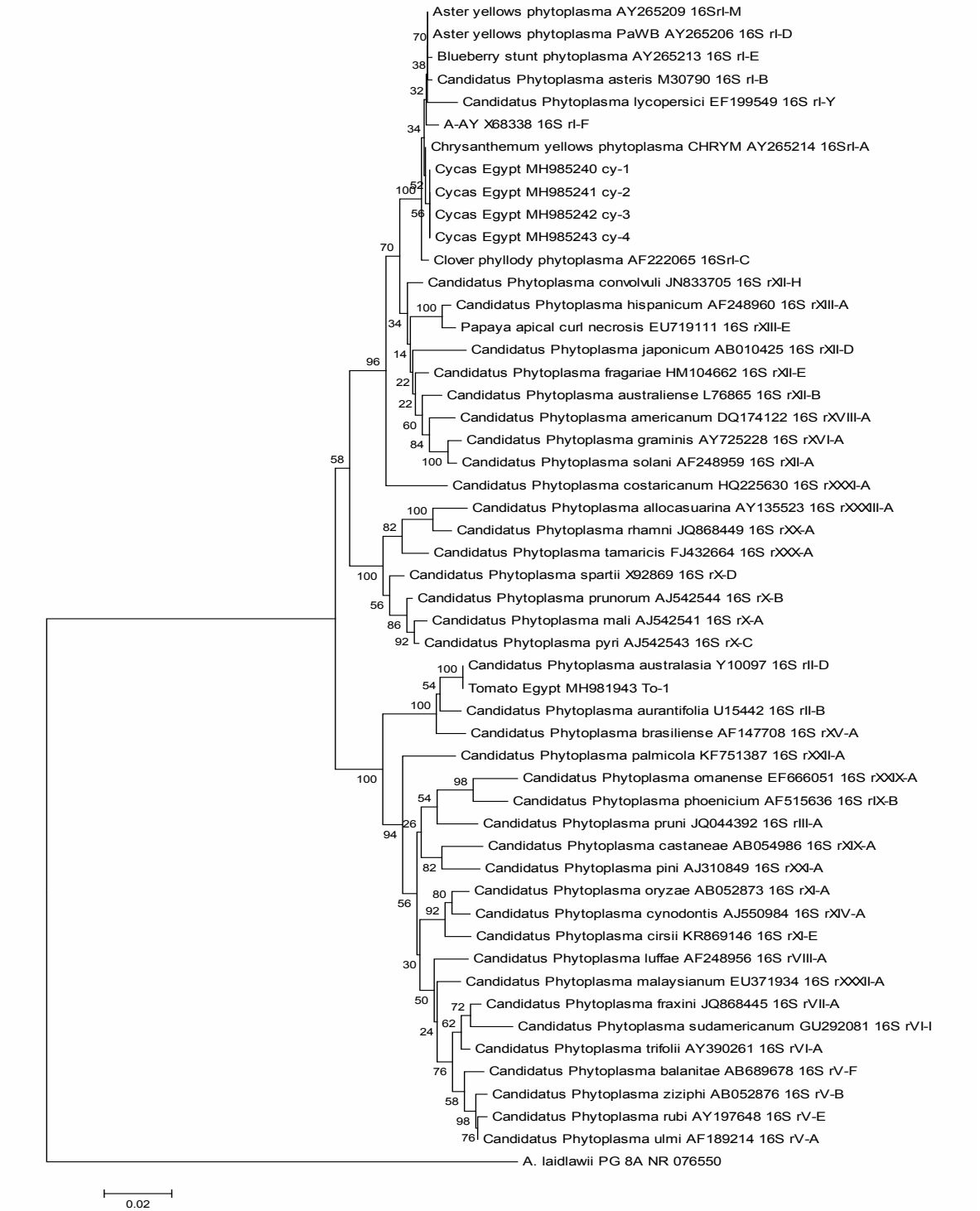

Fig. 2. A bootstrap rooted molecular phylogenetic tree with high values showing the maximum likelihood of aster yellows group 16SrI sequences and forty-seven '*Candidatus* Phytoplasma' closely related species. *Cycas* yellows phytoplasmas (Egypt) distinct clustered in one group. *Acholeplasma laidlawii* bacteria used to root the tree. On the branch far right the strains accession number and the 16S rRNA group.

REFERENCES

Behiry, S.I. and A. Bertaccini (2017). Detection and characterization of phytoplasmas infecting five plant species in Egypt. Phytopathogenic Mollicutes 7 (2), 101-105 doi: 10.5958/2249-4677. 2017. 00014 .7

Bertaccini , A. (2007). Phytoplasmas: diversity, taxonomy, and epidemiology. Frontieres in Bioscience, 12: 673-689.

Bertaccini, A. (2014). Phytoplasma collection. International Phytoplasmologists working group web. http://www. ipwgnet.org/ collection Accessed 10 Oct. 2017

Davis, R.E.; E.L. Dally; Y. Zhao; I-M. Lee; R. Jomantiene; A.J. Detweiler and M.L. Putnam (2010). First report of a new subgroup 16SrIX-E ('*Candidatus* Phytoplasma phoenicium'-related) phytoplasma associated with juniper witches' broom disease in Oregon, USA. *Plant Pathology* 59, 1161. [http:// dx. doi. org/ 10.1111 /j.1365-3059.2010.02294.x]

Deng, S. and C. Hiruki (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. Journal of Microbiology Methods, 14: 53-61.

Flores, D.; I.C. Haas; M.C. Canale and I.P. Bedendo (2013). Molecular identification of a 16SrIII-B phytoplasma associated with cassava witches' broom disease. European Journal of Plant Pathology, 137: 237–242.

Gibb, K.S.; A.C. Padovan; and B.D. Mogen (1995). Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plant species growing in Northern Australia. Phytopathology, 85: 169-174.

Gilbertson, L. and S.L. Dellaporta (1983). Molecular extraction DNA protocols. Pages 395-397 in: Molecular Biology of Plants. Cold Spring Harbor Laboratory, Cold Spring

Gundersen, D.E. and I-M. Lee (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopath. Medit. 35:144-151.

Gupta, M.K.; A.Samad; A.K. Shasany; P.V. Ajayakumar and M. Alam (2009). First report of a 16SrVI *'Candidatus* Phytoplasma trifolii' isolate infecting Norfolk Island pine (Araucaria heterophylla) in India. Plant Pathology 59, 399. [http: // dx.doi.org /10.1111 /j.1365-3059.2009.02136.x]

Harbor, N.Y.; D.E. Gundersen and I-M. Lee (1996). Ultrasensitive detection of phytoplasma by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea, 35: 114-151.

Kumar, S.; V. Singh and S. Lakhanpaul (2012). Molecular detection and phylogeny of phytoplasmas affecting cycads in India. New Disease Reports 26, 27. [http://dx.doi.org/ 10.5197/j.2044-0588.2012.026.027]

Lee, I-M.; D.E. Gundersen-Rindal; R.E. Davis and I.M. Bartoszik (1998). Revised classification scheme of phytoplasma based on RFLP analyses of 16S rDNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. 48: 1153-1169.

Omar, A.F. and S.A. Alsohiam (2016). Identification of new hosts of 16SrII group phytoplasmas in Saudi Arabia. Pathogenic Mollicutes 6(2), 71-76

Saghai-Maroof, M.A.; K.M. Soliman; R.A. Jorgenson and A.W. Allard (1984). Ribosomal spacer-length polymorphism in barley: Mendelian inheritance, chromosomal creation and population dynamics. Proceedings of the National Academy of Sciences of the United States of America 81, 8014-8018. [http:// dx.doi.org/ 10.1073/pnas.81.24.8014]

Schneider, B.; E. Torres; M.P. Martin; M. Schröder; H.D. Behnke and E. Seemüller (2005). '*Candidatus* Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis* . International Journal of Systematic and Evolutionary Microbiology 55, 303-307. [http: // dx. doi.org / 10.1099/ijs.0.63285-0]

Wei,W.; R.E. Davis; I.-M. Lee and Y. Zhao (2007). Computer simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. International Journal of Systematic and Evolutionary Microbiology, 57, 1855–1867.

دراسة الفيتوبلازما التابعة لمجموعة اصفرار الأستر والتى تصيب نبات الزينة السيكس ريفولتا فى مصر

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خلال عام 2015 لوحظ اصفرار شاحب على اوراق نبات السيكس ريفولتا فى بعض حدائق محافظة الأسكندرية التابعة لجمهورية مصر العربية ومن ثم تم أخذ عينات مصابة للكشف عن المسبب المرضى المسئول داخل المعمل وبأستخدام جهاز تضاعف البلمرة العشوائية المتسلسل وبعض الكواشف المتخصصة وكذلك تكنيك RFLP المتخلل بأنزيم القطع *Mse*I وبدراسة التتابعات الوراثية المتحصل عليها من جهاز Sequencer تم أثبات أن المسبب المرضى هو نوع من الفيتوبلازما يتبع مجموعة اصفرار الأستر –المجموعة الأولى التصنيفية وتتميز هذه الدراسة بأنها ولأول مرة يتم الكشف عن فيتوبلازما اصفرار الأستر فى نبات الزينة سيكس ريفولتا المنزرع فى مصر