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USE OF GENE SEQUENCING, SOIL CHEMICAL PROPERTIES AND MICROBIAL ANALYSIS TO CONFIRM THE NATURE OF THE SUGARCANE DISEASE *PHYTOPLASMA*S IN THAILAND

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ABSTRACT

Phytoplasmas are a group of plant pathogens that reduce yield in various plants including many crops, shrubs, and tree species. They have been classified into various 16Sr groups or '*Candidatus Phytoplasma* species' based primarily on the sequences of their 16S rRNA genes. However, other non-ribosomal sequences are often used to fine-tune their classifications. This research aimed to clarify the categorization of the sugarcane *phytoplasma*s that cause sugarcane white leaf and sugarcane grassy shoot symptoms in Thailand and determine whether they are different strains, based on non-16S ribosomal genes. Forty samples from plants exhibiting both sugarcane symptoms and from symptomless sugarcane were collected and PCR amplifications were done with primers for the 50S rRNA *Ser* and *SecA* genes moreover soil samples from non-symptom and symptom fields were collected for soil chemical properties and rhizospheric microorganism analysis. The phylogenetic tree results revealed that isolates displaying both symptom types grouped together based on all three sets of primers, including with isolates from other countries. Additionally, soil chemical properties and rhizospheric microorganism analysis results showed that soil chemical factors and rhizospheric microorganisms did not appear to correlate with the differences in symptoms. It is concluded that the two sugarcane symptoms are caused by the same *Phytoplasma* strain and that other, as yet unidentified factors, are responsible for this *Phytoplasma* giving different symptoms in different situations. This is the first evidence of soil chemical properties and rhizospheric microorganism not correlating with the diverse symptoms. As for further study, unidentified factors will be studied including quarantine methods to control the symptoms.

Keywords: sugarcane white leaf, sugarcane grassy shoot, *Phytoplasma*, phylogenetic tree, soil property.

INTRODUCTION

Sugarcane (*Saccharum* sp.) is a gramineous plant and a major crop, used to produce edible sugar and renewable energy. Sugarcane production covers 1.7 million hectares in Thailand and is cultivated in the central, north, north-east and east of the country, with the export trade being second only to Brazil. However, many sugarcane fields are faced with a problem of reduced sugarcane yield caused by *Phytoplasma* infections such as Thailand, Malaysia, Vietnam, Nepal, Pakistan Sri Lanka and India (Yadev *et al.,* 2017). Sugarcane white leaf (SCWL) and sugarcane grassy shoot (SCGS) are two disease symptoms that have been identified caused by *Phytoplasma*s in the 16srXI group, a group that also infects rice, Napier grass and Bermuda grass. Recently, the SCGS has been classified to a new taxon 16Sr XI-B and described as '*Candidatus Phytoplasma sacchari*' but no mention of SCWL was made in this report (Kirdat *et al.,* 2021). Therefore, there is still a question about whether the same or different strains cause the different sugarcane symptoms.

Phytoplasmas are bacteria that lack a cell wall, classified in the *Mollicutes*, and they infect numerous plants including fruits, crops and grasses and are transmitted via insect vectors. They were called *Mycoplasma-like organism (MLO)*

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until the 1980s, and have recently been grouped into the candidate taxon, '*Candidatus phytoplasma*', based on 16S rRNA sequences (Abesysinghe *et al.,* 2016; Yadev *et al.,* 2017). The 16S rRNA is the primary gene used to classify *Phytoplasma*s since it is extremely well conserved and there are now many groups of *Phytoplasma* based on this gene. However, the gene is poor for classification of closely related strains, so researchers have developed alternative techniques to classify closely related strains.

One of these potential techniques is Multilocus Sequence Typing (MLST) that uses non-16S ribosomal genes and sequencing of many other genes to classify, such as the housekeeping genes *dnaK, gryB, leuS, rpIV-rpsC, rpoB, recA, SecY, SecA, uvrB-degV, UvrB* (Arnaud *et al.,* 2007; Li *et al.,* 2014; Pilet *et al.,* 2019). These techniques have been used to show the genetic diversity based on geographic distribution and genetic exchange, and shown the ability of certain *Phytoplasma*s to infect several different plant species. For example, the Peanut witches' broom *Phytoplasma*, which belongs to the 16SrII group, was shown to infect *Crotalaria pallida*, *Tephrosia purpurea* (shrub) and *Cleome Viscosa* (weed) based on the MLST technique (Li *et al.,* 2014). Similarly, MLST distinguished '*CandidatusPhytoplasma* phoenicium', causing almond witches' broom (AlmWB) in Lebanon from strains infecting other host plants that shared 16S rRNA sequences where one gene (*imp*) distinguished AlmWB strains from different host plants (Quaglino *et al.,* 2015); and '*CandidatusPhytoplasma* palmicola' that infects coconuts could be separated into distinct geographically distributed strains using eight housekeeping genes (Pilet *et al.,* 2019)

As described above, SCWL and SCGS are caused by '*CandidatusPhytoplasma*' and there have been attempts to determine whether the diseases are caused by one or two strains. Previous studies reported SCWL and SCGS in Thailand as being caused by different *Phytoplasma*s based on 16S rRNA (Wongkaew *et al.,* 1997) whereas based on the 16S-23S intergenic region, *Phytoplasma*s found in China and India were shown to belong to the same group (Nasare *et al.,* 2007; Li *et al.,* 2014; Wang *et al.,* 2014). As describe above, 16S/23S rDNA of SCGS was closely related to SCWL, therefore, this study aimed to classify SCWL and SCGS based on sequencing of the *50S rRNA, Ser* and *SecA* genes in different regions of Thailand and determine whether different soil properties and soil microorganism might affect the different symptom expression. This finding will have noteworthy implications for defining host factors and lead to preventing the diseases.

MATERIALS AND METHODS

Sample collection: Forty sugarcane diseased samples, collected from one of two cultivars, Khon Kaen-3 (KK3) or Utong-3 (UT3) (which are the most extensively cultivated in Thailand), showing sugarcane white leaf and sugarcane grassy shoot, along with control uninfected samples, were collected from eleven provinces in Thailand; Udon Thani (UD, KK3), Khon Kaen (KK, KK3), Buriram (Br, KK3), Uthaithani (UT, KK3), Nakhon Sawan (NSK, UT3), Phitsanulok (Psk, UT3), Pethchabun (PB, UT3), Lop Buri, (LB, KK3), Kanchanaburi (KCB, KK3), Chon buri (CB, KK3), Sa Kaeo (SK, KK3) where there are many fields cultivated for sugar refining (Figure 1), and the samples were stored at -20°C for further study. Interestingly, KK3 and UT3 not only produce high yield of sugarcane stems including Commercial Cane Sugar, C.C.S, but also have unique character; KK3 is drought tolerant whilst UT3 is resistant to smut disease and red rot disease, so they are chosen for cultivation in different parts of Thailand depending on soil and temperature area.

DNA extraction : One gram of sugarcane disease leaves was used for total genomic DNA extraction using the cetyltrimethylammonium bromide (CTAB) method (Tiwari *et al.,* 2012). DNA samples were separated on 0.8% agarose gel for 20 min and stained with ethidium bromide before being placed on a gel documentation system (Cleaver Scientific micro DOC Gel Documentation System, Ireland). The total genomic DNA samples were diluted with 1x Tris-EDTA buffer to 10 ng/µl and stored at –20˚C for further study. In addition, DNA samples collected in previous studies in Sri Lanka (Abesysinghe *et al.,* 2016) and Vietnam (Quoc *et al.,* 2021) were also used for the sequence analyses.

Non-16S ribosomal RNA gene amplification: Primers used to analyze samples showing sugarcane symptoms are shown in Table 1. The PCR mixtures contained 10 µl of 2x Tiangen PCR mixture (Tiangen, China), 1 µl of each primer (10 mM) and 10 ng of template DNA, and then ddH_2O was added to obtain a final volume of 20 µl. Firstly, DNA samples were subjected to PCR with universal 16S rRNA primers, P1/P7 and R16mF2/ R16mR1, to confirm infected samples. P1 and P7 were used in the first round of the reaction and the reaction consisted of one cycle of 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 sec and one cycle of 72°C for 10 min (VeritiPro™ Thermal Cycler, USA). After that, 1 µl of the first-round reaction was used in the second round with R16mF2/R16mR1 primers at annealing temperature 60°C and subsequently the PCR products were visualized under gel documentation (Benchtop UV Transilluminator, Taiwan).

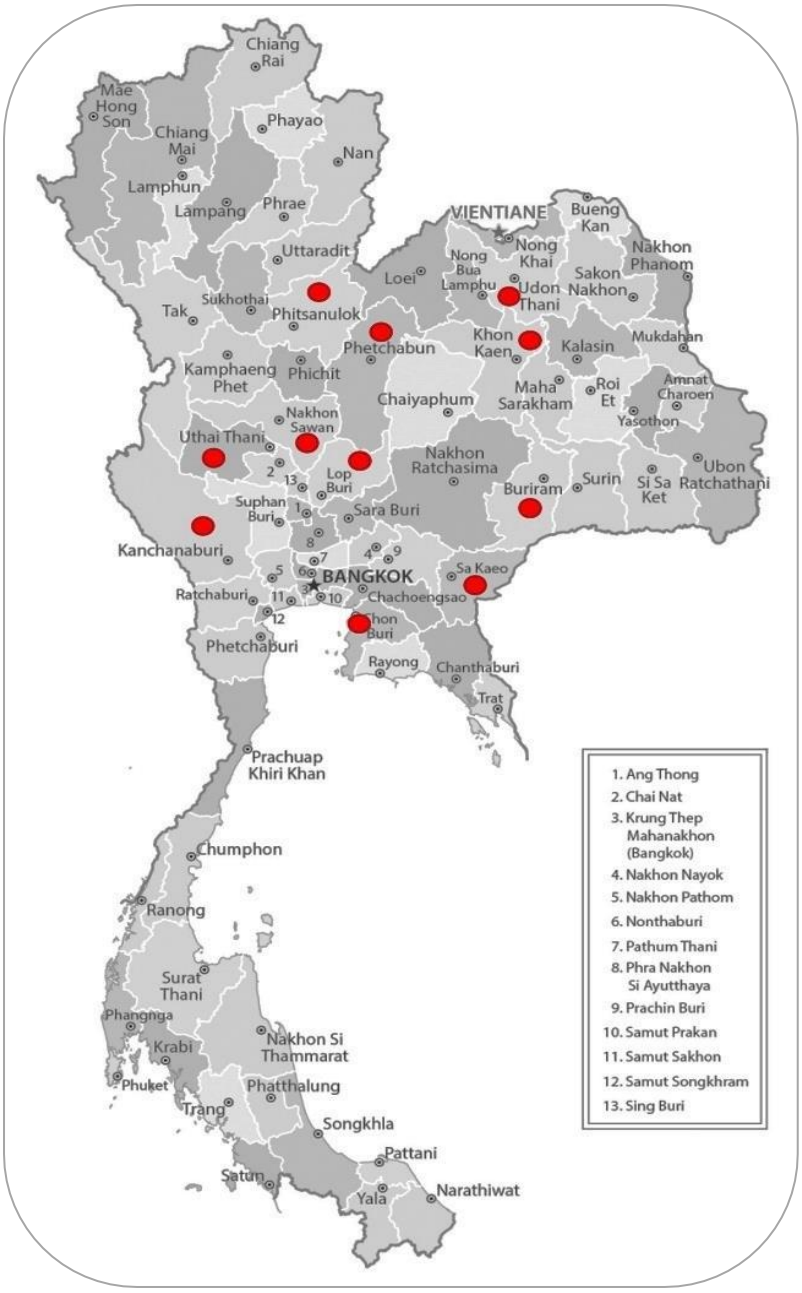

Figure 1. Geographical regions to collect samples in Thailand (red dots). Source : https://en.wikipedia.org/ wiki/ Provinces_of_Thailand

Positive samples and negative controls were then amplified with specific 50S, Ser and SecA primers. The first round of 50S PCR amplification consisted of one cycle of 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and one cycle of 72°C for 10 min with 50Sfor1/50Srev1 primers subsequently 2 µl of the first round was used in nested PCR with 50Sfor2/50Srev1 at the same temperature.

Serfor1 and Serrev1 first round PCR was done at 50°C and 2 µl of the PCR product was taken as template for the second round nested PCR, done with Serfor2 and Serrev2 primers at the same annealing temperature.

For the *SecA* gene, the samples and control first round PCR were done at annealing temperature 55°C with Secfor1/ SecArev3 primers, and the nested PCR was done with Secfor5/ SecArev2 primers at annealing temperature 55°C. The PCR products were analyzed using 2% agarose gel electrophoresis, stained with ethidium bromide before visualization on a gel documentation system and sequenced by ATGC Co. Ltd., Thailand.

Table 1. List of primer sequences used in this study.

Primer name	Primer sequences (5′-3′)	References
P1 forward	GTCGTAACAAGGTATCCCTACCGG	Deng and Hiruki,1991
P7 reverse	CGTCCTTCATCGGCTCTT	Smart *et al.,* 1996
R16mF2 forward	GAACGACTGTAAGACTGG	Lee *et al.,* 1993
R16mR1 reverse	TGACGGGCGGTGTGAVAAACCCCG	
50Sfor1	CCTACATCWAAYGGRCATCG	Sangsuwan, 2020
50Sfor2	AGACATCGYGGMGGWGG	
50Srev1	TACCCCAMGGMGTCATAGG	
Serfor1	TACCKAATWTACCWCATG	
Serrev1	GTTCTKCCTAYAGCTAAAGC	
Serfor2	TGAAASARATGGGAGC	
Serrev2	CMGMATTACTAATAGARGC	
SecAfor1	GARATGAAAACTGGRGAAGG	Abesysinghe *et al.,* 2016
SecAfor5	ASTCGTGAAGCTGAAGG + AGCTAAAAGAGAATTTGAAGG	
SecArev3	GTTTTRGCAGTTCCTGTCATNCC	
SecArev2	CCNTCRCTAAATTGNCGTCC	

Phylogenetic tree construction: Nucleotide sequences of each gene including sequences from NCBI GenBank were aligned using the ClustalW program (Bioedit program). Phylogenetic trees were constructed using Mega X software (www.megasoftware.net) with a neighbor-joining program. A thousand replicates were performed, and bootstrap values were calculated to represent the node stability and support the inferred clusters. Bootstrap values of 50 to 74% indicated weak support, 75 to 84% indicated moderate support and 85 to 100% indicated strong support (Richardson *et al.,* 2000).

Soil collection: Ten soil samples were collected from five provinces, Udonthani (UD), Khon Kean (KK), Pethchabun (PB), Kanchanaburi (KCB) and Chon Buri (CB) that showed SCWL and SCGS including one soil sample from non-symptom fields. Soil was collected at 15 cm depth under the sugarcane and wrapped with aluminum foil until analysis.

Soil chemical properties: Methods for soil chemical property analysis were selected from standardized procedures. Briefly, soil samples were analysed for pH (pH meter STARTER 2100, China), Electric conductivity (EC) (Electrical Conductivity Meter Model 11A, China) and available potassium following the methods of Nation soil survey centre (Soil survey staff, 1996). The available phosphorus analysis followed the BrayII method (Bray and Kurzt, 1945) whereas organic matter followed the Walkley-Black Titration (Walkley and Black, 1934; Nelson and Sommers, 1996).

Rhizospheric soil microorganism analysis: Ten grams of each sample of rhizosphere soil were dissolved in sterilized distilled water and then the diluted samples were put through 10-fold serial dilutions. The dilutions were plated on tryptic soy agar (TSA) (Himedia, India) medium by pour plated method for bacterial total count in triplicate and then the TSA plates were incubated at 35°C for 48 hr. Subsequently, total colony numbers were counted and calculated to colony forming unit.

For fungi and yeasts, the 10-fold dilutions were plated on Dicholan Rose Bengal Chloramphenicol Agar (DRBC) (HiMedia, India) by pour plated method and then incubated at room temperature for 3-5 days after which, total colony number was counted and converted to colony forming unit.

RESULTS

Sugarcane disease samples were collected in many areas in Thailand, showing sugarcane white leaf (SCWL) and sugarcane grassy shoot (SCGS) symptoms. The major symptom in Thailand is sugarcane white leaf, where the leaf is chlorotic, causing non photosynthesis, and the shape is slender (Figure 2A, 2B), whereas sugarcane grassy shoot disease shows numerous green tillers resulting in a failure to form mature stalks (Figure 2C). Additionally, some plants showed white leaf and stunted growth. Even though the pathogen for SCGS has previously been classified into a new taxon, *'Ca. Phytoplasma sacchari*', there is ongoing debate as to whether one or two strains cause the different diseases.

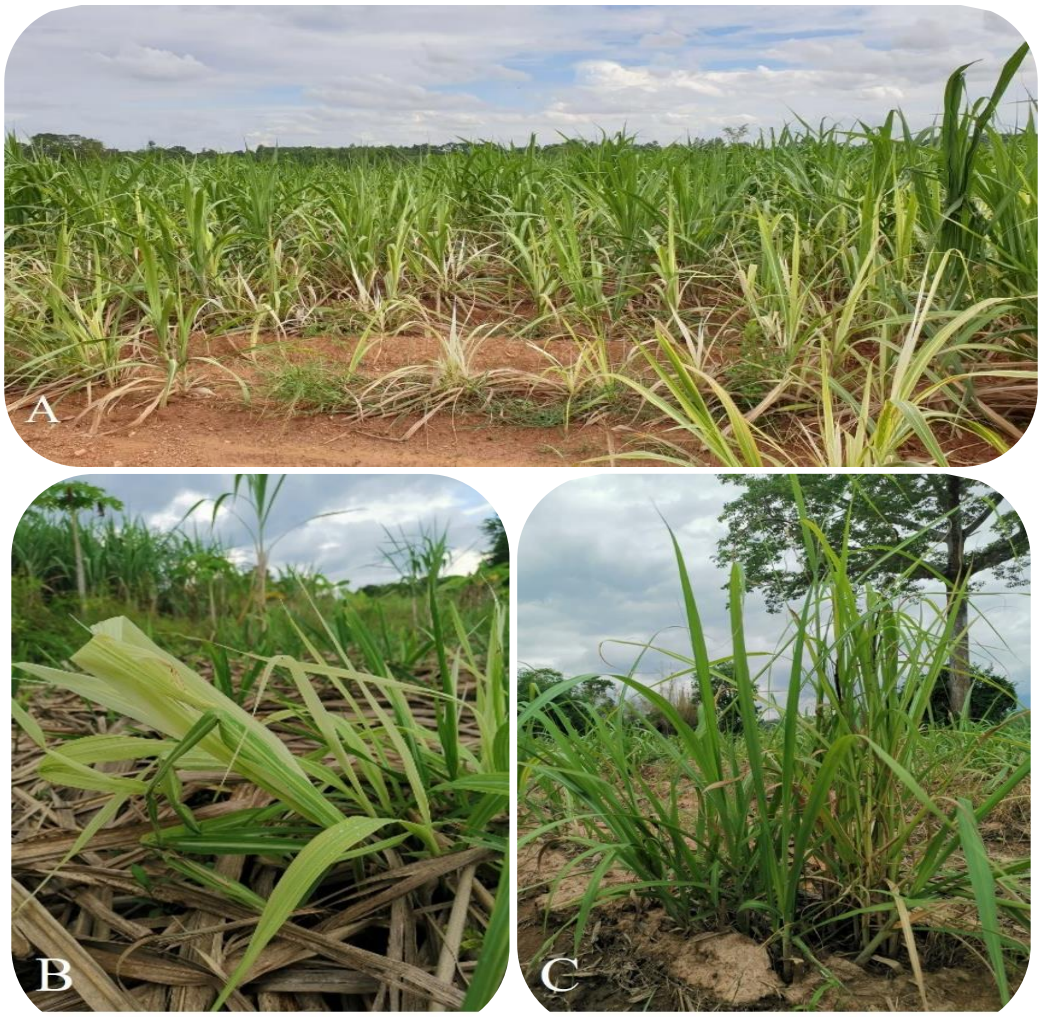

Figure 2 Sugarcane symptoms caused by *Phytoplasma*s. A and B: sugarcane white leaf; C: sugarcane grassy shoot.

Phylogenetic tree analysis: Forty samples including non-symptomatic samples were firstly tested in nested PCR with P1/P7 and R16mF2/R15mR1 primers, respectively (data not shown). The result showed a PCR band at 1600 bp from samples showing symptoms, whilst the control or no symptom samples did not give the PCR band. Samples were then tested with *50S, Ser* and *SecA* primers to determine the relationships between the *Phytoplasma*s, in particular with respect to the different sugarcane symptoms, and phylogenetic analyses were performed using the *Ser*, *50S* and *SecA* sequences. The nucleotide sequences were also combined with sequences obtained from other countries and used in previous studies (Abesysinghe *et al.,* 2019; Sangsuwan, 2020; Quoc *et al.,* 2021) Sugarcane white leaf from Vietnam (SCWLVN), Sugarcane white leaf from Sri Lanka (SCWLSri), Sugarcane grassy shoot from Vietnam (SCGSVN) and Sugarcane grassy shoot from Sri Lanka (SCGSSri).

50S gene analysis: Forty Thailand samples were tested with the 50S primers but only fourteen samples gave PCR bands (600 bp, data not shown), including one sample of sugarcane grassy shoot (SCGSKK2). All sample sequences were similar to the 50S DNA sequence of '*Ca.Phytoplasma sacchari*'. The phylogenetic tree was constructed using Aster yellows witches' broom (AY-WB) as an outgroup (Figure 3). Whilst the sequences were very similar, there were minor nucleotide differences, which grouped the samples into two clusters, one that included the samples of sugarcane white leaf and grassy shoot from Thailand, and the other that contained the sugarcane grassy shoot samples from Vietnam and Sri Lanka. The result indicates that there is greater variation between samples showing the same symptoms from different countries than from samples showing different symptoms in the same country, which would support the argument that the two types of symptoms are caused by the same *Phytoplasma*.

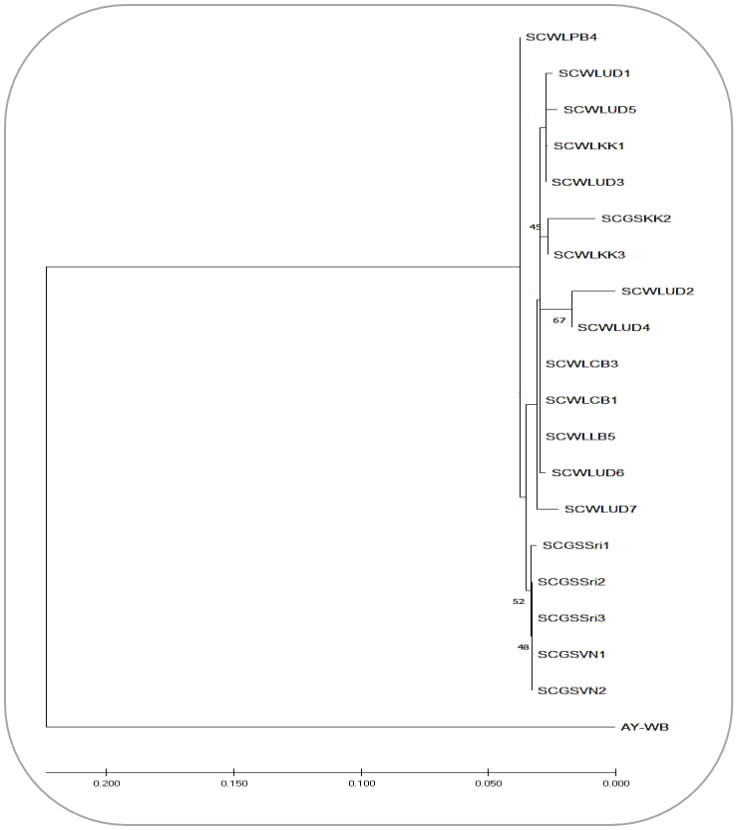

Figure 3. Dendrogram constructed by the neighbour-joining method with 1000 bootstraps, showing the relationship between SCWL and SCGS based on the *50S* sequences.

Ser gene analysis: Only one sample from Thailand gave PCR bands, SCWLBr2, so the phylogenetic tree (Figure 4) was constructed primarily with samples from other countries. The phylogenetic tree classified all the SCGS and SCWL samples into a single cluster with AY-WB as an outlier. There was no variation in *Ser* sequence between samples from the different geographic areas, and although the data is not sufficient to confirm that SCGS and SCWL in Thailand are caused by the same *Phytoplasma*, the lack of genetic variation between *Phytoplasma*s with these symptoms in other countries and between these and the SCWL sample from Thailand adds weight to the argument that the two symptoms are caused by the same *Phytoplasma* strain.

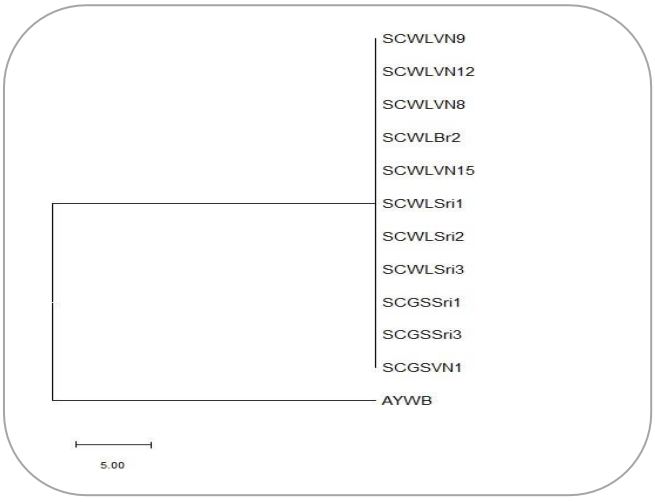

Figure 4. Dendrogram constructed by the neighbour-joining method with 1000 bootstraps, showing the relationship between SCWL and SCGS based on the *Ser* sequences.

SecA gene analysis: Only four samples from Thailand gave the PCR band (600 bp, data not shown) and the sequences showed that they were similar to *SecA* sequences of '*Ca.Phytoplasma sacchari*'. The phylogenetic tree was constructed with the *SecA* gene of sugarcane diseases in the database from other countries (JF754450, JF754452, JF754457 and DQ459440) and the phylogenetic tree essentially grouped all the samples from Thailand and other countries into the same group, whether they were SCGS samples or SCWS samples, with just minor nucleotide differences that did not affect the amino acid sequences (results not shown). The sugarcane samples were distinct from those of other 16SrXI*Phytoplasma*s, Bermuda grass white leaf and Napier grass stunt (Figure 5).

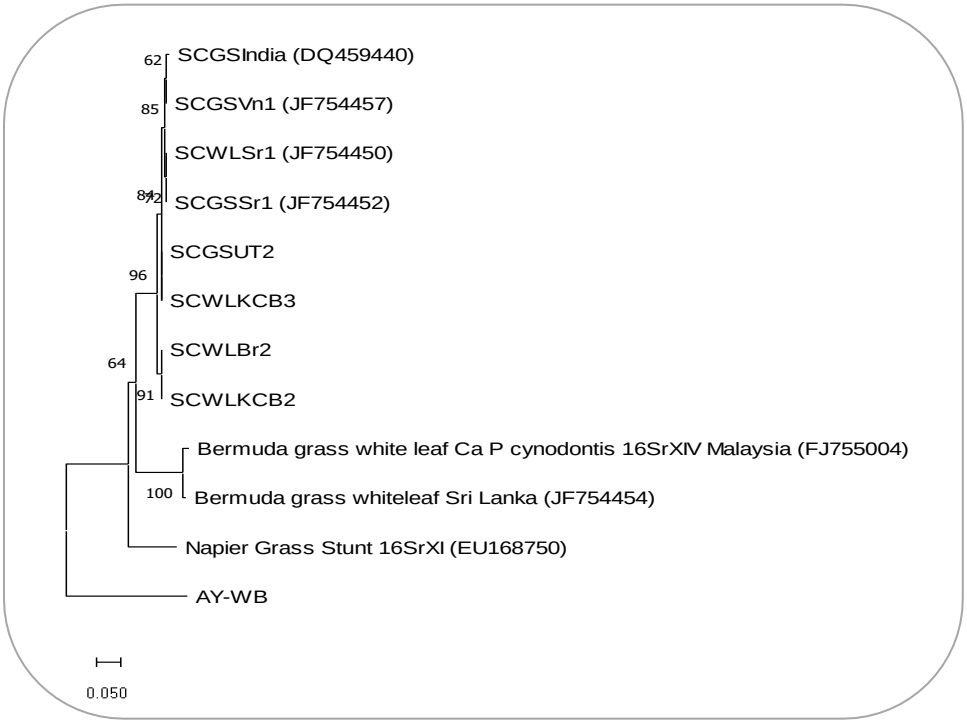

Figure 5. Dendrogram constructed by the neighbour-joining method with 1000 bootstraps, showing the relationship between SCWL and SCGS based on the *SecA* sequences.

Soil chemical property: Eleven soil samples collected from five provinces where the two symptoms occur, including from around symptomless plants were analyzed for their chemical properties and the results are shown in Table 2. The soil pH from around SCWL plants was between 5 and 8 and for SCGS was between 6 and 8 so was around the neutral pH that is optimal for sugarcane cultivation. The soil EC indicating mineral nutrients in the topsoil to form complexes with water (water-soluble salt) utilized by plants was lower than the optimal value (0.8-1.8) in all soil types, and these results were the same as OM values that were variable in both soil categories and lower than optimal values (Table 2).

Furthermore, the macro- and micro-nutrients P, K, Mg and Ca which are important for plant growth and plant disease resistance were analyzed. The results showed Available-P, available-K, Mg and Ca showed no correlation with the different soil origins which indicates that soil nutrients including soil chemical properties are not factors that account for the one *Phytoplasma* strain causing the two different sugarcane symptoms (Table 2).

Rhizopheric microorganisms: Soil microorganisms consist of bacteria, fungi, protists etc. that interact with environmental components such as plants, animals and humans to form food webs (Bonkowski *et al.,* 2009; Muller *et al.,* 2016). In particular, bacteria and fungi were the majority groups of microorganisms that were responsible for soil aggregation and nutrient restoration including decomposition.

Eleven soil samples were collected around the roots of sugarcane plants showing the different symptoms including soil from non-symptomatic sugarcane to analyze rhizospheric microorganism. Total counts of bacteria and fungi and yeasts are shown in Table 2 and the results suggest that numbers of rhizospheric microorganisms do not appear to account for the different symptoms being caused by the one strain of *Phytoplasma*.

Table 2 Soil chemical properties and rhizospheric microorganisms

symptom	pH	EC (dS/m)	OM (%)	Avail-P (mg/kg)	Avail-K (mg/kg)	Mg (mg/kg)	Ca (mg/kg)	Total Bacteria (cfu)	Total fungi and yeast (cfu)
Non-symptom	5.6	0.02	0.66	5	64	128	848	3.0×10^6	2.9×10^4
SCWL	8.6	0.16	2.4	6	45	828	9597	3.0×10^8	2.4×10^5
SCWL	6.3	0.07	0.53	60	102	115	445	4.4×10^6	2.0×10^4
SCWL	5.3	0.02	0.94	5	84	123	368	3.2×10^7	3.1×10^4
SCWL	8	0.14	1.48	11	172	341	5443	3.4×10^8	1.4×10^5
SCWL	7.8	0.07	0.68	14	28	-	-	2.9×10^6	1.1×10^5
SCGS	7	0.12	0.54	73	60	115	624	9.0×10^5	1.9×10^5
SCGS	6	0.04	0.92	6	71	73	813	3.4×10^7	2.3×10^5
SCGS	7.7	0.21	1.89	13	126	644	10458	3.5×10^7	2.5×10^4
SCGS	8.2	0.12	2.56	4	45	534	11299	3.0×10^6	2.9×10^4
SCGS	6	0.03	0.36	10	45	22	274	2.0×10^6	1.8×10^4

DISCUSSION

Sugarcane white leaf and sugarcane grassy shoot are caused by*Phytoplasma*s in the 16Sr XI group and whilst some previous studies have indicated that the two symptoms are caused by one strain, others have stated that two*Phytoplasma* strains are responsible for the different symptom types. Recently, the causal agent of SCGS has been given '*Ca.Phytoplasma sacchari*' status. In this current research forty samples have been collected across Thailand from sugarcane plants showing both SCGS and SCWL symptoms, and analysed by PCR and sequencing of three different non-rRNA genes. Whilst not all samples gave the PCR products, possibly due to low*Phytoplasma* levels in the plants or DNA instability during storage after extraction (Abesysinghe *et al.,* 2016; Youssef *et al.,* 2017), others did give PCR products that could be used to determine the nature of the*Phytoplasma*s present. Then, this research found that two sugarcane symptoms caused by one strain furthermore, soil chemical property and rhizospheric soil microorganisms were not factors to cause diverse symptoms.

Due to the large numbers of the 16S rRNA gene sequences in databases, this gene has been widely used for phylogenetic analysis of bacteria. However, whilst the gene is effective to characterize the relationship of prokaryotic cells from phylum to species it is less useful for discriminating between closely strains because of high conservation (Baker *et al.,* 2003; Pei *et al.,* 2009). One of the solutions has been to use other genes such as 23S rRNA. The 23S rRNA gene is a flexible region, but its core is conserved function at the level of secondary structure (Hunt *et al.,* 2006; Pei *et al.,* 2009). Moreover, the 23S rRNA gene is longer than 16S rRNA and it has unique indels and mutations resulting in variable sequences causing more genetic diversity and more useful diagnostic sequence than 16S rRNA (Pei *et al.,* 2009, Abdel-Lateif1 *et al.,* 2016). Moreover, 23S rRNA has been used successfully for classifications within*Phytoplasma*s, for example to show that the coconut lethal yellowing type diseases could be classified into three sub-clusters from 34*Phytoplasma* samples (Hodgetts *et al.,* 2008). In 2007, 16S-23S sequences were used to analyse*Phytoplasma*s causing sugarcane symptoms in India, and indicated that they were in the same cluster (Nasare *et al.,* 2007).

Other genes, such as 50S rRNA have also been used for*Phytoplasma* grouping (Pei *et al.,* 2012). The combination makes the 50S gene conserved at the regions to contact with other components as sites between the conserved regions were faster rates of mutation (Santoyo and Romero, 2005; Eickbush and Eickbush, 2007; Pei *et al.,* 2009). Previous studies showed that the 50S ribosomal protein; rplF and rplJ/rplL gene regions could identify Neisseria species and haplotypes of '*Candidatus Liberibacter solanacearum*' (Lso), respectively (Bennett *et al.,* 2014; Haapalainen *et al.,* 2019). In this current study, sequences of the 50S rRNA could not separate the*Phytoplasma*s causing the two sugarcane symptoms suggesting that it is probably just a single strain and that there may be any other factors responsible for the

different symptoms.

Other non-ribosomal genes have also been used to attempt to fine tune classifications of*Phytoplasma*s including the single copy genes such as *tuf, Sec* and *leuS* (Fraser *et al.,* 2004). In this study, *Ser* and *SecA* genes were used to categorize*Phytoplasma*s infecting sugarcane. In the case of the *Ser* gene, PCR only amplified from one sample in Thailand, but it was possible to construct a phylogenetic tree with other samples from other countries and the results showed that the *Ser* gene grouped the different diseases together.

SecA gene classified SCWL-Thailand samples from SCGS-Thailand samples and *SecA* gene also grouped the SCGS samples together. The *SecA* result was similar to the previous study that used *SecA* gene to classify*Phytoplasma*s by RFLP techniques and the result showed that *SecA* gene separated coconut lethal yellowing-type disease to three clusters meaning the disease could be caused by three strains (Hodgetts *et al.,* 2008) supported by Shaoshuai's result that showed *SecA* primer was better classified to resolve 16SrI group than *SecY* primer (Shaoshuai *et al.,* 2017). However, *SecY* gene also applied for classification of*Phytoplasma*s in China and Poland and the results showed that *SecY* was the potential marker to classified closely strains (Shaoshuai *et al.,* 2017; Cienslinska *et al.,* 2019). It should be noted that the *Sec* gene is an important gene for protein secretion into phloem and it is unique to bacteria and specific to*Phytoplasma* (Kakisawa *et al.,* 2001) resulting in *SecA* and *SecY* gene primers regularly being used for*Phytoplasma* detection including*Phytoplasma* classification (Hodgetts *et al.,* 2008; Sakuanrungsirikul *et al.,*2012). Consequently, gene specificity is one of the factors to consider for classification such as amino acid tRNA-synthetase specific to amino acid transference if the mutation occurs, it will affect*Phytoplasma* living.

Furthermore, the results of soil chemical properties and rhizospheric microorganism analysis were used alongside the phylogenetic tree results. According to soil result, the soil properties of pH, EC, OM, macro- and micro-nutrients did not show any indication of being associated with the different symptom types. None of the variations in the analysed factors were shown to correlate with the different symptom types.

As for soil microorganisms, rhizospheric microorganism associating with plant roots can be important and harmless or harmful, and can promote nutrient cycling and organic matter breakdown, or reduce plant growth including plant-defence systems. Previous studies have shown that plant secreted bioactive molecules, root exudates, into the rhizosphere can select or change soil microorganisms (Kawasaki *et al.,* 2016; Yin *et al.,* 2021). Additionally, different plant species, plant genotypes and developmental stages affect soil communities and are important factors to regulate soil microbial variation (Tkacz *et al.,* 2015, Mahoney *et al.,* 2017; Petrovic' *et al.,* 2018). Table 2 showed the results of total counts of bacteria and fungi and yeast and indicated that there were no clear correlations between numbers and symptom types although in some provinces SCWL bacterial counts were higher than SCGS bacterial counts.

Previous studies have suggested that the titre of the pathogens infecting plant hosts and plant age may be factors that result in the different disease symptoms (Larfeil *et al.,* 2010; Viswanathan *et al.,* 2011; Chaudhari *et al.,* 2019; Nithaya *et al.,* 2023) with infection in younger sugarcane resulting in yellow or white plants that died in a few weeks whilst infection in older sugarcane resulted in excessive numbers of tillers and sugarcane grassy shoot (Nithaya *et al.,* 2023). Additionally, environmental conditions have been suggested as an important factor along with host genotype/environment interactions (Abu Ahmad *et al.,* 2007; Larfeil *et al.,* 2010; Chaudhari *et al.,* 2019; Potts and Hunte, 2021). Both cultivars sampled in this study, KK3 and UT3 are susceptible and show both SCWL and SCGS symptoms, suggesting environment is probably a more important factor than the host genotype. Previous study showed that sugarcane propagate stems soaked in hot water moderately reducing disease so, environmental limitation by thermotherapy such as hot water and moist hot air may control sugarcane symptoms (Nithaya *et al.,* 2023).

CONCLUSION

Overall, the results from both the phylogenetic analyses, and from the soil properties and microbial analysis, provide strong and continuing support for the theory that sugarcane white leaf and sugarcane grassy shoot are caused by the same*Phytoplasma*, and that whilst there may be some genetic variability between isolates from different regions and countries, these are not the key factors that account for the different symptoms, since the genetics of the*Phytoplasma* and the host do not seem to be major contributing factors. Further study will identify any factors causing different symptoms and lead to find out quarantine techniques to protect sugarcane from*Phytoplasma*.

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