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Assessing genetic diversity of Yellow Star tree using SRAP markers and efficacy of their endophytic fungi in biological control

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Abstract The result showed that 52 fragments (64.22%) were polymorphic bands from seven combinations of Sequence-Related Amplified Polymorphism (SRAP) markers. The genetic relationship was analyzed using the UPGMA method by NTSYpc software version 2.11X showed a similarity coefficient ranging from 0.59 to 0.98. Endophytic fungi were isolated from fresh leaves of Yellow Star (*Schoutenia glomerata* King subsp. *peregrina* (Craib) Roekm. & Hartono and identified by morphology and DNA barcoding. The fungi were identified in *Alternaria alternata*, *Colletotrichum siamense*, *Diaporthe arengae*, *Phyllosticta capitalensis*, and *Xylaria cubensis.* The dual culture test evaluated the efficacy of endophytic fungi isolated from Yellow Star tree against *Colletotrichum gloeosporioides* and *Phomopsis asparagi* causing disease in asparagus. The results revealed that *X. cubensis* had the potential to inhibit the spore germination of the *C. gloeosporioides* ASGC06 and *P. asparagi* ASGP04, which inhibited spore germination of 99.97 and 100 percent, respectively. However, this fungus should be further studied for biological control and bioactive secondary metabolites.

Keywords: Yellow Star tree, Endophytic fungi, Biological control, SRAP markers

Introduction

Yellow Star (*Schoutenia glomerata* King subsp. *peregrina* (Craib) Roekm. & Hartono, known as Ruang Phueng, belongs to the Malvaceae family. The native range of this subspecies is Thailand. The Yellow Star tree has been chosen to symbolize the reign of His Majesty King Maha Vajiralongkorn Bodindradebayavarangkun, King Rama X of Thailand, which can be seen blossoming with fragrant and yellow flowers from July to October. The chemical contained in the plants of the Malvaceae family is used in medicine (Azizov *et al*., 2007), like *Alcea rosea*, which has been used as a diuretic. Only

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one report has previously been published on the antioxidant activity of Yellow Star leaves and flowers (Srikanlayanukul and Silapawattana, 2022). The Yellow Star tree has not been reported in genetic diversity, especially molecular markers. Sequence-related amplified polymorphism (SRAP) is a molecular approach that shows dominant molecular markers and amplifies coding regions in the genome (Li and Quiros, 2001). This technique has been successfully applied to genetic diversity in the *Alcea* genus (Badrkhani *et al*., 2014) and Durian (*Durio zibethinus* Murray) (Thinhuatoey *et al*., 2016), which is a member of the Malvaceae family.

Asparagus (*Asparagus officinalis* L.) has been used as a vegetable and medicine. However, phytopathogenic fungi decrease crop quality and quantity of plant products, such as stem blight caused by *Phomopsis asparagi*. Including *Colletotrichum gloeosporioides* is one of the major plant pathogens causing anthracnose. In asparagus, *Talaromyces siamensis* FKA-61 has been reported to inhibit the growth of *Phomopsis* strain FKA-62 (Nonaka *et al.*, 2015). Mangkalad *et al*. (2018) studied the antagonistic activity of *Neosartorya* and *Talaromyces* against *P. asparagi* in asparagus, which indicated that *N. hiratsuka* EU06 was the highest at 59.50% in biological control against controlling the stem blight of asparagus using the dual culture technique. This study aimed to evaluate the efficacy antagonistic activity of the endophytic fungi isolated from the Yellow Star trees to control diseases caused by *P. asparagi* and *C. gloeosporioides* in asparagus.

Materials and methods

Plant samples and DNA extraction

The fifteen samples of the Yellow Star tree were collected in Thailand from Chiang Mai (6 samples: SGKT01-06), Bangkok (8 samples: SGKT07-12 and 14-15), and only one sample (SGKT13) from Nonthaburi provinces. The fresh and healthy uninfected leaf samples were washed, air-dried and kept at -80 ℃. The genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). The precipitated DNA was purified using the GF-1 AmbiClean Kit.

SRAP markers

In a preliminary study, thirty SRAP primer combination sets of five forward primers (17 bases, ME1-ME5) and six reverse primers (18 bases, EM1-EM6) (Li and Quiros, 2001) were initially screened in sampling samples. Each

polymerase chain reaction (PCR) reaction was carried out in a final volume of 20 µL containing 200 ng of the high-quality DNA, 1.0 µM of each primer, 1.25 mM dNTP mix, 2.5 mM $MgCl_2$, 1U of *Taq* DNA polymerase and 1× of PCR buffer according to previously established protocols by Sabpayakom *et al*. (2016). The PCR program used two-step amplification involving the first five cycles at 35℃ annealing temperature followed by 35 cycles being increased to 50℃. The SRAP products were separated by electrophoresis on 2% agarose gel in 1× TBE buffer along with 100 bp of DNA marker. For SRAP profiles, DNA fragments in the same locus were scored as present (1) or absent (0) from each primer. The similarity coefficient was calculated, and the dendrogram was constructed using the NTSYSpc software version 2.11X.

Isolation of endophytic fungi

The Yellow Star leaves were collected in King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand. The endophytic fungi were isolated from the leaves by tissue transplanting technique following the method described by Mamat *et al*. (2018) with slight modifications. The leaves were rinsed several times with tap water and then air dried. The cutting leaf (1× 1 cm) was sterilized with 70% (v/v) ethanol for 2 min, followed by 3% NaOCl for 3 min, sequentially soaked in sterilized water two times and dried on sterile filter paper. The washed tissue was placed on Water Agar (WA) medium plate and incubated at room temperature for 2-3 days. After incubation, hyphal tips developed and grown from the leaf tissue were cut and placed on Potato Dextrose Agar (PDA) medium. The fungi were purified by transferring a single spore technique to a new PDA plate.

Morphological identification of endophytic fungi

The colony and conidia appearance on PDA, Malt Extract Agar (MEA) and Sabouraud Dextrose Agar (SDA) media were identified by morphological characteristics. The endophytic fungi were confirmed species by DNA barcoding. Mycelial grown on PDA for 4-7 days was used for DNA extraction (Suksiri *et al*., 2018). The PCR products were performed for sequencing analysis by Celemics, Inc. Korea. The sequences were identified by aligning the National Center for Biotechnology Information (NCBI) database sequence using Basic Local Alignment Search Tools (BLAST: https://blast.ncbi. nlm.nih.gov/Blast.cgi).

Dual culture assay

In vitro antifungal bioassays of endophytic fungi were carried out based on the dual culture assay. Two pathogen fungi, *Colletotrichum gloeosporioides* ASGC06, and *Phomopsis asparagi* ASPA04 were isolated from stem blight diseases of asparagus by tissue transplanting technique that was identified based on morphology and molecular analysis from previous experience in our team (Mangkalad *et al*., 2018). *C. gloeosporioides* ASGC06 and *P. asparagi* ASPA04 were prepared by grown on PDA and incubated at room temperature. An agar plug (5 mm diameter) with fungal mycelium of each endophytic fungi and plant pathogens fungi obtained from the edge of a 7-10 days old colony were placed apart on opposite sides of a PDA plate. The PDA plates were separately inoculated with a 5 mm agar plug of each endophyte and pathogen used as the control. Dual culture plates were incubated at room temperature for 25 days in a dark room. The percentage of spore germination was calculated using the following formula.

$$\text{Inhibition of spore germination} = \frac{R1-R2}{R1} \times 100$$

R1 and R2 are the conidia of the plant pathogenic fungus in the control and the dual culture plate, respectively. Therefore, some endophytic fungi with conidia shape and size the same as plant pathogens were not studied. Experiments were conducted in a completely randomized design (CRD). Four replications were made for each treatment. The statistical analysis of the dual culture test was done with ANOVA analysis ($p<0.05$).

Results

SRAP marker analysis

Initially, three samples of the Yellow Star tree were from Bangkok (SGKT07), Chiang Mai (SGKT05), and Nonthaburi (SGKT13) provinces, which have different areas were selected to screen 30 sets of primer combinations. Seven primer combinations (ME2/EM4, ME2/EM6, ME3/EM5, ME4/EM3, ME4/EM5, ME5/EM1, and ME5/EM3) were able to produce intense bands with clearly recordable and gave polymorphisms bands. Those primers combinations were used for SRAP analysis of 15 Yellow Star. SRAP profile from ME5/EM1 primers combination is shown in Figure 1. A total of 80 DNA bands were observed from 7 primer combinations. The number of SRAP bands per primer combination ranged from 9 (ME4/EM5) to 13 (ME4/EM3 and

ME5/EM1), with an average of 11.43 bands per primer combination. ME5/EM1 primers gave the highest number of polymorphic fragments (84.62%). Fifty-two polymorphic bands were scored with an average of 7.43, and the percentage of polymorphism (% polymorphism) is 65.00% (Table 1).

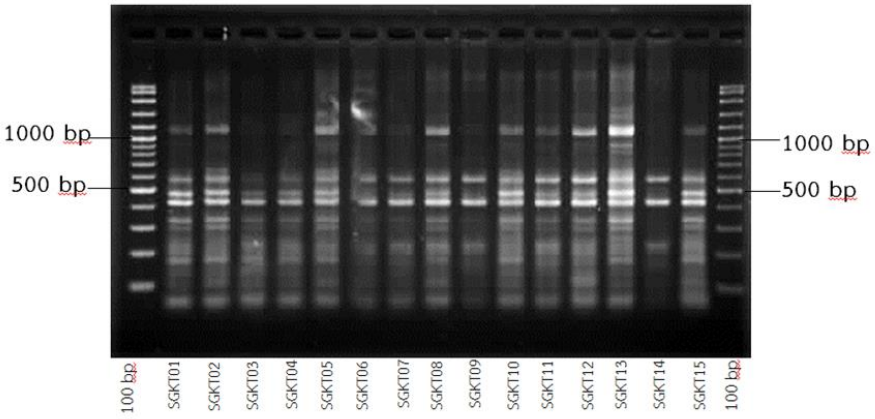

Figure 1. Example of the SRAP profiles from 15 Yellow Star trees samples revealed by ME5/EM1 primer combinations; VC 100 bp plus DNA ladder used as a marker

Table 1. Primer codes combination, the number of total DNA bands, the number of polymorphic bands and the percentage of polymorphism detected by the SRAP marker in 15 Yellow Star trees

Primer codes combination	Fragment size (bp)	No. of total DNA bands	No. of polymorphic bands	% of polymorphism
ME2/EM4	100-900	10	4	40.00
ME2/EM6	100-1100	11	7	63.64
ME3/EM5	120-1800	12	9	75.00
ME4/EM3	100-1000	13	8	61.54
ME4/EM5	100-720	9	6	66.67
ME5/EM1	100-1800	13	11	84.62
ME5/EM3	120-1200	12	7	58.33
Total	-	80	52	65.00
Average	**100-1800**	**11.43**	**7.43**	**65.00**

Fifty Yellow Star trees based on the SRAP bands were used to construct a dendrogram. The similarity coefficient ranged from 0.73 to 0.97 and was based on a cut-off point of 0.73, and the similarity coefficient was divided into two groups (Figure 2). The first group consisted of the nine Yellow Star trees,

namely SGKT01, 02, 05, 08, 10, 11, 12, 13 and SGKT15, which could be subdivided into two subgroups. SGKT13 sample came from Nonthaburi provinces and is part of this group. The other groups consisted of the six Yellow Star trees, namely SGKT03, 04, 06, 07, 09 and SGKT14, which could be subdivided into two subgroups. Genetic similarities between samples shown as simple matching coefficients ranged from 0.59 to 0.98 (data not shown).

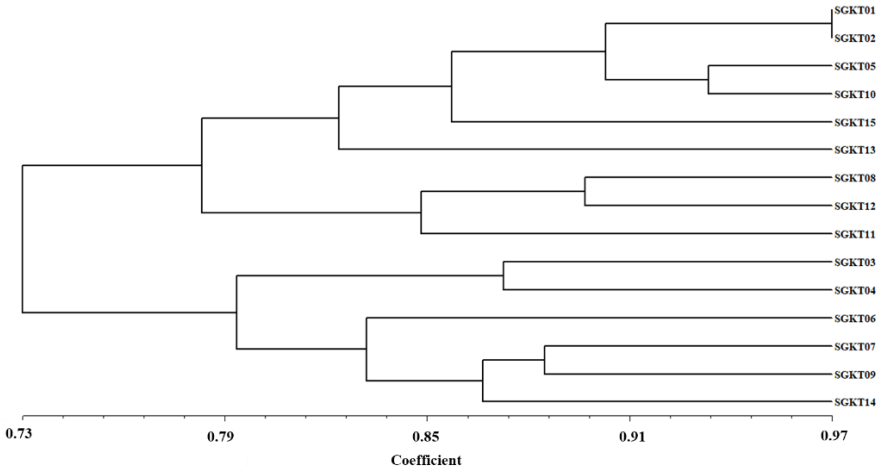

Figure 2. Dendrogram of 15 Yellow Star trees constructed from SRAP data using UPGMA method based on simple matching coefficient

Isolation, morphological identification, and DNA barcoding

Seven isolates of endophytic fungi (YS01-05, YS02-06, YS02-08, YS02-09, YS02-10, YS02-11, and YS02-16) were isolated from three samples of the Yellow Star leaves. All isolates were identified for their mycelium and colony color, spore or conidia shape and size, growth rate and sporulation on PDA, MEA and SDA medium after eight days of incubation. The morphological characteristics of five genera were identified, as shown in Table 2.

In the first group, the colony morphology of isolate PY01-05 is whiteish colonies with irregular edges after eight days. The reverse color was off-white to creamy yellow toward the center on PDA (Figures 3A and 3B), MEA (Figures 3C and 3D) and SDA medium (Figures 3E and 3F). The spore mass turned yellowish-orange in color with age (Figure 3G). Fast-growing colonies that the hyphae showed smooth-walled, septate and branched (Figure 3H). The white to light gray colony progressively turned to dark grey in the center, covered with predominantly dark brown acervulus with black setae (Figures 3I and 3J). Conidia were cylindrical shapes with rounded ends, and the size of conidia ranged from 2.80-4.75 × 8.50-14.31 μm (Figure 3K), which was

identified as *Colletotrichum*. The internal transcribed spacers (ITSs) sequence amplified with ITS1/ITS4 primers was confirmed to be *C. siamense* and *C. gloeosporioides* with 97.41 and 97.42 identity, respectively. However, the ITS sequence was unsuitable for precisely identifying this isolate. So, based on the ApMat intergenic region with AM-F and AM-R primers (Lio *et al*., 2018). This isolate revealed to *C. siamense* (MH713885) in GenBank, which is 97.83% identity.

Table 2. The morphological characteristics of five genera of endophytic fungi

Groups	Isolates	Colony pattern	Spore			Genus
			Shape	Width (μm)	Length (μm)	
1	01-05	white to light gray colony and yellowish to orange color spore mass	cylindrical shape	2.80-4.75	8.50-14.31	*Colletotrichum*
2	02-06	circular smooth	club-shaped or obclavate and long chains	5.41-16.05	10.84-33.48	*Alternaria*
3	02-08	irregular contoured	globose and ellipsoid	4.20-7.19	6.99-12.34	*Phyllosticta*
	02-16	irregular contoured	globose and ellipsoid	5.24-7.80	6.78-11.71	
4	02-09	irregular cottony	does not produce spores			*Xylaria*
	02-10	irregular cottony	does not produce spores			
5	02-11	irregular contoured	ellipsoid and fusiform	1.36-3.00	4.32-9.38	*Diaporthe*

In the second group, the colony morphology of isolate PY02-06 is gray-green with concentric growth rings on the PDA (Figures 4A and 4B), yellow-green with slight concentric growth rings and prominent white margins on the MEA medium (Figures 4C and 4D), and dark olive green colony on SDA medium (Figures 4E and 4F). Fast-growing colonies that the hyphae showed smooth-walled, septate and branched (Figure 4G). The conidia color was light green to dark green, irregular ellipsoid spatulate in shape with 1-5 transverse septa, but not clear with longitudinal septa. The size of conidia varied greatly, ranging from 10.84 to 33.48 μm in length and 5.41 to 16.05 μm in width showing long chains of single-celled conidia (Figures 4H and 4K). Based on these morphological characteristics and molecular identification showed 100% identity with ITSs sequence, which confirms to be *Alternaria alternata*.

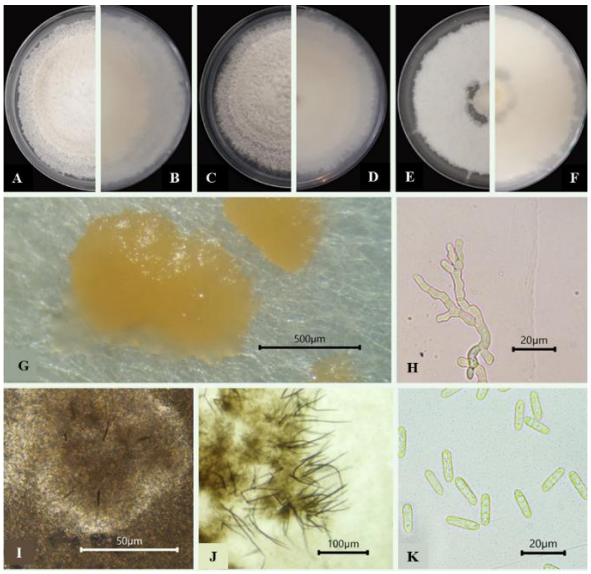

Figure 3. *Colletotrichum siamense* (PY01-05) (A-F) colonies on medium above and below after 8 days (A-B: PDA, C-D: MEA, E-F: SDA), (G) spore mass, (H) hyphae, (I-J) acervulus with black setae and (H) conidia

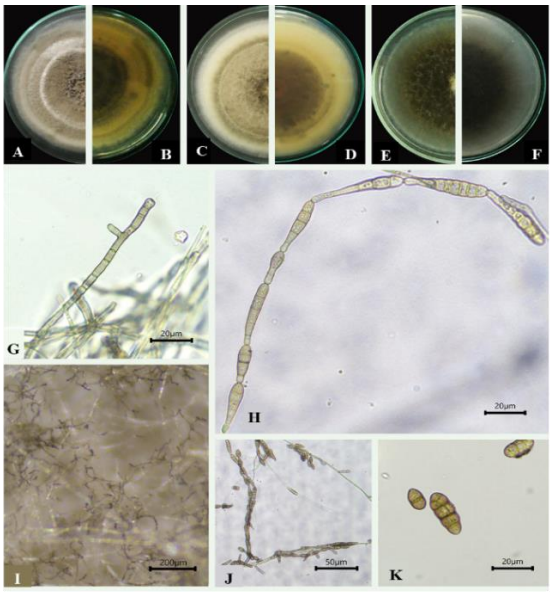

Figure 4. *Alternaria alternata* (PY02-06) (A-F) colonies on medium above and below after 8 days (A-B: PDA, C-D: MEA, E-F: SDA), (G) hyphae, (H-K) conidia

In the third group, colonies morphology of the culture isolates PY02-08 and PY02-16 showed initially white, gradually becoming gray-green to dark green with prominent white margins both on the PDA (Figures 5A and 5B) and MEA (Figures 5C and 5D). However, the growth grew slowly on PDA. The colonies were dark green on the SDA medium (Figures 5E and 5F). The hyphae showed smooth-walled, septate and branched (Figure 5 G). The conidia color was light green, ellipsoid in shape, and the size ranged from 6.99 to 11.71 μm in length and 4.20 to 7.80 μm in width. These morphological characteristics and phylogenetic relation from ITS regions showed that those isolates had an identity of 98.59%, confirmed to be *Phyllosticta capitalensis* corresponding to accession number MN958712.

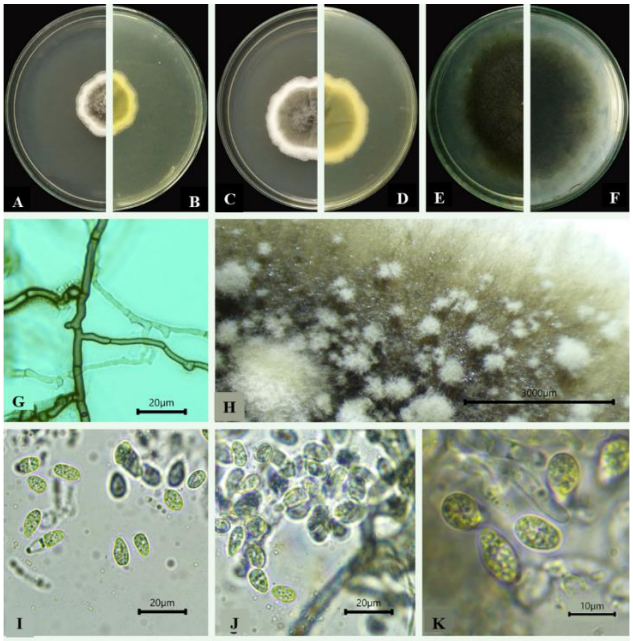

Figure 5. *Phyllosticta capitalensis* (PY02-08) (A-F) colonies on medium above and below after 8 days (A-B: PDA, C-D: MEA, E-F: SDA), (G) hyphae, (H) spore mass, (I-K) conidia

In the fourth group, colony morphology of the culture isolates PY02-09 and PY02-10 were irregular with white abundant cottony mycelium and gradually became yellow on three mediums. The cultures showed stromata without spores when cultured after thirty days. Morphology and DNA barcoding were used for identification. The *ß-tubulin* region with Bt2a/Bt2b primers was used to identify those isolates with an identity of 98.55%, which

was confirmed to be *Xylaria cubensis*, corresponding to accession number AB625373.

In the last group, the colony morphology of isolate PY02-11 showed white with an irregular shape that looks like a flower on both PDA and MEA and a dark green colony on the SDA. The hyphae showed smooth-walled and conidiogenous cells were cylindrical shapes with a slight taper towards the apex. The alpha conidia are ellipsoid shapes ranging from 4.32 to 9.38 μm in length and 1.36 to 3.00 μm in width, and the beta conidia are fusiform shapes ranging from 20.00-25.00 μm in length and 1.00-1.50 μm in width. These morphology characteristics and ITS regions resulted in the identity of 98.50% and were confirmed to be in *Diaporthe arengae*, corresponding to accession number MN651487.

Dual culture tests

The dual culture tested between two pathogenic fungi, *P. asparagi* ASPA04 and *C. gloeosporioides* ASGC06 and seven endophytic fungi: *C. siamense* YS01-05, *A. alternata* YS02-06, *P. capitalensis* YS02-08, *P. capitalensis* YS02-16, *X. cubensis* YS02-09, *X. cubensis* YS02-10 and *D. arengae* YS02-11. *C. siamense* YS01-05 was not studied because it has the same shape and size of the conidia as *C. gloeosporioides* ASGC06. *X. cubensis* YS02-09 and YS02-10 showed the highest effectiveness in controlling *C. gloeosporioides* ASGC06 with spore inhibition at 99.80 and 99.97%, respectively. *X. cubensis* YS02-09 and YS02-10 showed the highest effective isolates to inhibit *P. asparagi* ASPA04 with spore inhibition at 100.00 and 100.00%, respectively (Table 3).

Table 3. Dual culture test of seven endophytes fungi and two pathogenic fungi

Isolates of endophytes fungi	*C. gloeosporioides* ASGC06		*P. asparagi* ASGC04	
	No. of spore (10^6 spores per mL)	Spore inhibition (%)	No. of spore (10^6 spores per mL)	Spore inhibition (%)
Control	30.97	0.00	6.03	0.00
C. siamense YS01-05	1.71	94.49^{b} ±2.68	0.20	96.72^{b} ±1.99
A. alternata YS02-06	11.5	61.99^{d} ±1.49	0.65	89.29^{c} ±2.00
P. capitalensis YS02-08	12.20	60.61^{d} ±2.15	1.02	83.08^{d} ±0.89
P. capitalensis YS02-16	0.06	99.80^{a} ±0.14	1.13	81.35^{d} ±0.56
X. cubensis YS02-09	0.01	99.97^{a} ±0.07	0.00	100.00^{a} ±0.00
X. cubensis YS02-10	3.30	89.34^{c} ±2.90	0.00	100.00^{a} ±0.00
D. arengae YS02-11	1.71	94.49^{b} ±2.68	1.73	77.33^{e} ±2.85

Discussion

SRAP marker is a molecular approach that shows dominant molecular markers. This marker has been successfully applied in the *Alcea*, and *Durio* genus, a member of the Malvaceae family, the same as the Yellow Star trees family. Badrkhani *et al*. (2014) used seventeen SRAP primer combinations to generate 104 fragments, of which 97 (93.00%) were polymorphic, and two main clusters were detected among 14 species of *Alcea* collected from Northwest Iran. This report did not correspond to the geographical origin of the species. This result is consistent with the study of Thinhuatoey *et al*. (2016) that evaluated the genetic diversity of twenty-nine durian in Thailand based on the SRAP marker. The results indicate that the SRAP marker is efficient, stable, simple, and faster. However, the Yellow Star trees have no prior report on genetic diversity or genomic information about geographical distribution.

Fifty Yellow Star trees were used to construct a dendrogram with a similarity coefficient ranging from 0.73 to 0.97. With a cut-off point of 0.73, the samples were divided into two groups. Genetic similarities between the samples ranged from 0.59 to 0.98. The highest genetic similarity of 0.98 was seen in SGKT01 and SGKT02, which are more closely related. They have been growing up in the same place at Royal Park Rajapruek and the same age for two years, which suggests that they are of the exact origin with grafting propagated. The similarity value of SGKT13 and SGKT14 were the lowest genetic similarity between samples. This would select the parent in the hybridization process to create the new variety. However, the current study revealed a medium genetic diversity in the Yellow Star trees corresponding to sexual and asexual propagation. To Honor His Majesty King Rama X, people prepared the area for planting the Yellow Star tree to mark His Majesty the King. It will be planted from the grafting propagation because it has rapid growth and comes to develop a mature tree in a short period, which can cause a loss of genetic diversity. Including the results provide evidence that SRAP markers could be used in future determination in large Yellow Star tree populations.

Seven isolates of endophytic fungi were isolated from the Yellow Star leaves. The fungi were identified by both morphology and DNA barcoding. The fungi were identified in *C. siamense, A. alternata, P. capitalensis, X. cubensis* and *D. arengae*. *Colletotrichum* species are the most important plant pathogenic fungi (Zakaria, 2021) and endophytes fungi (Ma *et al*., 2018; Zheng *et al*., 2022) in various plant hosts. *C. siamense* was among the endophytic fungi isolated from *Dendrobium* orchids (Ma *et al*., 2018), *Musa* spp. leaves (Zakaria and Aziz, 2018) and guarana plant (Casas *et al*., 2021). Including *C. siamense* had

the potential for biocontrol in guarana seedlings (Casas *et al*., 2021). *A. alternata* is also pathogenic on numerous crop plants, including tobacco, strawberry, soybean, rice and citrus. *A. alternata* is also reported as endophytic fungi on these plants (DeMers, 2022). The mycelia of *A. alternata* could be used for biocontrol as a mycoherbicide to control *Eupatorium adenophorum* (Qiang *et al*., 2006).

Phyllosticta has often been reported as endophytes and plant pathogens with a broad host range, including *P. capitalensis* associated with the citrus black spot disease of citrus (Glienke *et al*., 2011; Wikee *et al*., 2013). On the other hand, *P. capitalensis* is isolated from non-pathogenic citrus leaves, and fruit shows the potential to inhibit *P. citricarpa* causes citrus black spot disease of citrus (Tran *et al*., 2019). *Diaporthe* and their *Phomopsis* asexual states have been reported as either pathogenic, causing diseases in different hosts, especially citrus, coffee and soybean (Dong *et al*., 2021) or harmless endophytes depending on the host and its health (Gomes *et al*., 2013). Its inhibition has not yet been reported, and endophytic *D. arengae* produces a variety of secondary metabolites (Patil *et al*., 2017).

X. cubensis is wood-decay fungi widely distributed in temperate, subtropical and tropical regions where it usually grows on decayed wood due to its capacity to degrade lignin and cellulose. *X. cubensis* is a frequent endophytic isolate known for its medicinal value and production of various bioactive compounds. For biocontrol, It was reported that piliformic acid and cytochalasin D produced by *Xylaria* have antifungal activity against *C. gloeosporioides*. In addition, many other secondary metabolites exhibited antimalarial activity, cytotoxicity, and antibacterial activity (Klaiklay *et al*., 2012; Sawadsitang *et al*., 2015).

In this study, the endophytic fungi isolated from fresh leaves of Yellow Star trees were identified and evaluated for biocontrol. *X. cubensis* YS02-09 and YS02-10 showed the highest effective isolates to control *C. gloeosporioides* with spore inhibition at 99.80 and 99.97% and to control *P. asparagi* with spore inhibition at 100.00 and 100.00%, respectively. The results indicate that *X. cubensis* had the potential as a biological control agent against diseases caused by *C. gloeosporioides* and *P. asparagi* in asparagus. Nevertheless, most endophytic fungi are common plant pathogens. So, research will be needed to address the safety of biocontrol agents because several factors, such as healthy and unstressed host plants, might contribute to endophytes becoming pathogenic.

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