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Analysis of genetic and virulence variability of *Stemphylium lycopersici* associated with leaf spot of vegetable crops

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Abstract *Stemphylium lycopersici* (Enjoji) W. Yamam was initially described from tomato and has been reported to infect different hosts worldwide. Sequence analyses of the internal transcribed spacer (ITS) regions 1 and 2, including 5.8S rDNA (ITS-5.8S rDNA) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR), as well as virulence studies were conducted to analyze 46 *S. lycopersici* isolates. *Stemphylium lycopersici* isolates used in this study were obtained from diseased tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.) and lettuce (*Lactuca sativa* L.) from major vegetable growing regions of Malaysia, including the three states of Pahang, Johor and Selangor between 2011 and 2012. Phylogenetic analysis of a combined dataset of the ITS-5.8S rDNA and *gpd* regions indicated that all isolates were clustered in the sub-cluster that comprised *S. lycopersici*, and were distinguished from other *Stemphylium* species. Cluster analyses using the UPGMA method for both RAPD and ISSR markers grouped *S. lycopersici* isolates into three main clusters with similarity index values of 67 and 68 %. The genetic diversity data confirmed that isolates of *S. lycopersici* are in concordance to host plants, and not geographical origin of the isolates. All *S. lycopersici* isolates were pathogenic on their original host plants and showed leaf spot symptoms; however, virulence variability was observed among the isolates. In cross-inoculation assays, the representative isolates were able to cause leaf spot symptoms on eggplant, pepper, lettuce and tomato, but not on cabbage.

Keywords *gpd* · ISSR · ITS-5.8S rDNA · Phylogeny · RAPD

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Introduction

The genus *Stemphylium* (anamorph: *Pleospora*, Dothideomycetes) was proposed by Wallroth (1833) with *Stemphylium botryosum* Wallr. as the type species. The number of described *Stemphylium* species is

estimated to be 150 (Wang and Zhang 2006). Most of these species are saprophytic (Ellis 1971; Simmons 1969; Wang et al. 2009), but various *Stemphylium* species, including *S. botryosum*, *S. lycopersici* (Enjoji) W. Yamam, *S. solani* G.F. Weber and *S. vesicarium* (Wallr.) Simmons have been reported to be common pathogens on vegetable crops causing severe disease in almost all regions where these crops are grown (Ellis 1971; Farr and Rossman 2014). Yield loss due to Stemphylium leaf spot (SLS) can be high and in some cases is 100 % when proper disease control measures are not adopted (Cedeño and Carrero 1997). *Stemphylium lycopersici* was first described from tomato in 1931 (*Solanum lycopersicum* L., Solanaceae, Enjoji 1931). Since then, this fungus has been reported to be the causal agent of leaf spot in more than 30 host genera worldwide, including Malaysia (Ellis and Gibson 1975; Farr and Rossman 2014). The hosts of this pathogen include tomato (Ellis and Gibson 1975; Min et al. 1995; Enjoji 1931), eggplant (Cho and Shin 2004; Gannibal 2012; Yu, 2001), pepper (Blazquez 1969; Cho and Shin 2004; Kim et al. 2004; Yu 2001) and lettuce (Anonymous 1979; Sawada 1959).

In recent years, molecular markers have been widely adopted to identify and characterize *Stemphylum* species in diverse environments (Câmara et al. 2002; Inderbitzin et al. 2009; Wang et al. 2010). Molecular approaches based on multi-locus phylogenetic analyses of the internal transcribed spacer (ITS) regions 1 and 2, including 5.8S rDNA (ITS-5.8S rDNA) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene have robustly defined the monophyly of *Stemphylum* in the ascomycete family Pleosporaceae, and revealed five distinct clades (A-E) (Câmara et al. 2002). Later, Inderbitzin et al. (2009) introduced *S. lancipes* (Ellis et Everhart) Simmons as a new distinct clade (F).

The efficacy of control strategy on the plant pathogen populations are inhibited by limited information on genetic variability (McDonald and Linde 2002). The most common adopted effort is the use of fungicides and resistant cultivars. However, Milgroom and Peever (2003) expressed their concern as the efforts directly may alter genetic variability. Hence, understanding genetic variations within the pathogen populations is imperative and should be considered as one of the first steps for the delineation of disease management programs (McDonald and Linde 2002). Genetic variation in populations of *Stemphylium* species has been characterized by random amplified polymorphic DNA (RAPD) (Chaisrisook et al. 1995; Mehta 2001). Recent studies have suggested that virulence testing should be used in conjunction with other molecular diagnostic tools to establish relationships within *Stemphylium* species (Köhl et al. 2009). In Malaysia, genetic diversity and virulence variability of *S. lycopersici* populations remains unknown due to the absence of studies for assessing the genetic variation among isolates. Therefore, the main objective of the present study was to estimate the virulence and genetic variability among *S. lycopersici* isolates associated with vegetable crops in Malaysia, including tomato, eggplant, pepper, and lettuce.

Materials and methods

Fungal isolates

Forty-six isolates of *Stemphylium* spp. were obtained from the Plant Pathology Laboratory, Plant Protection Department, University of Putra Malaysia (UPM). The isolates were collected from infected vegetable crops, including tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.) and lettuce (*Lactuca sativa* L.) showing leaf spot symptoms from major vegetable growing regions of Malaysia, including the three states of Pahang, Selangor and Johor between 2011 and 2012 (Table 1). All isolates were purified using a single spore isolation technique (Ricker and Ricker 1936) prior to further studies.

Fungal DNA extraction

Pure cultures of 46 *Stemphylium* spp. isolates were subcultured by placing mycelial plugs (5 mm^2), taken from actively growing margins of 10-day-old cultures, at the center of potato dextrose agar (PDA) media. The PDA media were incubated at 25±2 °C with 12 h photoperiod conditions. When the mycelia covered the PDA plates (after 12–14 days), sterile distilled water containing 0.05 % (v/v) Tween-80 was added into the plates and mycelia were collected by gently scrubbing with a sterile spatula and transferred to 1.5 ml micro tubes. The micro tubes were centrifuged at 3,000×g, at 4 °C for 5 min and the supernatant were discarded and pellets with about 100 mg mycelia were obtained. Total genomic DNA was extracted from all isolates using the 3 % SDS method as described by Gonzalez-Mendoza et al.

Table 1 Origins of *Stemphylium lycopersici* isolates, reference *Stemphylium* isolates and *A. tenuissima* used in the study

No.[a,b,c]	Isolate	ITS Acc. No.	*gpd* Acc. No.	Species	Host	Location	Year
1	SSN-T01	This study	This study	*S. lycopersici*	*Solanum lycopersicum*	Lojing, Pahang	2011
2	SSN-T02	This study	This study	*S. lycopersici*	*S. lycopersicum*	Lojing, Pahang	2011
3	SSN-T03	KF483117	KF483121	*S. lycopersici*	*S. lycopersicum*	Lojing, Pahang	2011
4	SSN-T04	This study	This study	*S. lycopersici*	*S. lycopersicum*	Bertam, Pahang	2011
5	SSN-T05	This study	This study	*S. lycopersici*	*S. lycopersicum*	Bertam, Pahang	2011
6	SSN-T06	This study	This study	*S. lycopersici*	*S. lycopersicum*	Bertam, Pahang	2011
7	SSN-T07	This study	This study	*S. lycopersici*	*S. lycopersicum*	Boh Road, Pahang	2011
8	SSN-T08	This study	This study	*S. lycopersici*	*S. lycopersicum*	Kampo Raja, Pahang	2011
9	SSN-T09	This study	This study	*S. lycopersici*	*S. lycopersicum*	Kampo Raja, Pahang	2011
10	SSN-T10	This study	This study	*S. lycopersici*	*S. lycopersicum*	Keama Farms, Pahang	2011
11	SSN-T11	This study	This study	*S. lycopersici*	*S. lycopersicum*	Keama Farms, Pahang	2011
12	SSN-T12	This study	This study	*S. lycopersici*	*S. lycopersicum*	Tanah Rata, Pahang	2011
13	SSN-T13	This study	This study	*S. lycopersici*	*S. lycopersicum*	Tangkak, Johor	2012
14	SSN-T14	This study	This study	*S. lycopersici*	*S. lycopersicum*	Tangkak, Johor	2012
15	SSN-T15	This study	This study	*S. lycopersici*	*S. lycopersicum*	Serdang, Selangor	2012
16	SSN-E01	This study	This study	*S. lycopersici*	*Solanum melongena*	Lojing, Pahang	2011
17	SSN-E02	This study	This study	*S. lycopersici*	*S. melongena*	Lojing, Pahang	2011
18	SSN-E03	This study	This study	*S. lycopersici*	*S. melongena*	Bertam, Pahang	2011
19	SSN-E04	This study	This study	*S. lycopersici*	*S. melongena*	Bertam, Pahang	2011
20	SSN-E05	This study	This study	*S. lycopersici*	*S. melongena*	Boh Road, Pahang	2011
21	SSN-E06	This study	This study	*S. lycopersici*	*S. melongena*	Boh Road, Pahang	2011
22	SSN-E07	This study	This study	*S. lycopersici*	*S. melongena*	Keama Farms, Pahang	2011
23	SSN-E08	This study	This study	*S. lycopersici*	*S. melongena*	Keama Farms, Pahang	2011
24	SSN-E09	This study	This study	*S. lycopersici*	*S. melongena*	Tanah Rata, Pahang	2011
25	SSN-E10	This study	This study	*S. lycopersici*	*S. melongena*	Tangkak, Johor	2012
26	SSN-E11	This study	This study	*S. lycopersici*	*S. melongena*	Tangkak, Johor	2012
27	SSN-E12	KF483118	KF483122	*S. lycopersici*	*S. melongena*	Tangkak, Johor	2012
28	SSN-E13	This study	This study	*S. lycopersici*	*S. melongena*	Serdang, Selangor	2012
29	SSN-E14	This study	This study	*S. lycopersici*	*S. melongena*	Serdang, Selangor	2012
30	SSN-P01	This study	This study	*S. lycopersici*	*Capsicum annuum*	Lojing, Pahang	2011
31	SSN-P02	This study	This study	*S. lycopersici*	*C. annuum*	Bertam, Pahang	2011
32	SSN-P03	This study	This study	*S. lycopersici*	*C. annuum*	Bertam, Pahang	2011
33	SSN-P04	This study	This study	*S. lycopersici*	*C. annuum*	Boh Road, Pahang	2011
34	SSN-P05	KF483119	KF483123	*S. lycopersici*	*C. annuum*	Boh Road, Pahang	2011
35	SSN-P06	This study	This study	*S. lycopersici*	*C. annuum*	Tangkak, Johor	2012
36	SSN-P07	This study	This study	*S. lycopersici*	*C. annuum*	Tangkak, Johor	2012
37	SSN-P08	This study	This study	*S. lycopersici*	*C. annuum*	Tangkak, Johor	2012
38	SSN-P09	This study	This study	*S. lycopersici*	*C. annuum*	Tangkak, Johor	2012
39	SSN-P10	This study	This study	*S. lycopersici*	*C. annuum*	Serdang, Selangor	2012
40	SSN-L01	This study	This study	*S. lycopersici*	*Lactuca sativa*	Lojing, Pahang	2011
41	SSN-L02	This study	This study	*S. lycopersici*	*L. sativa*	Boh Road, Pahang	2011
42	SSN-L03	This study	This study	*S. lycopersici*	*L. sativa*	Keama Farms, Pahang	2011
43	SSN-L04	This study	This study	*S. lycopersici*	*L. sativa*	Keama Farms, Pahang	2011
44	SSN-L05	KF483120	KF483124	*S. lycopersici*	*L. sativa*	Tangkak, Johor	2012

Table 1 (continued)

No.[a,b,c]	Isolate	ITS Acc. No.	*gpd* Acc. No.	Species	Host	Location	Year
45	SSN-L06	This study	This study	*S. lycopersici*	*L. sativa*	Tangkak, Johor	2012
46	SSN-L07	This study	This study	*S. lycopersici*	*L. sativa*	Serdang, Selangor	2012
47	**EGS 46-001**	**AY329216**	**AY317020**	***S. lycopersici***	***Lycopersicon esculentum***	**Dominican Republic**	-
48	**EGS 17-137**	**AY329206**	**AY317010**	***S. xanthosomatis***	***Xanthosoma sagittifolium***	**New Caledonia**	-
49	**EGS 41-135**	**AY329214**	**AY317018**	***S. solani***	***Solanum lycopersicum***	**USA**	-
50	**EGS 36-138**	**AY329169**	**AY316969**	***S. herbarum***	***Medicago sativa***	**India**	-
51	**EGS 37-067**	**AY329212**	**AY317016**	***S. vesicarium***	***Medicago* sp.**	**South Africa**	-
52	**EGS 08-069**	**AY329168**	**AY316968**	***S. botryosum***	***Asparagus officinalis***	**USA**	-
53	**EGS 46-182**	**AY329203**	**AY317007**	***S. lancipes***	***Aguilegia* sp.**	**Auckland, New Zealand**	-
54	**EGS 34-015**	**AF347032**	**AY278809**	***A. tenuissima***	-	-	-

[a] *Stemphylium lycopersici* isolates collected from vegetable crops in Malaysia (1 to 46)

[b] Reference *Stemphylium* isolates used in this study (47 to 53)

[c] *Alternaria tenuissima* served as the out-group taxon (54)

(2010). A NanoDrop spectrophotometer (ND-1000, LMS Co., Ltd., Tokio, Japan) was used to check the quality and concentration of Genomic DNA.

PCR amplification and sequencing

PCR amplification of the ITS-5.8S rDNA and *gpd* regions of all *Stemphylium* spp. isolates were conducted using universal primers ITS5 and ITS4 (White et al. 1990) and gpd1 and gpd2 (Berbee et al. 1999), respectively. PCR amplification was carried out in a 25 ml volume containing 0.5 μM primer, 2.5 μl of a 10x buffer (200 mM Tris–HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U *Taq* DNA polymerase (Fermentas Co. Biosyntech Sdn Bhd, Selangor, Malaysia) and 2 μl of DNA template (10 ng). PCR amplification for both regions was conducted in a thermocycler (DNA Engine® Peltier Thermal Cycler PTC-200, MJ research, USA) programmed with the following parameters: 35 cycles of 94 °C for 1 min denaturing, 55 °C for 40 s annealing and 72 °C for 2 min extension. The initial denaturing at 94 °C was extended to 4 min and final extension was at 72 °C for 10 min. PCR products were resolved in 1 % agarose gel under 1×TAE buffer (40 mM Tris, 20 mM Acetic acid and 1 mM EDTA) at 70 V for 45 min at room temperature, stained with ethidium bromide, and visualized under UV light. PCR products were purified using Gene JET™ commercial PCR Purification Kit, according to the manufacturer's instructions, and sequenced by a commercial sequencing service provider (First Base Laboratories Sdn. Bhd., Selangor, Malaysia).

Sequence alignment and phylogenetic analysis

DNA sequences of each isolate were refined using BioEdit sequence Alignment Editor (Hall 1999), in which the sequences obtained from reverse primers were transformed to the reverse complement orientation and aligned with the sequences obtained from forward primers to obtain consensus sequences. BLASTn alignment (Altschul et al. 1997) was conducted to identify and analyze homologous sequences with those of *Stemphylium* species deposited in the GenBank by Câmara et al. (2002) and Inderbitzin et al. (2009). To analyze the relationship of the isolates to known *Stemphylium* species, the 46 sequences from this study and sequences of seven reference *Stemphylium* species (Inderbitzin et al. 2009; TreeBASE study S9931; Table 1) were initially aligned using the Clustal W Multiple alignment (Thompson et al. 1994), checked visually, and improved manually where necessary. Phylogenetic analysis of combined dataset of the ITS-5.8S rDNA and *gpd* regions using the Maximum Likelihood method was performed with Jukes-Cantor model in MEGA 5.0 (Tamura et al. 2011). Branch support of the trees obtained from the maximum likelihood analysis was assessed by boot-strapping with 1,000 replications to estimate the reliability of inferred monophyletic groups. All positions containing gaps were treated as

missing data. *Alternaria tenuissima* (Nees) Wiltshire served as the out-group taxon in the analysis.

Random amplified polymorphic DNA (RAPD) analysis

Seven primers OPA-03, OPF-20, OPG-05, OPJ-20, OPJ-21, OPX-04 and OPY-02 (Operon Technologies Inc., Alameda, CA) with high polymorphism and reproductive profiles were chosen among 19 primers to perform RAPD analysis on *S. lycopersici* isolates based on the results of initial screening against a set of representative studied isolates (Table 2). The primers were synthesized by First BASE Laboratories Sdn Bhd, Malaysia. PCR amplification of RAPD loci was carried out in a 25 ml containing 0.5 μM primer, 2.5 μl of a 10x buffer (200 mM Tris–HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U *Taq* DNA polymerase (Fermentas Co. Biosyntech Sdn Bhd, Selangor, Malaysia) and 2 μl of DNA template (10 ng). RAPD analysis was carried out as described by Pryor and Michailides (2002). PCR amplification was conducted in a thermocycler (DNA Engine® Peltier Thermal Cycler PTC-200, MJ Research, USA) programmed with the following parameters: 45 cycles of 94 °C for 1 min (denaturation), 35 °C for 1.5 min (annealing) and 72 °C for 2 min (extension) with the initial denaturing of 94 °C for 4 min and final extension of 72 °C for 10 min. All PCR reactions were performed in three replications to confirm the consistency of amplification.

Inter simple sequence repeat (ISSR) analysis

Two microsatellite primers ($[ACA]_5$ and $[CCA]_5$) and two minisatellite primers (M13 and T3B) with high polymorphism and reproductive profiles were chosen among 18 studied primers based on the results of initial screening against a set of representative studied isolates (Table 2). The primers were synthesized by First BASE

Table 2 RAPD and ISSR primers utilized to identify and assess interspecific genetic diversity among *Stemphylium lycopersici* isolates

RAPD primers[a]	Sequence	References[b]	ISSR primers	Sequence[a]	References[c]
OPA-01	CAGGCCCTTC	This study	_	$(AAG)_6$	Vitale et al. (2011)
OPA-02	TGCCGAGCTG	Mehta (2001)	_	$(AC)_8T$	Vitale et al. (2011)
OPA-03*	AGTCAGCCAC	Mehta (2001)	_	$(ACA)_5$*	Park et al. (2008), Vitale et al. (2011)
OPA-04	AATCGGGCTG	This study	_	$(AG)_8TA$	Vitale et al. (2011)
OPA-05	AGGGGTCTTG	This study	_	$(AG)_8TC$	Vitale et al. (2011)
OPA-07	GAAACGGGTG	This study	_	$(CAA)_5$	Vitale et al. (2011)
OPA-09	GGGTAACGCC	This study	_	$(CCA)_5$*	Vitale et al. (2011)
OPA-11	CAATCGCCGT	This study	_	$(CTC)_4$	Vitale et al. (2011)
OPA-13	CAGCACCCAC	This study	_	$(GA)_6GG$	Vitale et al. (2011)
OPF-20*	GGTCTAGAGG	This study	_	$(GA)_8C$	Vitale et al. (2011)
OPG-05*	CTGAGACGGA	This study	_	$(GA)_8T$	Vitale et al. (2011)
OPG-07	GAACCTGCGG	This study	_	$(GACA)_4$	Vitale et al. (2011)
OPJ-17	ACGCCAGTTC	Mehta (2001)	_	$(GAG)_4GC$	Vitale et al. (2011)
OPJ-20*	AAGCGGCCTC	Mehta (2001)	_	$(GT)_6CC$	Vitale et al. (2011)
OPJ-21*	ACGAGGGACT	This study	_	$(GTC)_6$	Vitale et al. (2011)
OPK-01	CATTCGAGCC	Mehta (2001)	_	$(GTG)_5$	Vitale et al. (2011)
OPX-04*	CCGCTACCGA	Mehta (2001)	M13	GAGGGTGGCGGTTCT*	Park et al. (2008), Vitale et al. (2011)
OPY-02*	CATCGCCGCA	Mehta (2001)	T3B	AGGTCGCGGGTTCGAATCC*	Park et al. (2008), Vitale et al. (2011)
OPY-03	ACAGCCTGCT	Mehta (2001)	_	_	_

[a] Primers with an asterisk (*) were utilized to identify and assess interspecific genetic diversity among *Stemphylium lycopersici* isolates

[b] Eight RAPD primers with larger numbers of bands as described by Mehta (2001) were selected for this study. Other primers were used in this study

[c] All ISSR primers used by Vitale et al. (2011), including three primers with high polymorphism and reproductive profiles as described by Park et al. (2008) were selected for this study

Laboratories Sdn Bhd, Malaysia. PCR amplification of ISSR was carried out in a 25 ml containing 0.5 μM primer, 2.5 μl of a 10x buffer (200 mM Tris–HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U *Taq* DNA polymerase (Fermentas Co. Biosyntech Sdn Bhd, Selangor, Malaysia) and 2 μl of DNA template (10 ng). ISSR analysis was carried out as described by Park et al. (2008). PCR amplification was conducted in a thermocycler programmed with the following parameters: 45 cycles of 94 °C for 1 min denaturing, 55 °C for 1.5 min annealing and 72 °C for 2 min extension. Initial denaturing at 94 °C was extended to 5 min and the final extension was at 72 °C for 10 min. All PCR reactions were performed in 3 replications to confirm the consistency of amplification.

Gel electrophoresis and staining

PCR products of RAPD and ISSR analyses were size-separated in 1 % agarose gel under 1×TAE buffer (40 mM Tris, 20 mM Acetic acid and 1 mM EDTA) at 70 V for 45 min at room temperature. Gels were stained with ethidium bromide, visualized under UV light and photographed using a gel documentation system (GeneSnap Ver 6.03, Syngene Laboratories, Cambridge, United Kingdom). The sizes of amplified and digested DNA fragments were estimated using GeneTools (Ver 3.00.13, Syngene Laboratories) by comparison with a 2-Log DNA Ladder (0.1–10 kb) marker (Fermentas Co. Biosyntech Sdn Bhd, Selangor, Malaysia).

RAPD and ISSR analyses

Monomorphic and polymorphic bands for both RAPD and ISSR analyses were considered as binary characters and were scored as 1 for presence and 0 for absence of DNA bands. The scores were then entered into a matrix for analysis by the numerical taxonomy and multivariate analysis system, NTSYS-pc 1.8 program (Applied Biostatistics Inc., Setauket, NY, USA) (Rohlf 1993). The similarity matrix was calculated using Jaccard's similarity coefficient. Clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA) to generate the dendrogram.

Virulence and cross-inoculation assays

Two sets of experiments were carried out to test the virulence and cross-inoculation of *S. lycopersici* isolates using the detached leaf technique (Pryor and Michailides 2002; Sujatha et al. 1997). All isolates were used to determine their virulence on detached leaves of tomato (*Solanum lycopersicum* L. cv. 152177-A), eggplant (*Solanum melongena* L. cv. 125066-X), pepper (*Capsicum annuum* L. cv. BBS010) and lettuce (*Lactuca sativa* L. cv. BBS012) (provided by MARDI, Malaysia) as original hosts. For the cross-inoculation assays, one representative isolate with high virulence obtained from each host was used to inoculate the other host plants, and cabbage (*Brassica oleracea* L. cv. BBS040) as a non-host plant. All the experiments were arranged in a completely randomized design in four replications. Each replication consisted of six detached leaves of each tested crop. The 20-μl drops of conidial suspension (10^5 conidia ml^{-1}) containing 0.05 % of Tween 20 were used to inoculate the detached 45-day-old leaves at three spots per leaf. The inoculated leaves were placed on moist filter paper in petri dishes and incubated in humid chambers at 25±2 °C with 95 % RH and a 12 h photoperiod. Control leaves were inoculated with sterile distilled water under the same conditions as the inoculated leaves. Seven days after inoculation, disease rating was scored based on a modified scale of 0–4 points described by Pryor and Michailides (2002), where: 0=no lesion, 1=lesions<1 mm in diameter, 2=lesions 1 to 5 mm in diameter, and 3=lesions>5 mm in diameter. The experiments were repeated twice. Koch's postulate was fulfilled by re-isolation of the inoculated fungi. Percent disease severity (PDS) in each replication was calculated using the following formula proposed by Kempe and Sequeira (1983).

$$\text{Percent disease severity (PDS)} = (\text{Sum of numerical level}) /(6 \times 3) \times 100$$

In this formula: number 6 is the number of leaves observed in each replication and 3 is the highest level of infection.

Results

Sequence alignment and phylogenetic analysis

PCR amplification of the ITS-5.8S rDNA and *gpd* regions of all isolates produced fragments of size 567 and 591 bp, respectively. BLASTn queries based on the ITS-5.8S rDNA and *gpd* regions indicated that sequences of

all isolates were 100 % identical to those of *S. lycopersici*, and nearly identical (100 and 99 %, respectively) to those of *S. xanthosomatis* B. Huguenin available in the GenBank. Sequences of the ITS-5.8S rDNA and *gpd* regions for the representative isolates obtained from each host (SSN-T03, SSN-E12, SSN-P05 and SSN-L05) were deposited in the GenBank (Table 1). Phylogenetic analysis inferred from a combined dataset of the ITS-5.8S rDNA and *gpd* regions indicated that all isolates were clustered in a distinct cluster which included *S. lycopersici* and *S. xanthosomatis* with a strong bootstrap value of 100 %, and the isolates were clustered in the sub-cluster that comprised *S. lycopersici* (Fig. 1). The isolates were distinguished clearly from other *Stemphylium* species used in this study. *Alternaria tenuissima* was phylogenetically distant to *Stemphylium* and clustered as the sister taxon.

RAPD analysis

A total of 65 consistently amplified DNA bands were generated from seven RAPD primers, in which 55.38 % were polymorphic. The average number of bands per primer was 9.2 which ranged in size from approximately 100 to 3,000 bp. The dendrogram produced from UPGMA analysis based on Jaccard's coefficient grouped 46*S. lycopersici* isolates into three main clusters (Fig. 2). Cluster A included 29 isolates from tomato

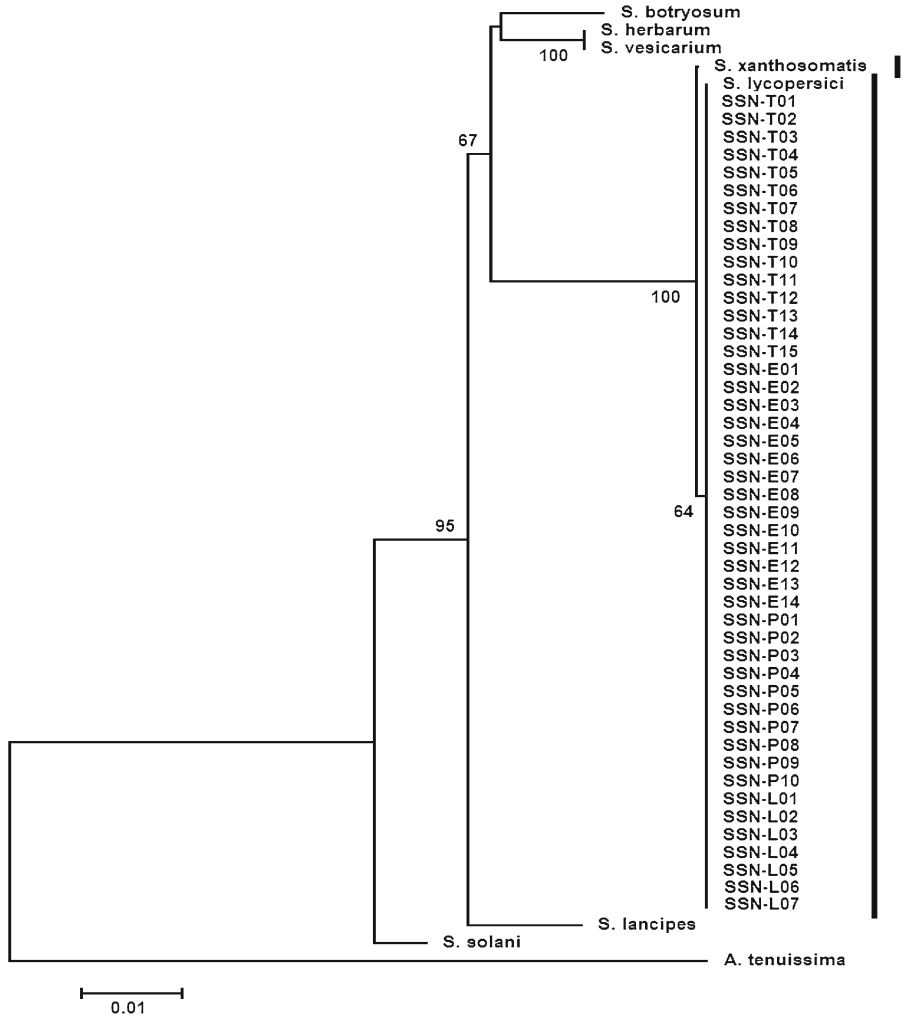

Fig. 1 Phylogenetic tree generated from maximum likelihood analysis of combined dataset of ITS-5.8S rDNA and *gpd* sequences of the 46 isolates from this study, and reference *Stemphylium* isolates (Inderbitzin et al. 2009; TreeBASE study S9931). The tree was rooted with *Alternaria tenuissima*. Numbers of bootstrap support values≥50 % based on 1,000 replicates. The bar indicates nucleotide substitutions per site

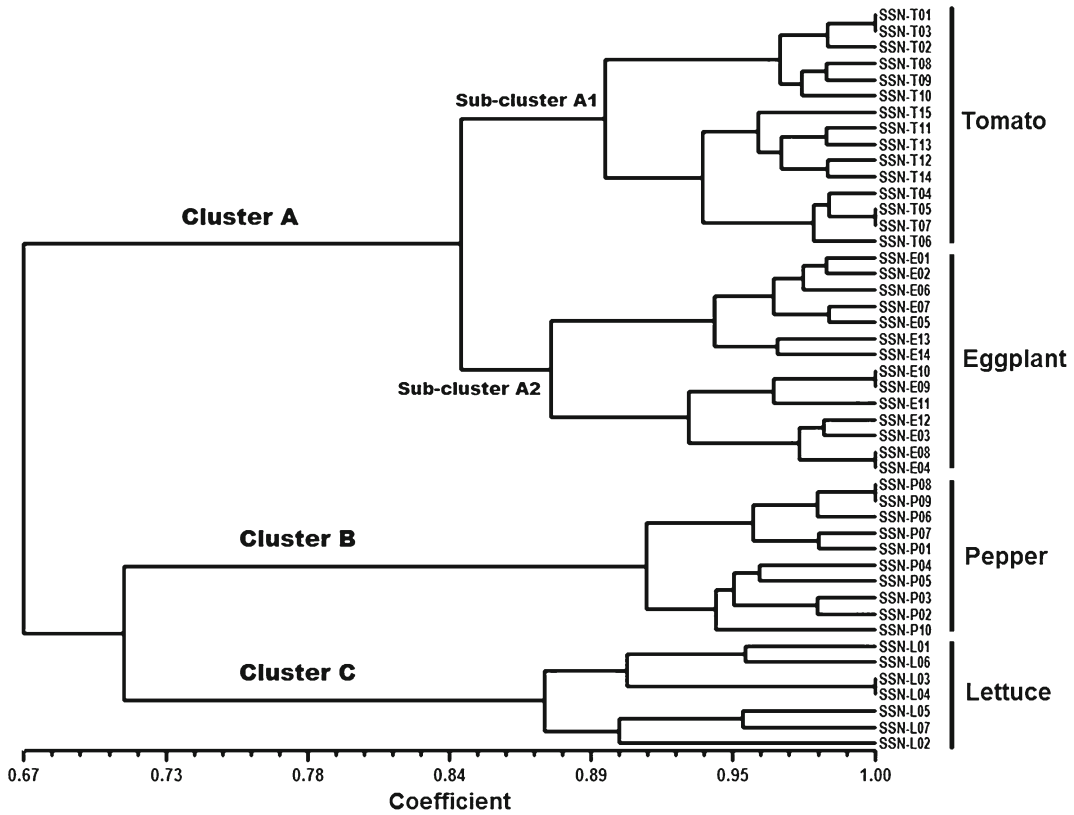

Fig. 2 UPGMA dendrogram generated by RAPD fingerprint analysis of *Stemphylium lycopersici* from different vegetable crops using a combination of seven primer sets (OPA-03, OPF-20, OPG-05, OPJ-20, OPJ-21, OPX-04 and OPY-02). Clusters A, B and C indicate the three groups of *S. lycopersici*. Isolate numbers and hosts are shown

and eggplant. This cluster was split into two sub-clusters (A1 and A2). Sub-clusters A1 contained 15 isolates from tomato, and A2 comprised of 14 isolates from eggplant. Clusters B contained 10 isolates from pepper, and C was comprised of seven isolates from lettuce. The similarity index was calculated at 67 % between all *S. lycopersici* isolates. The isolates in sub-clusters A1 and A2 showed approximately 84 % similarity, and the isolates in clusters B and C exhibited approximately 70 % similarity.

ISSR analysis

A total of 47 consistently amplified DNA bands were generated with the four ISSR primers, in which 69.23 % were polymorphic. The average number of bands per primer was 11.7 which ranged in size from approximately 100 to 2,500 bp. The dendrogram produced from UPGMA analysis based on Jaccard's coefficient grouped 46 *S. lycopersici* isolates into three main clusters (Fig. 3). Cluster A included 29 isolates from tomato and eggplant. This cluster was split into two sub-clusters (A1 and A2). Sub-clusters A1 contained 15 isolates from tomato, and A2 comprised of 14 isolates that were obtained from eggplant. Clusters B and C contained the isolates from pepper and lettuce, respectively. The similarity index was calculated at 68 % between all *S. lycopersici* isolates. The isolates in sub-clusters A1 and A2 showed approximately 80 % similarity. The isolates obtained from lettuce had the lowest similarity of 68 % compared to the rest of the isolates examined.

Virulence and cross-inoculation assays

All *S. lycopersici* isolates were pathogenic on their original host plants and showed leaf spot symptoms; however, virulence variability was observed among the isolates. The spots began to appear 1–2 days after inoculation of detached leaves of the four vegetable crops

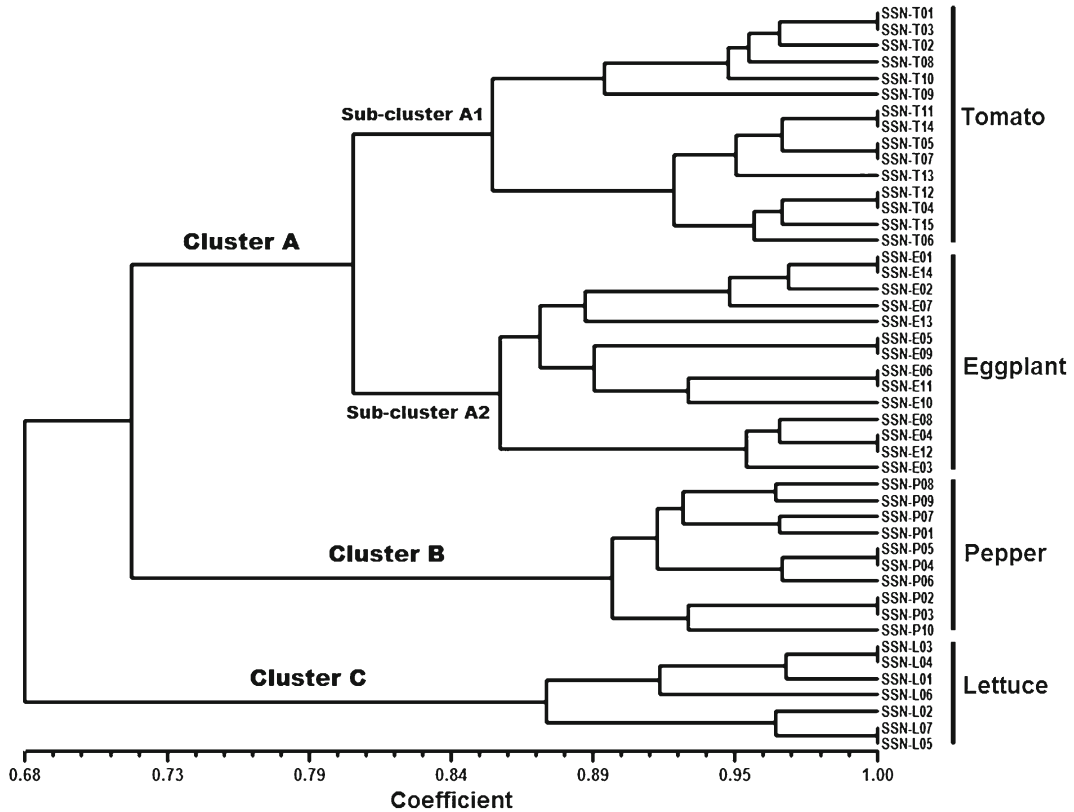

Fig. 3 UPGMA dendrogram generated by ISSR fingerprint analysis of *Stemphylium lycopersici* from different vegetable crops using a combination of four primer sets (M13, T3B, $[ACA]_5$ and $[CCA]_5$). Clusters A, B and C indicate the three groups of *S. lycopersici*. Isolate numbers and hosts are shown

examined. After 7 days, symptoms similar to those observed in infected fields and greenhouses developed on the inoculated leaves. No symptoms were observed on control leaves inoculated with sterile distilled water. *Stemphylium lycopersici* isolates with the same molecular characteristics were re-isolated from inoculated leaves, but not from control leaves. Therefore, the results of this experiment confirmed that *S. lycopersici* isolates were the causal agent of leaf spot on tomato, eggplant, pepper and lettuce. In the cross-inoculation assays, detached 45-day-old leaves of tomato, eggplant, pepper and lettuce as original host plants, and cabbage as a non-host plant were separately inoculated in all possible pairwise combinations with highly virulent isolates originally obtained from each host (SSN-T12, SSN-E06, SSN-P07 and SSN-L07). The cross-inoculation assays revealed that the representative isolates were able to cause leaf spot symptoms on tomato, eggplant, pepper and lettuce, but not on cabbage as a non-host plant (Table 3). All isolates used in this study were highly virulent (>50 % PDS) on the four host plants. No symptoms of the disease were observed on cabbage leaves as the non-host plant inoculated with the representative isolates. Control leaves also remained healthy without symptoms of the disease.

Discussion

Molecular markers have been widely adopted to determine the genetic characteristics of fungi, plants and animals. Molecular techniques based on detection, such as sequencing of different DNA genes have been used as alternatives to morphological identification of *Stemphylium* species (Câmara et al. 2002; Inderbitzin et al. 2009; Mehta et al. 2002; Zheng et al. 2008). The ITS-5.8S rDNA sequence is being widely used to identify phylogenetic relationship among fungal taxa, especially at the generic and lower levels (Callac and Guinberteau 2005; Sotome et al. 2009; Wyk et al. 2009). However, the relatively high level of variability in the *gpd* sequence (Smith 1989) makes this gene more

Table 3 Cross-inoculation assays and percent disease severity of representative *Stemphylium lycopersici* isolates (SSN-T12, SSN-E06, SSN-P07 and SSN-L07) on different vegetable crops by artificial inoculation

Isolate	Origin	Disease severity on different vegetable crops[a,b,c]				
		Tomato	Eggplant	Pepper	Lettuce	Cabbage
SSN-T12	Tomato	HV	HV	HV	HV	NV
SSN-E06	Eggplant	HV	HV	HV	HV	NV
SSN-P07	Pepper	HV	HV	HV	HV	NV
SSN-L07	Lettuce	HV	HV	HV	HV	NV
Control	…..	NV	NV	NV	NV	NV

[a] Cross-inoculation of representative *Stemphylium lycopersici* isolates on vegetable crops 7 days after inoculation

[b] Virulence was rated based on percent disease severity (PDS) recorded on different vegetable crops (Santha Lakshmi Prasad et al. 2009). non virulent (NV), no symptom; weakly virulent (WV), < 20 % PDS; virulent (V), 20 to 50 % PDS; highly virulent (HV), > 50 % PDS

[c] PDS for all isolates were 100 %, except for isolates **SSN-L07** on tomato (PDS=95.5 %), **SSN-E06** and **SSN-P07** on eggplant (PDS=99.5 and 95.2 %, respectively), **SSN-T12**, **SSN-P07** and **SSN-L07** on pepper (PDS=61.2, 98.6 and 64.2 %, respectively)

[*] PDS was calculated based on the lesion diameter on leaves using standard formula; (Sum of numerical level)/(6×3)×100; number 6 is the number of leaves observed in each replicate and 3 is the highest level of infection

appropriate for phylogenetic comparison at the species taxonomic level (Berbee et al. 1999). In this study, phylogenetic analysis inferred from combined dataset of the ITS-5.8S rDNA and *gpd* regions confirmed the identification of all isolates as *S. lycopersici*. The isolates differed from *S. xanthosomatis* by substitution in one locus at position 126 (A/G) at the *gpd* gene. These results also revealed that sequences of the ITS-5.8S rDNA and *gpd* could not differentiate 46 *S. lycopersici* isolates obtained from different host plants and geographical origins, as the isolates were clustered in the same subclade that comprised reference *S. lycopersici* obtained from tomato in the Dominican Republic. This result is in agreement with a previous study that was conducted by Câmara et al. (2002).

RAPD and ISSR markers are extremely powerful tools to separate individuals having intraspecific and interspecific variability. These markers provided comprehensive information regarding the intra and interspecific variations in *Stemphylium* species (Chaisrisook et al. 1995; Mehta 2001), and other plant pathogenic fungi (Achenbach et al. 1996; Nghia et al. 2008; Park et al. 2008; RuiQian et al. 2009; Sharma et al. 2013; Zhou et al. 2001). To the best of our knowledge, there is no report on the use of ISSR markers to analyze intraspecific and interspecific variability in the *Stemphylium* genus, and this is the first report on the use of RAPD markers to analyze *S. lycopersici* isolates from different hosts and geographical regions. Mehta (2001) analyzed 33 *Stemphylium* spp. isolates obtained from cotton and tomato plants in Brazil using RAPD markers, in which two *S. lycopersici* isolates associated with tomato from the same geographical region (Location: Botucatu) were clustered in a distinct clade from *S. solani* isolates. Several researchers have reported correlations between RAPD and ISSR groups and features of isolates such as virulence, geographical origin and host plant genotype from which the isolates were collected (Atan and Hamid 2003; Köhl et al. 2009; Lourenço et al. 2011; Mehta 2001; Silva et al. 1998; Silva et al. 2003), but some others have found no correlation (Darmono et al. 1996; Romruensukharom et al. 2005; Sharma et al. 2013). The advantage of the ISSR technique compared to RAPD lies in the effective multilocus markers used for diversity analysis, fingerprinting and genome mapping. They are easy to employ and are highly reproducible (Goldwin et al. 1997). In the present study, 55.38 % and 69.23 % of the bands generated using RAPD and ISSR respectively, were polymorphic, which reflects the relatively high level of genetic variation that exists among the isolates. Genetic diversity was observed among all isolates using both markers with the identification of three main RAPD and ISSR profiles. The results suggested a relatively low similarity index value among the isolates collected from the various vegetable crops (67 and 68 % for RAPD and ISSR markers, respectively). The clustering based on RAPD and ISSR markers showed concordance with host plants of the isolates originating

from tomato, eggplant, pepper and lettuce, which were represented in distinct clusters. The results between RAPD and ISSR markers were congruent. However, the effective multilocus marker ratio and subsequent to that the number of private alleles was greater with ISSR markers. The results of ISSR markers were very similar with RAPD markers, and with both markers, the isolates obtained from tomato and eggplant were clustered in a distinct cluster, and the isolates obtained from pepper and lettuce were grouped into other distinct clusters. This separation of the isolates obtained from tomato and eggplant into a distinct cluster with high values of similarity index (approximately 84 and 80 % in RAPD and ISSR, respectively) implies that this group of isolates belongs to the near-physiological group compared to the isolates collected from pepper and lettuce. Based on RAPD and ISSR markers, *S. lycopersici* isolates obtained from various vegetable crops originating from the three states of Pahang, Johor and Selangor in Malaysia showed no geographical variation. The high level of polymorphism within the isolates may, however be attributed to the fact that the isolates were obtained from different host plants, thus reinforcing the hypothesis of diverse hosts being more important than geographical regions. This result is in agreement with the results of Mehta (2001) and Köhl et al. (2009), who did not observe any clear relationship between genetic variability and geographical origin of *Stemphylium* species, while the isolates represented host specialization. Köhl et al. (2009) indicated that the variation among *S. vesicarium* isolates obtained from different hosts was high (approximately 30 % similarity index), and the variation showed significant concordance with host plants. Mehta (2001) also revealed that *S. solani* isolates obtained from cotton and tomato in Brazil had a high variation (approximately 60 % similarity index), and the variation was according to host plants, not geographical origin of the isolates.

Virulence assays revealed that all isolates were pathogenic on the original hosts and leaf spot symptoms were observed on the detached leaves of tomato, eggplant, pepper and lettuce similar to those observed in the infected greenhouses and fields, 7 days after inoculation. Re-isolation of the fungus with the same morphological characters described earlier on potato dextrose agar (PDA) confirmed Koch's postulates. Thus, the results of the present study confirmed that *S. lycopersici* isolates were the causal agents of leaf spot on the four vegetable crops. In the cross-inoculation assays, the representative isolates with high virulence selected from each crop were able to cause leaf spot on the inoculated leaves of the four host plants found in this study, but not on cabbage as a non-host plant. This finding suggested that *S. lycopersici* can spread between these four economically important crops, and hence a comprehensive management is required to manage this disease. These results are in agreement with previous reports on the pathogenic ability of *S. lycopersici* to infect different hosts (more than 30 host genera) all over the world (Farr and Rossman 2014).

In conclusion, both RAPD and ISSR markers proved to be useful in differentiating *S. lycopersici* isolates. However, the effective multilocus marker ratio and subsequent to that the number of private alleles was greater with ISSR markers. Moreover, the current outcome indicated that the makers are able to respond onto the limitation of molecular sequence method. This study also confirmed that isolates of *S. lycopersici* existing in Malaysia are in concordance to host plants, and not geographical origin of the isolates. Hence, it is recommended that for better control of the pathogen each vegetable crop be investigated separately. The data of RAPD and ISSR markers could also be expanded for a wider genetic diversity of *S. lycopersici* on different host plants from different geographical regions.

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