Molecular Identification of *Bipolaris cactivora* on Dragon Fruit in Thailand

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ABSTRACT: A fruit rot disease observed in white-fleshed dragon fruit, *Hylocereus undatus* (Haworth) Britton & Rose, collected from Loei province, Thailand was studied to identify the causative agent. Common symptoms on the surface of the fruits were water-soaking brown spots with powdery signs. The diseased fruits were then isolated for the fungal pathogen. According to the morphology, *Bipolaris cactivora* (Petr.) Alcorn was the most possible one that caused the disease. To confirm this, the molecular identification and phylogenetic analysis using two gene sequences, internal transcribed spacer (ITS) amplified with ITS1 and ITS4 primers and elogation factor (EF) using 983F and 2218R primers were thus employed. Due to the statitical supports of high bootstrap score at 99% derived from Neighbour-joining method and potential interior propability, 100% calculated fron Baysian analysis, the fungal pathogen of rot disease on the dragon fruits was *B. cactivora*.
Keywords: brown rot, *Hylocereus undatus*, ITS, elongation factor, water-soaking, phylogenetic trees

Introduction

Fungi in genus *Bipolaris* Shoemaker has been reported as saprophyte, endophyte and significant plant pathogens commonly found worldwide (Manamgoda et al., 2012). The most frequent ones are *Bipolaris maydis* (Y Nisik. & C. Miyake) Shoemaker, *B. oryzae* (Breda de Haan) Shoemaker and *B. sorokiniana* (Sacc.) Shoemaker that have caused damages on economic food crops (Manamgoda et al., 2012, Manamgoda et al., 2014). Recently, according to morphology, *B. cactivora* (Petr.) Alcorn has been officially reported in Thailand that isolates of this pathogen were obtained from dragon fruits throughout the country (Oeurn et al., 2015). It has been also widely found in Japan (Taba et al., 2007), Israel (Ben-Ze'ev et al., 2011), South-Florida (Tarnowski et al., 2010), Vietnam (He et al., 2012) and Europe (Durbin et al., 1955). This causal pathogenic agent is not only identified from the dragon fruits but also distributed in areas growing cactus plants as a fungus causing stem rot disease (Kim et al., 2004). Loei province where dragon fruit trees are grown and the fruit rot diseases were seen. In preliminary investigation, there were some plant-pathogenic fungi derived from the collected dragon fruit plants. *Alternaria* sp. and *Phomopsis* sp. were isolated on the plant stems. *Cladosporium cucumerinum* Ellis & Arthur, *Fusarium* sp., *Alternaria* sp. and *Colletotrichum gloeosporiodes* (Penz.) Penz. & Sacc. were additionally obtained from rotten flowers. *B. cactivora*, *C. gloeosporiodes* and *Rhizopus stolonifer* (Ehrenb.) Vuill. were isolated from both rotten flowers and fruits.

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The identification of *Bipolaris* species is generally based on their characteristics as they possess variation of morphology and physiology in their mycelium and conidia (Chand et al., 2003, Banerjee et al., 2014). The identification of *B. cactivora* using its morphology is sometimes unable to distinguish it from other *Bipolaris* and closely-related species (Manamgoda et al., 2011). That is why a molecular techniques, polymerase chain reaction has been applied to identify plant pathogens using unique genomic regions (Banerjee et al., 2014). To overcome morphology boundary, molecular phylogenetic analysis using different DNA sequences of taxonomically informative regions were employed for identification of phylogenetic relationships (Miller and Buyck, 2008). The DNA sequences used to study the molecular phylogeny of genus *Bipolaris* were ribosomal genes, internal transcribed spacers (ITS), glyceraldehyde-3-phosphate dehydrogenasee (GPDH) and translation elongation factor 1-alpha (TEF) regions. (Manamgoda et al., 2014). As dragon fruit is not a predominant crop in Thailand, records related to diseases in dragon fruits are then not fully documented. Therefore, the objectives of this study were additionally to confirm the existence of *B. cactivora* isolated from the dragon fruits via phylogenetic analysis using ITS and TEF regions.

Materials and Methods

Fungal isolates and Morphology

Single conidia of *B. cactivora* on dragon fruits collected from Phu Ruea district, Loei province, Thailand were isolated and maintained on potato dextrose agar (PDA) for morphological investigation. In order to observe conidia and conidiophores, the fungal mycelia were placed on surface-sterilized dragon fruits using Clorox 10% for 5 mins and incubated at 25±1 °C for 4 days. Conidia and conidiophores were mounted in distilled water and observed under a Carl Zeiss Axioplan2 compound light microscope. The measurement of conidial width was indicated by the widest part of each conidium. The lengths and widths were measured using Axiovision Rel. v. 4.8.2 software (Carl Zeiss Microscopy, Thornwood, NY, USA). One hundred conidia were measured then calculated for mean, minimum, maximum, 5th and 95th percentiles and standard deviation (Damm et al., 2008; Gramaje et al., 2012).

Molecular study

Fungal culture: The germinated mycelia of *B. cactivora* on PDA for 5 days were to potato dextrose broth (PDB) for 4 days at 25±1 °C. The mycelium was harvested using filter paper then directly kept in the freezer (-20 °C) for DNA extraction.

DNA extraction: According to White et al (1990), the frozen mycelia samples were ground in liquid nitrogen by using sterile mortar and pestle. Lysis buffer (200 mmol/l Tris-HCl, pH 8.0; 250 mmol/l NaCl, 25 mmol/l EDTA, pH 8,0; 1% sodium dodecyl sulfate) 700 µL was added with 3 µL ß-mercaptoethanol. The tubes were incubated at 60 °C for 30 min. After incubation, the samples were added with chloroform: isoamyl alcohol (24:1) for 700 µL then centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was collected and transferred to new tubes. Isopropanol, 0.7 time of collected supernatant was added and

placed in the freezer (-20 °C) for 20 min. After that the tubes were spun at 12,000 rpm for 5 min to obtain DNA pellets then the pellets were cleaned with 70% ethanol 500 µL two times and air-dried. The DNA pellets were dissolved in TE buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA) 50 µL for 10 min then RNase A 1 µL (10 ng/µL) and Proteinase K 1 µL (10 ng/µL) were respectively added and incubated for 20 min. To clean the DNA, additional 100 µL of chloroform: isoamyl alcohol (24:1) was added, centrifuged 12,000 rpm for 4 min and the supernatant collected and transferred to new tubes. The tubes were added with 3 µL of 3M Sodium acetate and 150 µL of absolute ethanol and kept in the freezer (-20 °C) for 20 min. Then, they were again centrifuged at 12,000 rpm for 10 min to derive the cleaned DNA pellets. Lastly, the tubes were cleaned with 70% ethanol with amount 400 µL then kept the pellet dry and re-suspended in TE buffer. The genomic DNA in TE was kept at -20 °C for further use.

Polymerase chain reaction (PCR) and DNA sequence for phylogenetic analysis: The amplification of ITS region was done using primers ITS1 and ITS4 (White et al., 1990). The partial region of the translation elongation factor (EF-1α) was amplified with EF 983/2218R primer. Both of the regions ITS and EF-1α were in a 50 µl reaction final volume which contained 1 µl dNTPs, 5 µl PCR buffer, 4 µl $MgCl_2$, 1 µl of each primer, 1 µl gDNA, 0.5 µl Taq DNA polymerase (Thermo Scientific) and 13.5 µl dH_2O. The condition for both primers (ITS and EF-1α) PCR amplification program was carried out conditions; according to these 1 min of initial denaturation at 95 °C followed by 30 cycles of 95°C for 1 min, 59°C for 1 min, 74 °C for 50 s and final extension at 74 °C for 7 min.

To check whether the amplification was successful, 1 µl PCR products was loaded into 1% agarose gel and the electrophoresis was carried out with TBE buffer (1 M Tris, 0.9 M boric acid, and 0.01 M EDTA, pH 8.3). The running program was set for 45 min before stained with ethidium bromide then visualized in gel documentation. Then, the PCR products were set to purify and sequence using a BigDye® Terminator v3.1 cycle sequencing kit by First BASE Laboratories, Seri Kembangan, Selangor, Malaysia.

Phylogenetic trees: Prior to the construction of phylogenetic trees, the chromatograms of ITS and EF-1α sequences were edited via BioEdit version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to check for unclear signals which were then manually removed. The generated sequences were edited and aligned together with from sequences of fungal species (**Table 1**) obtained from GenBank (http://www.ncbi.nlm.nih.gov/) using MEGA 6.06. Maximum parsimony analysis was used to construct a phylogenetic tree using program MEGA 6.06 for constructing Neighbor Joining trees. One-thousand bootstrap replications with model Number of Differences were applied. Additionally, Bayesian analysis using

Table 1 List of fungi used with accession number, cited from GenBank

Identity	Locality and GenBank Access Number			
	locality	ITS	locality	EF-1α
Alternaria alternata	NL*	AF071346	India	DQ677927
			Russia	KJ443203
Aureobasidium pullulans			NL	APU19723
Bipolaris australiensis	Australia	AJ853762		
Bipolaris cactivora	China	GU390882, HQ896484		
	USA	HM598677, HM598679		
	Italy	KF041822		
Bipolaris coffeana	Kenya	KJ415525		
	Thailand	KJ922385, KM230386		
	USA	KM230385		
Bipolaris maydis	Japan	KM230388, KJ909769	Japan	KM093792
	USA	KM230389	USA	KM093794, KM093795
Bipolaris micropus	USA	HE792933, HE792934, HE792935	USA	HE792957, HE792958
Bipolaris oryzae	Thailand	JX256416, JX256417	Thailand	KM093790
	Japan	KJ922383	Japan	KM093789
	USA	KM230393	USA	KM093786
Bipolaris ovariicola	NL	AF163092		
Bipolaris sacchari	Taiwan	KJ020916		
	India	KJ174418		
	New Zealand	KJ922386		
Bipolaris sorokiniana	Italy	KJ909776	Italy	KM093762
	Japan	KJ909792	Japan	KM093767
	USA	KJ922381	USA	KM093763
Bipolaris spicifera	China	JN695634, JN695635, JN695636		
Curvularia australiensis			Australia	KC503939, KC503952, KJ415452
Curvularia lunata			NL, USA	JQ965130, JX266596
Curvularia perotidis			Australia	JN601021, KM230407

NL* = Non Identified Location

MrBayes 3.2.4, using Markov Chain Monte Carlo (MCMC) algorithm (Ronquist et al., 2003) with four chains was run simultaneously applying the sixth substitution model that was general time-reversible (GTR) with rate variation of invariable site (I). The analyses were run until the average standard deviation of split frequency was below 0.01. Four MCMC chains were run simultaneously with 100,000 generations for the first data sets and 900,000 generations for dataset 2 with phylogenetic trees sampled every 100 generations applied to all searches. The posterior probabilities and consensus tree were determined after 25% burn in (Huelsenbeck and

Rannala, 2004). The significance of tree nodes were indicated by bootstrap values and posterior probabilities equal to or greater than 70% and 0.95 respectively (Spatafora et al., 2006).

Results and Discussion

Morphology

The observation of the morphology of *B. cactivora* isolated from Leoi province has been reportedly in previous study (Oeurn et al., 2015). The symptoms appeared on the fruits brown spots with water soak and black powdery spot (**Figure 1**). Due to the asexual morph of *B. cactivora* on the fruit, conidiophores were pale to light brown, caespitose, straight or flexuous. Conidia with a basal hilum were straight, ellipsoidal, fusiform or obclavate, 2-4 septa, pale light brown to brown (**Figure 1**), and conidia size were (23.78-)25.30 - 47.30(-50.95) x (5.73-)5.97 - 8.84(-9.84) µm (av=36.26, SD=6.47, n=100; av=7.13, SD=0.75, n=100).

Figure 1 A: rotten flower of dragon fruit plant, B and C: disease symptom caused by *B. cactivora* on the dragon fruit, D: conidia attached with conidiophore, E: young spore without septa , F: conidia , G and H: conidiophores . Scale bars: C = 100 mm; D- I = 20 µm.

Phylogenetic Study

DNA sequences had been uploaded to GenBank. The accession numbers of these sequences are KT287105-KT287115. Moreover, the culture of *B. cactivora*, Thailand isolates, has been deposited in CBS fungal biodiversity center and CBS accession numbers are CBS140067-CBS140072.

According to the results of the phylogeny based on DNA sequences of ITS, the fungal pathogen was agreeably indicated as *B. cactivora*. Supported by bootstrap value, 99, it was grouped in the sample clade as *B. cactivora* shown in **Figure 2** and an outgroup was *Alternaria alternata* (AF071346). Apart from bootstrap analysis, Bayesian analysis was also run the same sequence data. The high posterior probability, 100% illustrated in **Figure 3** confirmed that fungal isolates of this study *B. cactivora* as they were clustered in the same branch as those retrieved from GenBank. Although the protein coding gene sequences (EF-1α) of *B. cactivora* was unavailable in GenBank, the trees constructed by using these sequences ascertained that the fungal isolates were similar according to bootstrap value at 100 and 98% posterior probability (**Figure 4** and **5**) rooted with *Aureobasidium pullulans*.

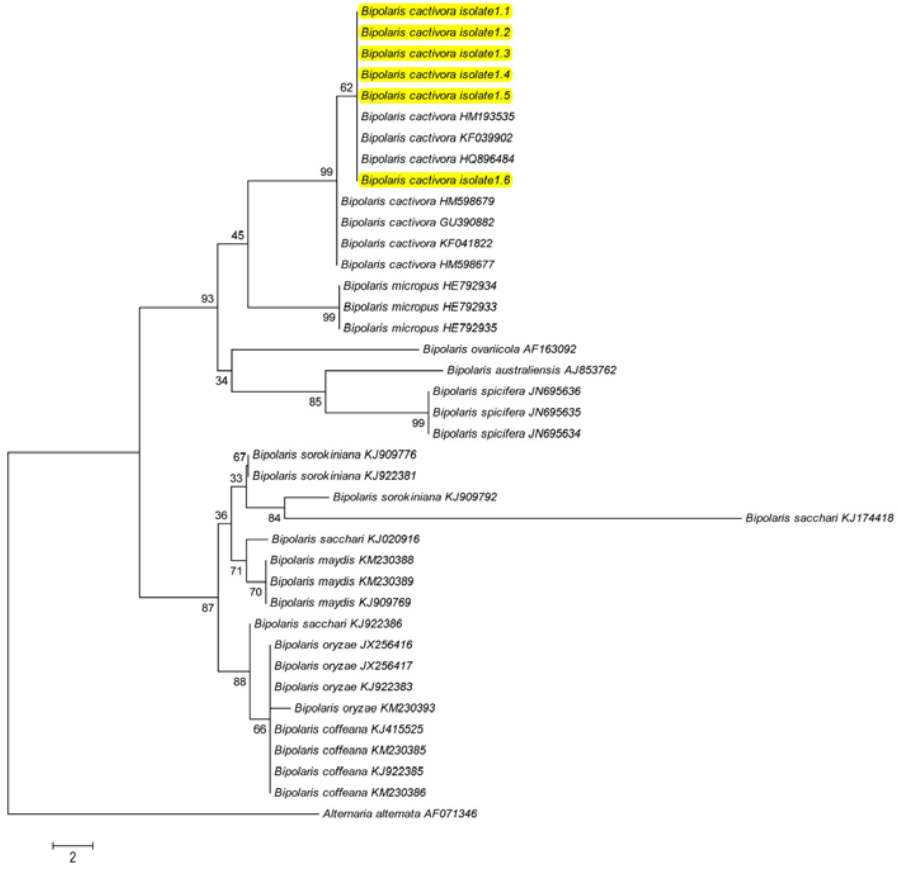

Figure 2 The phylogenetic tress derived from Neighbor Joining analysis using ITS sequences suggests that the fungus from this study is clustered in the same branch with *Bipolaris cativora* with supportive bootstrap score, 99. The outgroup is *Alternaria alternata*.

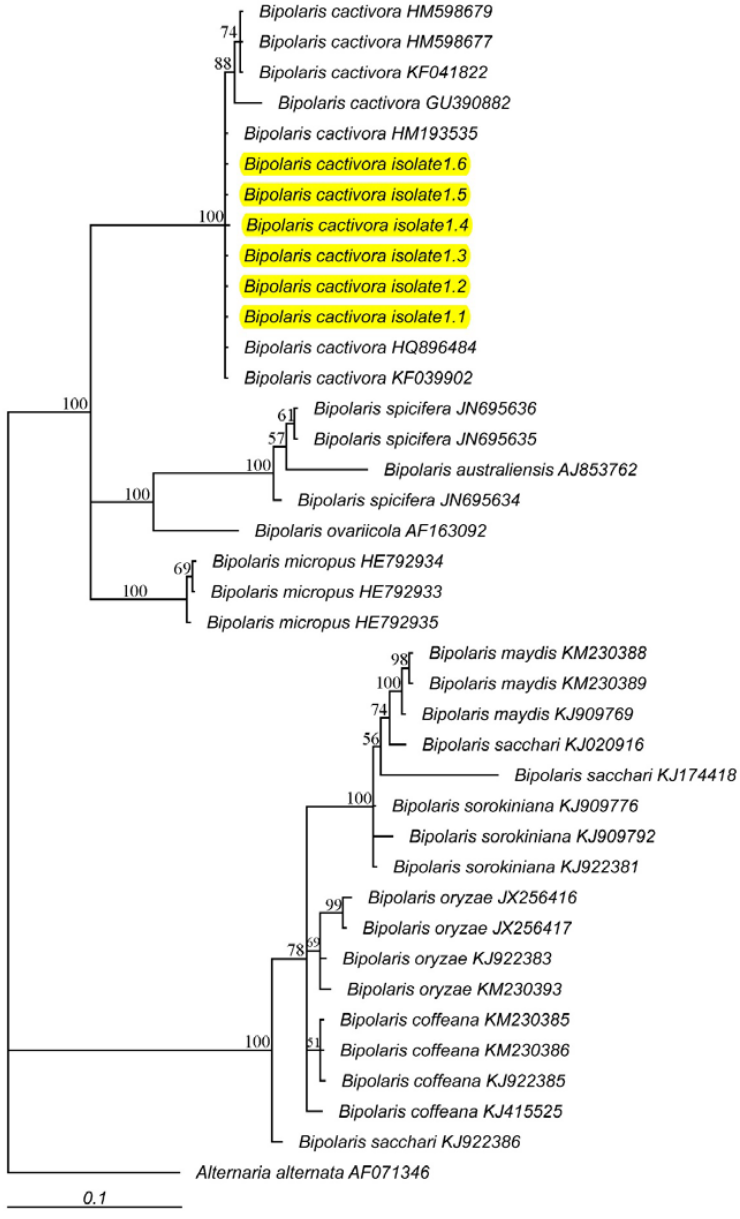

Figure 3 The phylogenetic tress derived from Bayesian analysis using ITS sequences suggests that the fungus from this study is clustered in the same branch with *Bipolaris cativora* with high posterior probability, 100. The outgroup is *Alternaria alternata*.

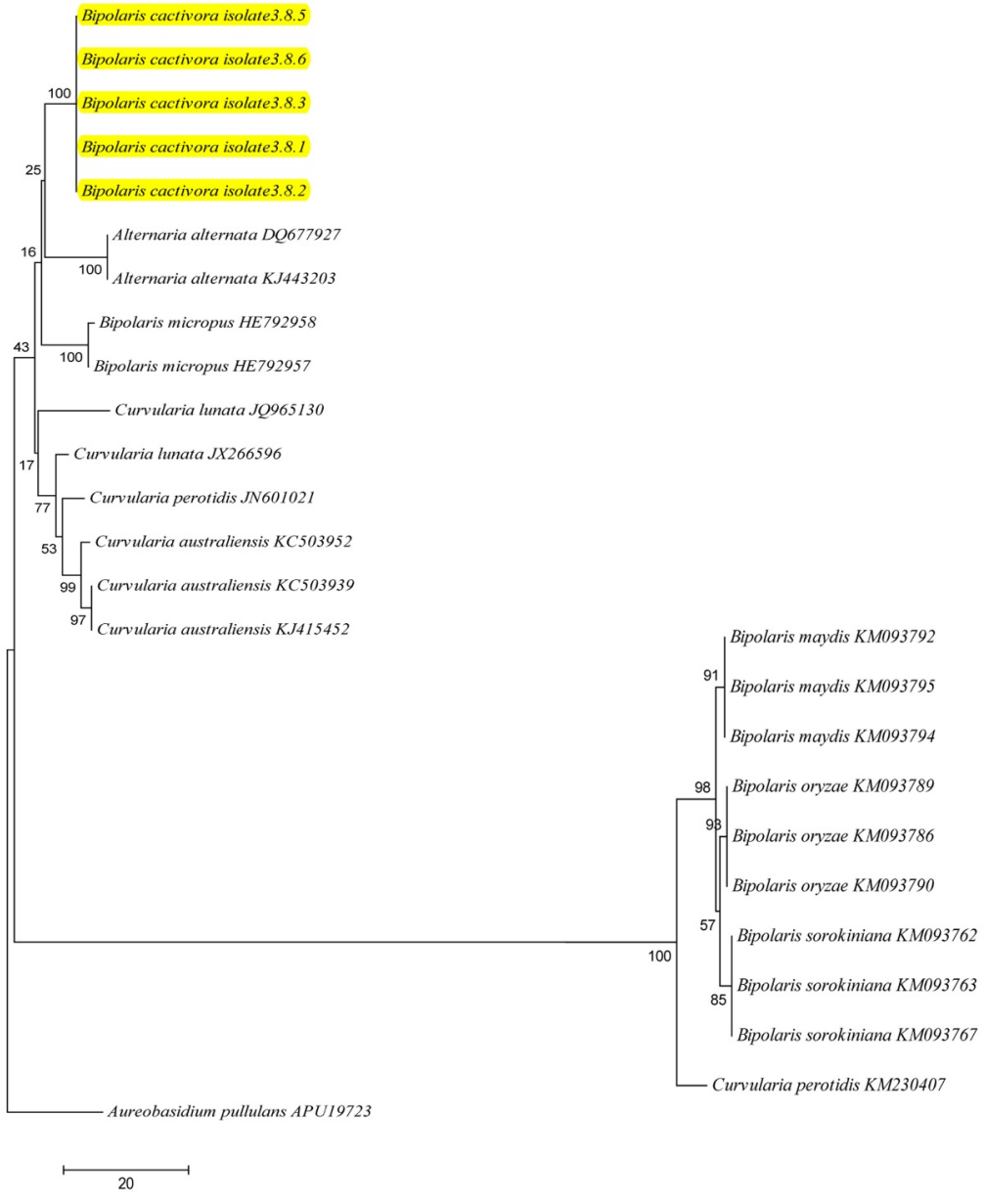

Figure 4 The phylogenetic tress derived from Neighbor Joining analysis using EF-1α sequences suggests that the fungus from this study is distinctively separated out of other *Bipolaris* and its relative species. The outgroup is *Alternaria alternata*.

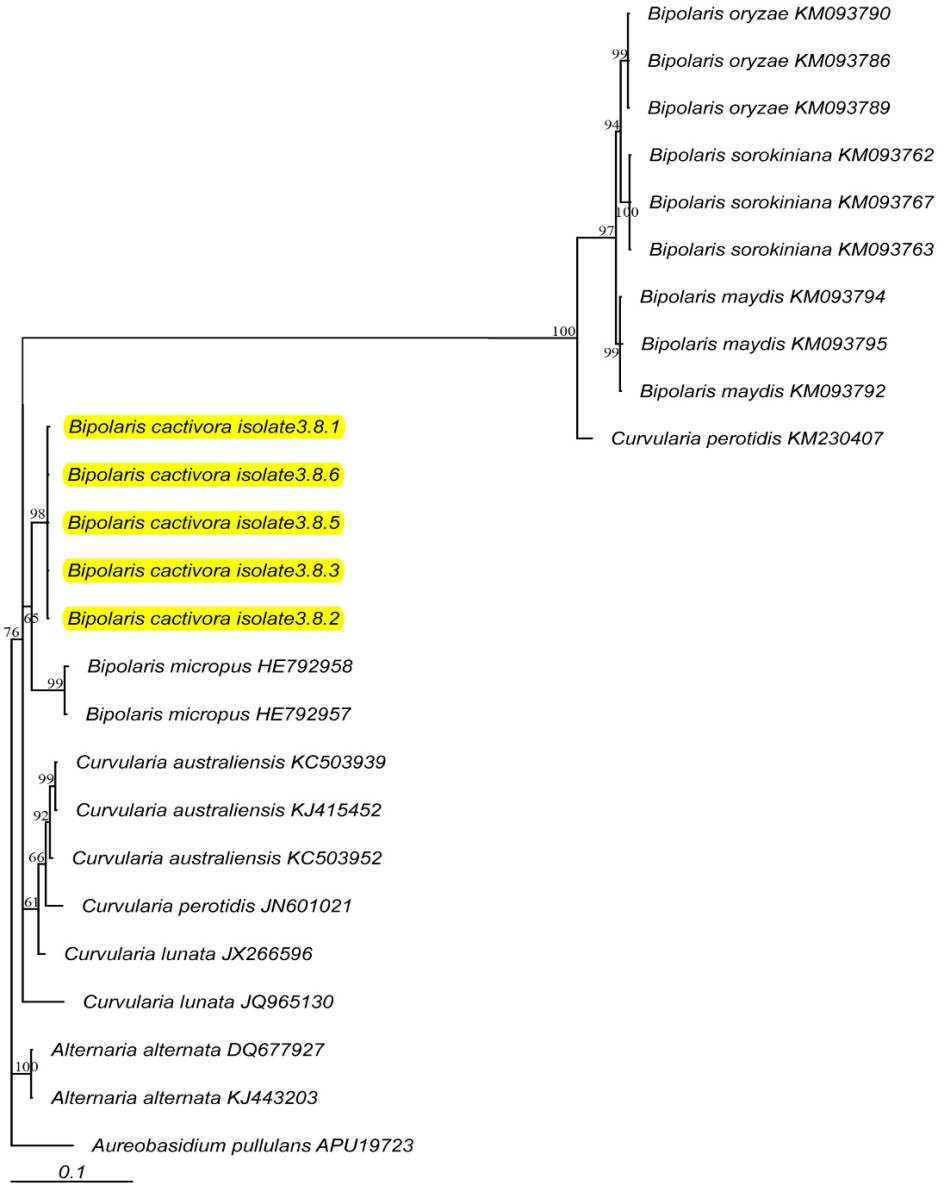

Figure 5 The phylogenetic tress derived from Neighbor Joining analysis using EF-1α sequences suggests that the fungus from this study is distinctively separated out of other *Bipolaris* and its relative species. The outgroup is *Alternaria alternata*.

Discussion

Fruit rot disease on dragon fruit caused by *B. cactivora* has been wildly reported in slightly cool area such as Japan, Israel, South-Florida, Vietnam and Europe (Taba et al., 2007; Tarnowski et al., 2010, Ben-Ze'ev et al., 2011; He et al., 2012). In Thailand, *B. cactivora* associated with dragon fruits has also been documented (Athipunyakom et al., 2009, Oeurn et al., 2015) as it identified by using morphological features. Two species of *Bipolaris* and *Curvularia* have been difficult to distinguish based on their morphology of conidia (Sivanesan, 2003) and *B. cactivora*, which is lack of molecular data (Manamgoda et al., 2014). Analysis of genetic diversity of the plant pathogens have been developed via molecular study and it could be able to strengthen the understanding of taxonomy while morphological characteristics are not completely able to determine fungal strains (Sharma, 2003, Banerjee et al., 2014). Therefore, this study was then conducted to confirm the presence of and identify *B. cactivora* that caused the rot disease on the dragon fruits, which was morphologically similar to some *Curvularia* species (Manamgoda et al., 2014)).

The results of conidial morphology were closely matched with previous studied by Tarnowski et al. (2010) and Taba et al. (2007) who worked on *B. cactivora* in terms of the conidial size, 24-51 x 9-13 µm and 35-45 x 8-10 µm (Taba et al., 2007, Tarnowski et al., 2010) respectively. The characteristics of the genus *Bipolaris* have been described i.e. brown conidiophore, conidia in fusoid, straight or curved shape and germ tube germinates at each end (Manamgoda et al., 2014). Tarnowski et al. (2010) also mentioned that *B. cactivora* on dragon fruit had its conidia formed at the tips of pale golden brown conidiophore with blackish brown colonies. Conidia was pale-to-medium golden brown, smooth and clavate with a protuberant hilum and 2-4 septa (Tarnowski et al., 2010). *Helminthosporium* stem rot of cacti in Japan caused by *Helminthosporium cactivora* was similarly described that its conidia were dark brown, straight or slight or slightly curve, obtuse ellipsoidal but did not mention about how a number of conidial septa. Accordingly, in some occasions, the confusion led by morphological features was occurred to identify the rot-disease pathogen in the cacti plant. The molecular data was therefore employed to resolve this issue (Valente et al., 1999, Mendoza et al., 2001).

DNA sequences of ITS and EF-1α regions have been used to build phylogenetic trees in order to assist the identification of interested taxa (Manamgoda et al., 2014)). The trees obtained from this study using ITS sequences aligned with the *Bipolari*s taxa most confirmed that the isolates derived from the dragon fruits was *B. cactivora* as they were clustered in the same branch with *B. cactivora* (HM193535, HQ896484 and KF039902) retrieved from GenBank with supportive bootstrap and posterior probability scores. Furthermore, Tarnowski et al. (2010) reported the use of ITS and Glycerol-3-phosphate dehydrogenase (GPDH) DNA sequences compared with previously published sequences of *Bipolaris* (Tarnowski et al., 2010). The result showed with the great confidence of the analysis, 99.7% that the fungal isolates belonged to

B. cactivora. Although, no DNA sequence of EF-1α in GenBank for this species, ITS was the strong witness that all isolates obtained from the dragon fruits were *B. cactivora*.

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