International Journal of Agricultural Technology 2018 Vol. 14(6): 845-860
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

Morphological and Molecular Based Identification of Corn Downy Mildew Distributed in Thailand

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Janruang, P. and Unartngam, J. (2018). Morphological and molecular based identification of corn downy mildew distributed in Thailand. International Journal of Agricultural Technology 14(6):845-860.

Abstract In Thailand, corn downy mildews are reported to cause by six species of fungi including *Peronosclerospora sorghi*, *P. philippinensis*, *P. sacchari*, *P. spontanea*, *Sclerophthora rayssiae* var. *zeae* and *P. maydis*. The fungal species of corn downy mildew distributed in different geographic areas of Thailand based on morphological and molecular identification was determined. The disease samples were collected for morphological and DNA sequence observations. The results showed that all fungal isolates possessed various conidial shapes with 15.0-19.8×15.0-32.0 µm in size and different conidiophore types using septum position. Therefore, morphological characteristics of conidia and conidiophore type were similar in range to *P. maydis* and *P. sorghi*. Phylogenetic analysis was carried out using the 28S rDNA gene and internal transcribed spacer 1 (ITS1) region. The results of ITS1 region analysis indicated that 31 sequences were grouped together with *P. maydis* obtained from the GenBank database (NCBI) at a 99% similarity coefficient and separated from other sequences. Therefore, the present study suggested that four species in Pest List of Thailand should be removed from the list of corn downy mildew, including *P. philippinensis*, *P. sacchari*, *P. spontanea* and *Sclerophthora rayssiae* var. *zeae*.

Keywords: Corn downy mildew, *Peronosclerospora*, 28S rDNA, ITS1 region

Introduction

Corn downy mildew fungi are obligately biotrophic pathogens, cause one of the most important corn diseases in Thailand, which significantly decreases corn production. Starting in the late 1960s, this disease was rapidly distributed in several provinces of Thailand. Therefore, the development of resistant cultivars and the use of chemical protectants were initiated (Carlos, 1976). Several fungal species cause corn downy mildew and outbreaks of the disease have occurred worldwide. Six species have been reported in Thailand, belonging to two genera: *Peronosclerospora sorghi* (W. Weston & Uppal) C.G. Shaw, *P. philippinensis* (W. Weston) C.G. Shaw, *P. sacchari* (T. Miyake)

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Shirai & Hara, *P. spontanea* (Weston) C.G. Shaw, *Sclerophthora rayssiae* var. *zeae* Payak & Renfro (Bonman *et al*.,1983; Chamswarng, 1976; Pupipat, 1976; Watanavanich *et al.*, 1976; White, 1999) and *P. maydis* (Racib.) C.G. Shaw (Perumal *et al*., 2008). Indeed, the Pest list of Thailand previously reported that five species of the genus *Peronosclerospora* caused corn downy mildew in Thailand.

In the past, fungal species identification only used morphological characteristics (Chamswarng, 1976; Pitakspraiwan and Giatgong, 1976; Pupipat, 1976; Renfro and Pupipat, 1976). Interestingly, various species of the genus *Peronosclerospora* are rather similar in morphological characters. Furthermore, since oospores of *P. maydis, P. sorghi* and *P. philippinensis* have not been observed, accurate species identification based on morphology is still ambiguous (Murray, 2009; Shaw, 1978; White, 1999; Widiantini *et al*., 2015). Subsequently, host range observation has been used for accurate species identification (Bock *et al*., 1996; Bonman *et al*., 1983). Specific host species have been designated to separate the fungal species, including *Sorghum* spp., *Saccharum* spp., and other Gramineae (Bonde and Peterson, 1983; Shaw, 1978; White, 1999). Detection and accurate identification of plant pathogens is one of the most important strategies for plant disease management.

The fungal morphological characteristics may be of limited usefulness because they can vary according to environmental factors (Bonde *et al*., 1992; Kimigafukuro, 1979; Widiantini *et al*., 2015). At present, molecular markers are applied for genetic study and are important techniques for identification when combined with pathogenicity and morphology (Telle *et al*., 2011). In most fungi, ribosomal DNA includes the small subunit (SSU, 18S), internal transcribed spacer (ITS1+5.8S+ITS2), and large subunit (LSU) 25-28S regions, which have been successfully used as tools for phylogenetic study (Fell *et al*., 2000; Porter and Golding, 2012; Riethmüller *et al*., 2002). Therefore, the purpose of the present study was to determine the distribution of fungal species causing corn downy mildew in different geographic areas of Thailand.

Materials and methods

Sample Collection

Downy mildew fungi were surveyed and collected from 17 provinces in Thailand during 2012-2015 covering the central (C), northern (N), northeastern (NE), western (W) and southern (S) regions.Disease severity and disease index were evaluated and recorded. Five symptom levels of disease severity (applied from Shekhar and Kumar, 2012) were recorded as: 0 was no symptoms or

healthy plant (A), 1 was light infection (1-25%) (B), 2 was moderate infection (26-50%) (C), 3 was heavy infection (51-75%) (D) and 4 was very heavy infection (≥76%) (E). Then, the disease severity level and disease index (applied from MaMaugh, 2008) were calculated as follows:

$$\text{Disease Index (\%)} = \frac{((0\times A)+(1\times B)+(2\times C)+(3\times D)+(4\times E))}{4\times \text{all corn number were surveyed in field)}}\times 100$$

Morphological observations

Infected leaves were thoroughly washed, air-dried, and incubated in plastic containers with free moisture and darkness at temperatures below 24℃ for 8 h. After that, the fresh conidiophore and conidia produced on the upper and lower corn leaf were collected and placed onto a glass slide containing a drop of lactophenol mixed with acid fuchsin. Morphological characterization was carried out under a compound microscope for species identification. A total of 50 conidia and 10 conidiophores were assessed. The dimensions of conidia (length and width, ×200) and conidiophores (basal cell, ×200) were measured and recorded (Chamswarng, 1976; Watanavanich *et al*., 1976; Widiantini *et al*., 2015).

DNA extraction

Infected corn leaves were incubated as indicated above, the fresh conidiophores and conidia occurred on the upper and lower corn leaf. Conidiophores and conidia were isolated using sterilized small syringes under a stereo-microscope and transferred to 50 ml of 5% (w/v) Chelex 100 buffer using sterilized distilled water in 1.5 ml Eppendorf tubes. Then, the solution was boiled in a water bath for 8 min, and then mixed by vortexing three times. The DNA was amplified in the regions of Domain 1- Domain 2 (D1/D2) of 28S rDNA and internal transcribed spacer 1 using PCR.

PCR reaction and sequencing

The DNA was amplified in D1/D2 region of the 28S rDNA using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 primers (5'-GGTCCGTGTTTCAAGACGG-3') (Porter and Golding, 2012). PCR reactions were carried out in 50 µl containing 5 µl of genomic DNA, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM dNTP mixed, 20 pmol for forward and reverse primers and 1U *Taq* polymerase. PCR was performed in a Tprofessional Thermocycler (Biometra) as follows: initial denaturation at 96℃ for 1 min, followed 30 cycles of denaturation at 95℃ for 30 sec,

annealing at 60 ℃ for 30 sec and 72 ℃ for 30 sec with a final extension of 4 min at 72 ℃.

The amplification of the internal transcribed spacer1 (ITS1) region was conducted coupled with the 28S rDNA region using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 primers (5'-GCTGCGTTCTTCATCGATGC-3') (T. J. White *et al*., 1990). PCR reactions were carried out in 40 µl containing 5 µl of genomic DNA, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM dNTP mixed, 10 pmol for forward and reverse primers and 1U *Taq* polymerase. PCR was performed in a Tprofessional Thermocycler (Biometra) as follows: initial denaturation at 95 ℃ for 1 min, followed by 35 cycles of denaturation at 95 ℃ for 30 sec, annealing at 55 ℃ for 1 min and 72 ℃ for 1 min with a final extension of 10 min at 72 ℃. After amplification, 5 µl of all PCR products were electrophoresed in a 1% (W/V) agarose gel and purified using Gene JET PCR purification kit (Thermo Scientific, Lithuania) and sent to Solgent Co., Ltd. (Korea) for sequencing.

Data analysis

All sample sequences were deposited in the DNA Data Bank of Japan (DDBJ). Multiple sequence alignment was performed using Muscle in MEGA6.0 (Tamura *et al*., 2013) and analyzed together with sequences obtained from GenBank. Phylogenetic analysis was clustered by the unweighted pair-group method with arithmetic means (UPGMA), the bootstrap value was calculated with 1000 heuristic replicates to estimate support for the clade stability of the consensus trees (Sneath and Sokal, 1973).

Results

Sample collection

Ninety-seven isolates were observed in 12 provinces with a high severity of corn downy mildew disease (Table 1). The fungal isolates were distributed as follows: in the northern region including Chiang Mai (7 isolates) and Chiang Rai (2 isolates) provinces, the western region including Kanchanaburi (18 isolates) and Ratchaburi (9 isolates) provinces, the central region including Nakhon Pathom (18 isolates), Nakhon Sawan (1 isolates), Saraburi (16 isolates), Sukhothai (3 isolates), Suphan Buri (12 isolates) and Uthai Thani (3 isolates) provinces, and the northeastern region including Nakhon Ratchasima (2 isolates) and Nong Khai (3 isolates) provinces. While,

the disease was not observed in five provinces including Phetchabun (C), Khon Kaen (NE), Loei (NE), Nakhon Si Thammarat (S) and Songkhla (S). High disease indexes were found on sweet corn and waxy corn in the rainy season (Figure 1), which corn had been grown continuously by the farmer, especially in the western, central and northeastern regions (Table 1). High disease indexes were found on sweet corn and waxy corn in the rainy season (Figure 1), which corn had been grown continuously by the farmer, especially in the western, central and northeastern regions.

Table 1. Sample collection of corn downy mildew disease at different geographic areas in Thailand.

Regions	Provinces	Corn varieties	Number of isolates	Disease index (%)
Northern	Chiang Mai	Sweet corn	7	2-80
	Chiang Rai	Waxy corn	2	70-80
Western	Kanchanaburi	Baby corn	10	2-50
		Waxy corn	6	2-70
		Sweet corn	2	50-70
	Ratchaburi	Baby corn	4	2-10
		Waxy corn	5	2-90
Central	Nakhon Pathom	Baby corn	3	7-20
		Waxy corn	12	2-100
		Sweet corn	3	2-70
	Nakhon Sawan	Field corn	1	80
	Saraburi	-	16	40-90
	Sukhothai	Field corn	3	40
	Suphan Buri	Waxy corn	12	20-70
	Uthai Thani	Sweet corn	3	70-80
Northeastern	Nakhon Ratchasima	Waxy corn	2	70-80
	Nong Khai	Waxy corn	3	70-80

Remarks: - : no data apperance of corn varieties (in field of corn breeding program)

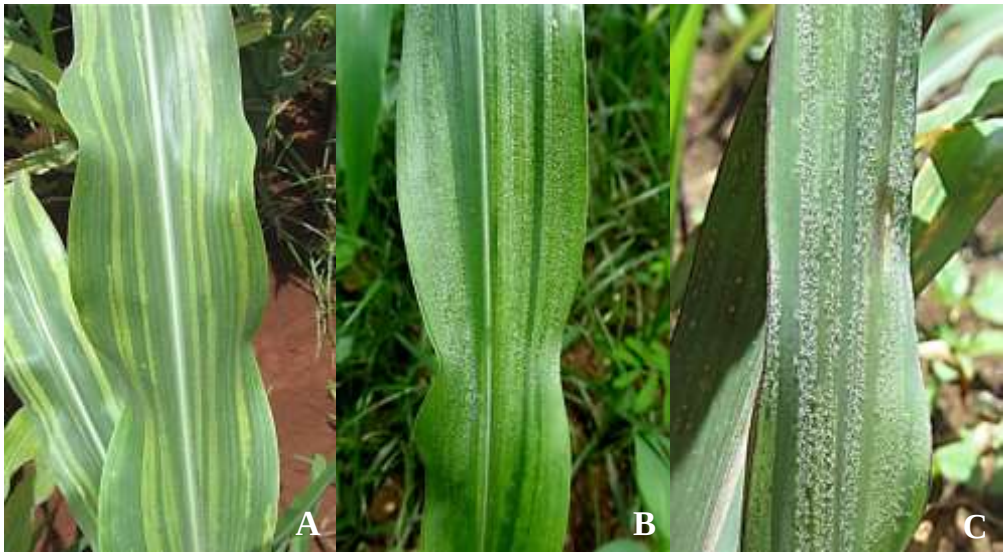

Figure 1. The symptoms/signs of corn downy mildew disease: elongated chlorotic streaks on sweet corn (A), conidial masses on upper leaf of sweet corn (B) and conidial masses on lower leaves of waxy corn (C).

Morphological observations

Ninety-seven isolates were observed using morphological characteristics which included conidia and conidiophore characters. All isolates possessed conidiophores which were erect, large, hyaline, with long and expanded apexes, dichotomously branching two to three times, and emerged singly or in groups from stomata. The conidiophore length was 172.26-412.12 µm (Table 2) and morphological characteristics of conidiophore shape and length were similar in range to *P. maydis* (D. G. White, 1999; Widiantini *et al*., 2015). The conidiophore type of all isolates were divided to three types using septa position, including non-septate conidiophores (17 isolates), basal septum (97 isolates), and 1-3 septa in conidiophores (97 isolates) (Figure 2). The septum at the basal cell (basal septum) was 10-166.55 µm in length. Based on the presence of the basal septum Pande *et al*. (1997) reported that sorghum downy mildew could be caused by *P. sorghi* in India. Additionally, both of the latter conidiophore types were observed in one isolate. The conidia were hyaline, with a thin cell wall latter, ovoid, oblong, elliptical, and spherical to sub-spherical in shape with dimensions of 15.0-19.8×15.0-32.0 µm produced on sterigma (Figure 2).

Table 2. Comparison of corn downy mildew isolates in Thailand with other species of downy mildew on corn.

Corn downy mildew		Conidiophores lenth (µm)	Septum of conidiophores	Conidial sizes (µm)	Conidial shape
Pathogen	**Other host**				
P. sorghi	Sorghum, Corn	180-300	basal septum	14.4-27.3×15.0-28.9[/1]	Oval to almost spherical
P. maydis	Corn	150-550	No report	17.0-23.0×27.0-39.0[/1] 10.0-17.5×12.5-22.5[/1,2]	Globose, oval, elongate and spherical to sub-spherical
P. philippinensis	Corn	150-400	Septum	17.0-21.0×27.0-39.0[/1]	Elongate or ellipsoid or oval
P. sacchari	Corn Sugarcane	160-170	No report	15.0-23.0×25.0-41.0[/1]	Elliptical and oblong to ovoid
P. spontanea	Corn	350-550	No report	11.0-21.0×25.0-65.0[/1]	Elongate or ellipsoid and cylindrical
Present study	Corn	172.26-412.12	non-septum, basal septum, and 1-3 septa	15.0-19.8×15.0-32.0	Oval, oblong, elliptical, spherical to sub-spherical

[2]/D. G. White, 1999
[2]/Widiantini *et al*., 2015

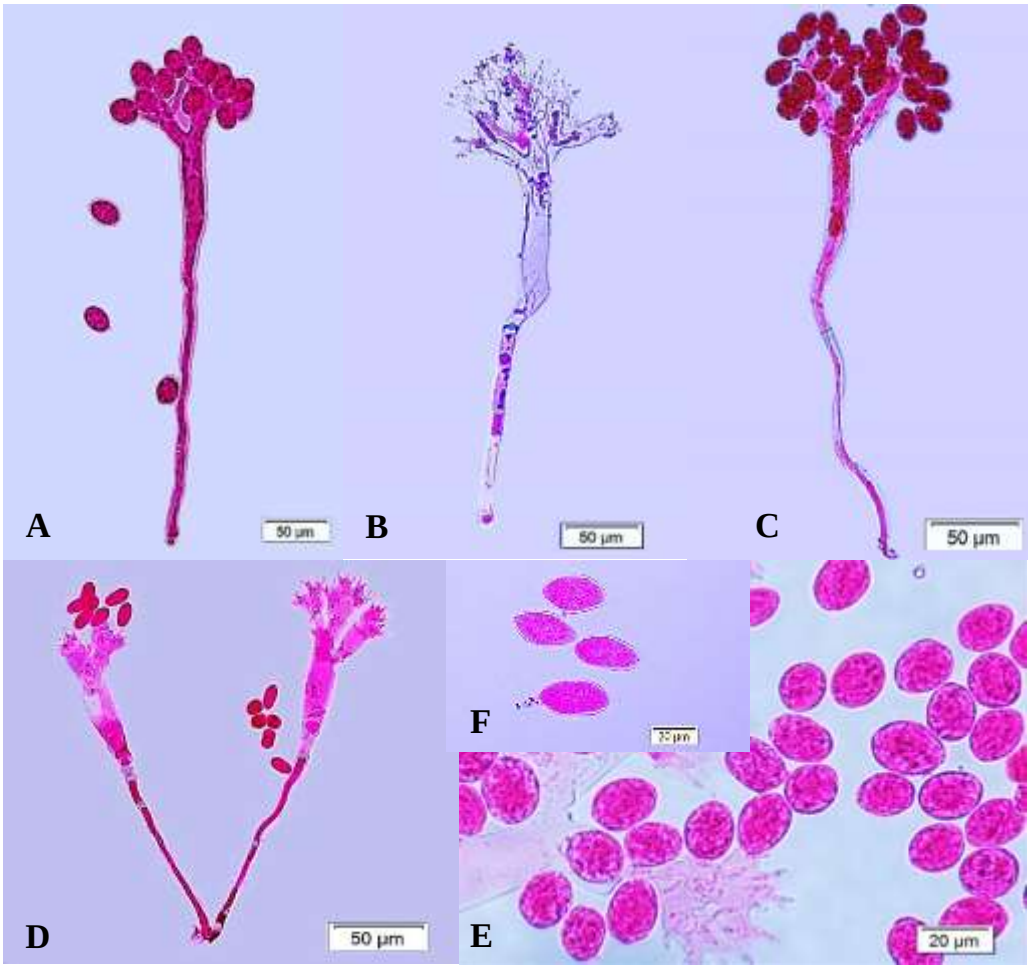

Figure 2. Morphological observation of *Peronosclerospora* species under a compound microscope, including three types of conidiophores; no septa in conidiophore (A), a basal septum in conidiophore (B), 1-2 septa in conidiophore (C and D), and conidial shape (E and F); the black arrow indicate the septum.

DNA sequence analysis

Twenty-eight sequences were obtained from DNA amplification of the D1/D2 region of the 28S rDNA containing 737-759 bp (Table 3). Multiple alignment was done together with nucleotide sequences from GenBank database (NCBI), including *Peronosclerospora sorghi* containing 394-407 bp (KX721504, HQ261763), *Peronosclerospora philippinensis* containing 395 bp (KX721503), *Sclerospora graminicola* containing 742 bp (AY273987),

Table 3. Corn downy mildew isolates used for D1/D2 of 28S and ITS1 region of rDNA analysis

Sample Number	Locations	GenBank accession number	
		LSU	ITS
DM-NP05	Nakhon Pathom	LC179770	-
DM-NP06	Nakhon Pathom	LC179771	-
DM-NP08	Nakhon Pathom	LC179773	-
DM-NP09	Nakhon Pathom	LC179772	-
DM-NP12	Nakhon Pathom	LC179774	-
DM-NP13	Nakhon Pathom	LC179775	LC128603
DM-RB03	Rachaburi	LC179776	-
DM-RB08	Rachaburi	LC179777	-
DM-KB05	Kanchanaburi	LC179764	LC128600
DM-KB06	Kanchanaburi	LC179765	LC128599
DM-KB07	Kanchanaburi	LC179766	-
DM-KB08	Kanchanaburi	LC179767	-
DM-KB09	Kanchanaburi	LC179768	LC128602
DM-KB10	Kanchanaburi	LC179769	-
DM-CM05	Chiang Mai	LC179762	-
DM-CM06	Chiang Mai	LC179763	LC128601
DM-NR01	Nakhon ratchasima	LC179790	-
DM-NR02	Nakhon ratchasima	LC179791	LC128608
DM-SP01	Suphanburi	LC179778	-
DM-SP02	Suphanburi	LC179779	LC128606
DM-SB01	Saraburi	LC179780	LC128605
DM-SB02	Saraburi	LC179781	LC128613
DM-SB03	Saraburi	LC179782	LC128615
DM-SB08	Saraburi	-	LC128609
DM-SB09	Saraburi	-	LC128610
DM-SB10	Saraburi	-	LC128611
DM-SB11	Saraburi	-	LC128612
DM-SB13	Saraburi	-	LC128614
DM-SB15	Saraburi	-	LC128616
DM-SK01	Sukhothai	-	LC128619
DM-SK02	Sukhothai	-	LC128618
DM-SK03	Sukhothai	-	LC128617
DM-NK01	Nong Khai	LC179785	LC128597
DM-NK02	Nong Khai	LC179786	-
DM-NK03	Nong Khai	LC179787	-
DM-UT01	Uthaithan	LC179789	LC128607
DM-CR01	Chiang Rai	LC179783	LC128604
DM-CR02	Chiang Rai	LC179784	LC128598
DM-NKS01	Nakhon sawan	LC179788	LC128620
DM-Laos01	Laos PDR	-	LC128596
DM-Laos02	Laos PDR	-	LC128594
DM-Laos03	Laos PDR	-	LC128595
Pm3	Indonesia	-	LC322282

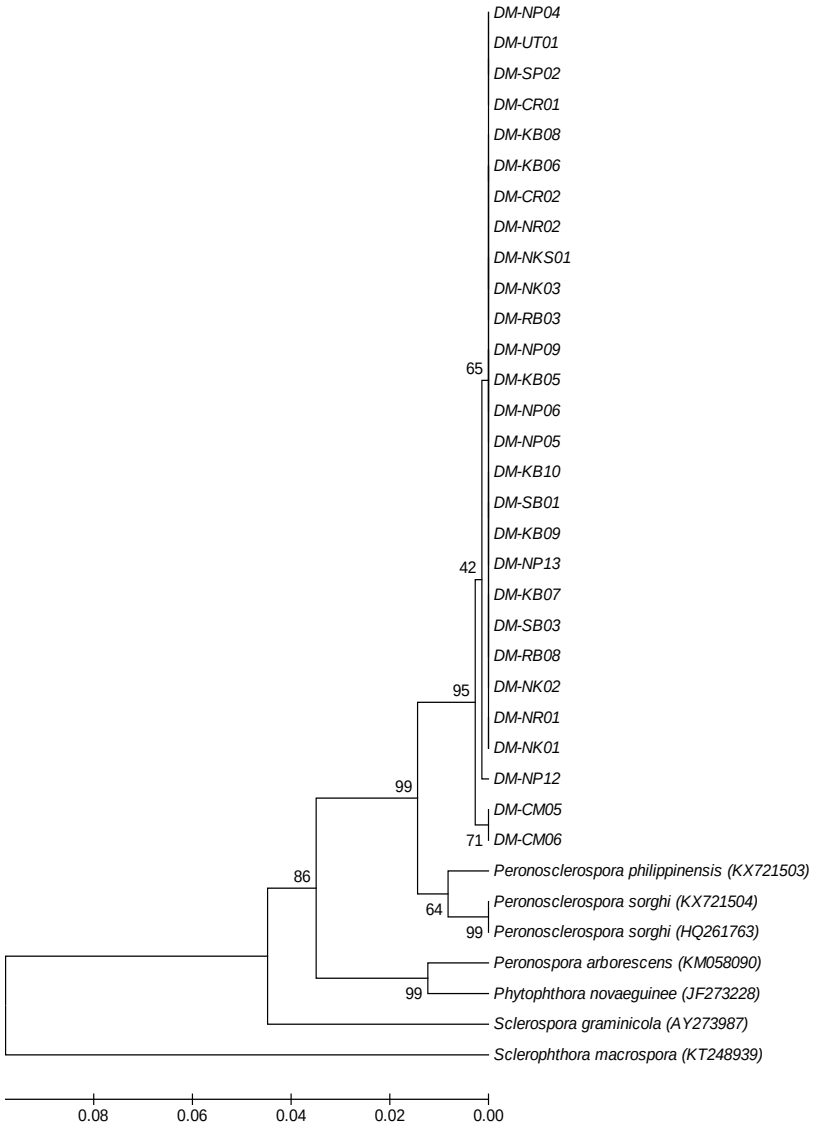

Figure 3. Phylogenetic dendrogram of *Peronosclerospora* species isolates compared with other sequences of the 28S rDNA gene from the GenBank database (NCBI), using the UPGMA algorithm (MEGA 6 program). Bootstrap values above 50% from 1000 replicates are indicated on the corresponding branch

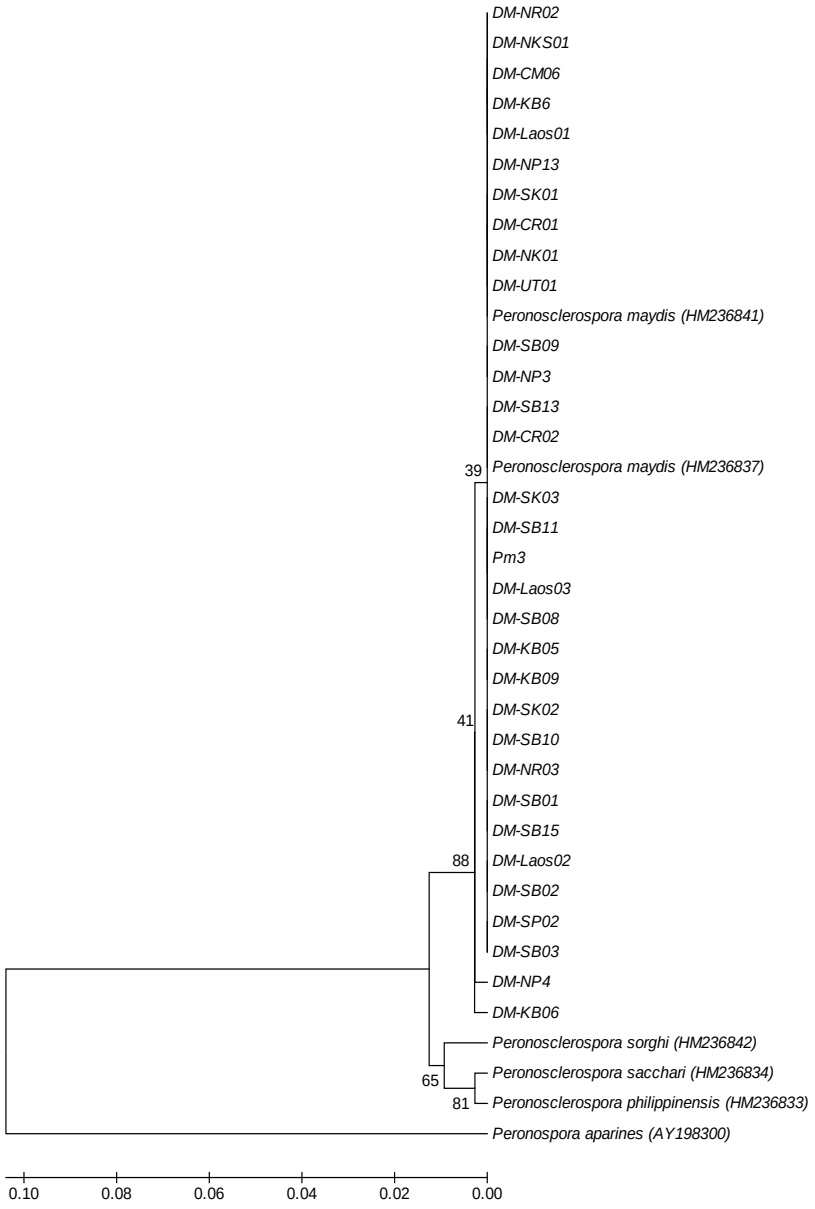

Figure 4. Phylogenetic dendrogram of *Peronosclerospora* species isolates compared with other sequences of ITS1 region from GenBank database (NCBI), using the UPGMA algorithm (MEGA 6 program). Bootstrap values above 50% from 1000 replicates are indicated on the corresponding branch.

Sclerophthora macrospora contained 408 bp (KT248939), *Peronospora arborescens* containing 761 bp (KM058090) and *Phytophthora novaeguinee* containing 759 bp (JF273228). The results showed that all isolates from four regions of Thailand clustered together (same group) with *Peronosclerospora sorghi* (KX721504, HQ261763) and *Peronosclerospora philippinensis* (KX721503) with a 98% similarity coefficient and with a 99% supporting boostrap value, and separated from other *Peronosclerospora* species with a 99% similarity coefficient with the UPGMA clustering method. All isolates were separated from *S. graminicola* (AY273987), *S. macrospora* (KT248939), *P. novaeguinee* (JF273228) and *P. arborescens* (KM058090) (Figure 3). These results suggest that corn downy mildew fungi in Thailand are species of *Peronosclerospora* and genetically separated from *Sclerospora*, *Sclerophthora*, *Peronospora* and *Phytophtora* based on 28S rDNA sequences (conserved sequence) obtained from GenBank database (NCBI).

Thirty-one sequences of ITS1 region were 237-265 bp in size. Multiple alignment was done with sequences obtained from the GenBank database (NCBI). The result showed that all isolates were grouped together with the *P. maydis* sequence (Pm3: Indonesia) which contained 231 bp (LC322282), and *P. maydis* obtained from the GenBank database (NCBI) which contained 208 bp. (HM236837 and HM236841) with a 99% similarity coefficient and with a 88% supporting bootstrap value, and separated from other sequences including *P. sorghi* containing 208 bp. (HM236842), *P. sacchari* containing 208 bp. (HM236834), *P. philippinensis* containing 208 bp. (HM236833), and *Peronospora aparines* containing 264 bp. (AY198300) (Figure 4).

Discussion

The systemic symptoms of corn downy mildew disease typically included chlorotic streaks with white conidial masses on lower and upper leave, and becoming necrotic lesions. Susceptible corn varieties normally showed stunting, phyllody, and sometimes barren and deformed cobs on sweet corn and waxy corn. Corn had been grown continuously by the farmer, especially in the western, central and northeastern regions. These regions are an original source of corn production in Thailand. Additionally, corn downy mildew disease was observed on baby corn that was moderately resistant to these pathogens. The disease severity of downy mildew fungi was characterized by different symptoms depending on the corn variety and environmental conditions (USDA, 2006; D. G. White, 1999; Widiantini *et al*., 2015). Morphological observations based on conidia characters, the isolates were grouped as follows: *P. maydis* (8 isolates) and *P. sorghi* (89 isolates) (Chamswarng, 1976; Pupipat, 1976;

Watanavanich *et al*., 1976; Widiantini *et al*., 2015). Formation of the basal septum coincides with achievement of the final size of conidia. These sizes can vary even within a same isolate when grown under different conditions (C. H. Bock *et al*., 2000; Bonde *et al*., 1992; Widiantini *et al*., 2015).

The variation of fungal conidia and conidiophore sizes depended on the corn variety, environmental conditions and locality. The morphological variation within fungal species may be caused by genetic variation (C. H. Bock *et al*., 2000; Maltese *et al*., 1995; Nath *et al*., 2015). Morphological variation of corn downy mildew fungi was reported among *P. maydis* isolates from some localities in Africa and in Java-Indonesia (C. H. Bock *et al*., 2000; Widiantini *et al*., 2015). Additionally, three species of sugarcane downy mildew caused by *Peronosclerospora* varied morphologically in Philippines (Husmillo and Reyes, 1980). Furthermore, six species of corn downy mildew have been recorded in Thailand using morphological characteristics. The morphological observations of our research indicated that four fungal species of corn downy mildew should be removed from Pest list of Thailand including: *P. philippinensis*, *P. sacchari, P. spontanea* and *Sclerophthora rayssiae* var. *zeae*.

Species discrimination in the genus *Pernosclerospora* on corn is difficult due to overlapping morphological characteristics, and corn downy mildew cannot be diagnosed using only symptoms and morphological criteria (Widiantini *et al*., 2015). In the past, sorghum downy mildew caused by *P. sorghi* had been incorrectly recorded using morphological characteristics in Australia but is now considered to be caused by *P. maydis* using morphology characters and nucleotide sequence (Wang *et al*., 2000). Subsequently, re-examination of downy mildew in northern Australia using morphological observation and DNA sequence analysis reported the presence of *P. australiensis*. This fungal species was also found on cultivated maize, while oospores were found on the native *Sorghum spp*. (Shivas *et al*., 2012). In 2008, corn downy mildew in Thailand have been classified as *P. maydis* that three isolates were tested using simple sequence repeat markers combined with sorghum downy mildew and related species (Perumal *et al*., 2008). In addition, *Peronosclerospora maydis* was determined using isozyme markers that are typified by unique banding patterns, but the banding patterns for *P. sacchari* and *P. philippinensis* are similar (Micales *et al*., 1988). Although, we found that single nucleotide sequences can be useful to separate genera of corn downy mildew fungi, they were inadequate to differentiate species. Currently, few sequences of the genus *Peronosclerospora* are available in the database making genetic comparison difficult. However, our research indicated that *P. maydis* is the dominant pathogen of corn downy mildew in Thailand identified by morphology and nucleotide sequence analyses. Four of the six species of corn

downy mildew fungi in the Pest list of Thailand should be removed including: *P. philippinensis*, *P. sacchari*, *P. spontanea* and *Sclerophthora rayssiae* var. *zeae*.

Acknowledgement

We would like to thank the Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen and the Center for Agricultural Biotechnology (CAB), Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand for providing equipment for this research. We are sincerely grateful to Inseechandrastitya Institute for Crop Research and Development, Kasetsart University, Thailand for providing corn seeds. This research was financially supported by the National Science and Technology Development Agency (NSTDA), in Thailand Graduate Institute of Science and Technology (TGIST) project (TGIST 0157051), Thailand.

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(Received: 25 August 2018, accepted: 31 October 2018)