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Genetic Diversity of *Puccinia polysora* in Thailand based on Inter Simple Sequence Repeat (ISSR) markers analysis

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Corn rust fungi are considered as major pathogens in corn production of Thailand. Two corn rust species, *Puccinia polysora* (southern rust) and *P. sorghi* (common rust) which are different in their life cycles and preferred environment have been reported in Thailand. In this study, genetic diversity of southern rust from various locations of corn plantation in Thailand was investigated. The genetic diversity of corn rust was analyzed using PCR amplification of inter simple sequence repeat (ISSR) region. Morphological-based identification determined that all of the rust specimens were *P. polysora*. A high variety of 38 polymorphic bands were found from the specimens by PCR amplification using five primers: GAG $(TCG)_5$, GAG $(CGA)_5$, $(CGA)_5$, $(GTC)_5$ and $(CAG)_5$. These bands were used to construct UPGMA dendogram, which cluster analysis divided the 38 specimens into 13 groups at 75% Dice's similarity coefficient with cophenetic correlation (r) =0.826. The isolates from different localities are presented in the same groups, supporting that *P. polysora* from different provinces in Thailand are expressed genetic characteristic that resulted from the spore migration.

Keywords: corn rust, southern rust, inter simple sequence repeat, *Puccinia polysora*

Introduction

Thailand is one of the world's leading exporters of corn (*Zea may* Linn.). According to current statistics of Food and Agriculture Organization of the United Nation (2007), exported values of corns by Thailand to the world market were worth 103.2 million USD (3.67 million tons) in 2007. Many high yields varieties were developed and introduced to the farmers in order to increase the production. However, many of these varieties are susceptible to pests and diseases in particular corn rust fungi. The corn rust fungi are considered major pathogens in corn production worldwide. Two corn rust species, *Puccinia*

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polysora Underw (southern rust) and *P. sorghi* Schr (common rust) have been recognized being different in their life cycles and preferred environment. These two species have been reported in Thailand based on urediospore's morphology-based identification (Giatgong, 1980 ; Lorsuwan, 1984). The common rust fungus requires the alternate host (*Oxalis* sp.) to complete its life cycle while the southern rust fungus does not. Since the morphology of southern rust and common rust is similar, the morphology-based identification of these two species is difficult. Therefore, advance identification techniques are necessary to develop in order to distinguish both corn rust fungi species. The advantage of the molecular phylogenetic analysis methods have been preceded successful application on the rust fungi. The authors have been studied the variation of nucleotide sequence in DNA of rust, fungi although they are obligate parasites that are difficult to culture and maintain on synthetic medium. The molecular phylogenetic analysis is now possible performed by Bruns *et al* (1990), and Lee and Taylor (1990) who extracted DNA from a single spore of dry herbarium specimens and amplified target DNA by PCR. Virtudazo *et al.* (2001) modified DNA extraction methods from Suyama *et al.* (1996) and extracted genomic DNA from spores obtained from single uredium or telium and then amplified target DNA by PCR (Pfunder *et al.,* 2001; Roy *et al.*, 1998; Zambino and Szobo, 1993; Weber *et al.*, 2003; Vogler and Bruns, 1998)

Molecular markers such as RAPD, AFLP, SSR and ISSR marker have been successfully used as tools for studying the phylogenetic relationships and diversity of rust fungi (Villareal *et al.*, 2002; Hovmoller *et al.*, 2002; Becerra *et al.*, 2007; Manuela *et al.*, 2005; Dracatos *et al.*, 2006; Yu *et al.*, 2006). ISSR marker is a simple and rapid technique, requires no sequence information and using a single primer for detection and random amplification like RAPD marker. Only small amounts of DNA template are required and the results are clearly scorable demonstrated (Ratanacherdchai *et al.*, 2010). Therefore, this technique is suitable for detection of genetic diversity of rust fungi which is a limitation of DNA concentration. Moreover, the genetic diversity of corn rust fungi in Thailand has not been reported, although the rust disease is a main problem of corn production in Thailand. The purpose of this study was to evaluate the genetic diversity of *P. polysora* from various corn plantations in Thailand using ISSR markers.

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Materials and Methods

Morphological observation

The corn rust specimens were collected from various localities in 12 provinces of Thailand as following Chiang-Mai, Lampang, Tak, Petchabun, Nakhon Sawan, Nakhon Ratchasima, Saraburi, Lop-Buri, Khon-Kaen, Chaiyaphom, Si Sa Ket and Songkhla. The herbarium specimens were examined and identified using morphology of uredium, urediospore, telium and teliospore under a microscope. For each specimen, three morphometric traits, including length, width and wall thickness of 30 spores were measured. The specimens have been deposited in the Fungal Collection, Department of Plant Pathology, Faculty of Agriculture at Kamphaengsaen, Kasetsart University, Kamphaengsean Campus (KKFC).

DNA extraction

DNA was extracted from single uredium (100-200 urediospores). Spores were crushed between two sterile glass slides and suspended in 20 µl extraction buffer containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% SDS, incubated at 37°C for 60 min, and then at 95 °C for 10 min (Suyama *et al.*, 1996; Virtudazo *et al.*,2001).

PCR amplification of ISSR region

DNA specimens were amplified using 20 µl PCR reaction each containing 5 µl. of DNA, 10 pmole of single primer, 2.5 units of Taq DNA polymerase and the supplied dNTP mixture (containing 2 mM of each dNTP) and Ex Taq reaction buffer (containing 2 mM Mg2+). PCR was carried out using T professional Standard Gradient (Biometra)under the following condition: 95°C for 5 min, then 30 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min, and final step of 72°C for 10 min. The PCR amplification of the ISSR regions were amplified using five primers as following $GAG(TCG)_5$, $GAG(CGA)_5$, $(CGA)_5$, $(GTC)_5$ and $(CAG)_5$. After amplification, 3 µl of the reaction product was electrophoresed on 1% (w/v) agarose gels containing 0.1 µl /ml GelStar (Nucleic Acid Gel Satin , 10000X concentrate in DMSO) in TBE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.0).

Data collection and analysis

All bands of each primer were manually recorded polymorphic band as binary data by 1 (present) or 0 (absent). The binary data was analyzed with the computer program NTSYSpc version 2.02 (Rohlf, 1993). An unweighted pair group arithmetic mean method (UPGMA) cluster analysis was performed using the DICE's similarity coefficient. Dendrogram was generated with the tree option (TREE) and a cophenetic value distance matrix was derived from dendrogram with a COPH program in NTSYSpc. The cophenetic value distance matrix was compared for level of correlation with the original matrix with the MXCOMP NTSYS program. Bootstrap values were calculated with 1000 replicate by Winboot program (Yap and Nelson, 1996).

Results and discussion

Morphological observation

The rust disease is the main problem of corn production in Thailand. The rust fungi can distribute and survive in the various corn plantation localities. There are three species of corn rust fungi which are reported in the world such as *P. sorghi* (common rust) *P. polysora* (southern rust) and *Physopella zeae* (tropical corn rust) (Melching, 1975). The two former species have been reported in Thailand using uredium and urediospore morphology for identification (Giatgong, 1980 ; Lorsuwan, 1984). *Puccinia sorghi* could be differentiated from *P. polysora* by its larger, less densely occurring, elongated uredia, darker and rounder urediospores (White, 2000). Moreover, the teliospore morphology is important and useful for distinguishing between *P. sorghi* and *P. polysora*. However, Thailand locates in the tropical with hot climate. It is difficult to find the telial stage of rust fungi. In this survey, we found teliospores on only one specimen collected from Tak province. This specimen was identified as *P. polysora* using teliospore based identification.

One hundred and eighty-six specimens were collected from twelve provinces of Thailand. Morphological study on the characteristics of rust pustules on the infected leaves and stems demonstrated the diversity on the symptomatology including colors, shapes and the distribution of the pustules.(Fig. 1) The shapes of uredium on leaf and stalk were circular to elongate. Most of urediospores occurs with paraphysis in uredium. Interestingly, one of the examined specimens produces dark teliospores with one septum in uredia. The urediospores were mostly ovoid-oblong with

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cinnamon-brown color. Morphological-based identification determined that all of the rust specimens were *P. polysora*. (Fig. 2)

Fig 1. Symptom diversity of southern rust caused by *Puccinia polysora* on corn leaves and stems

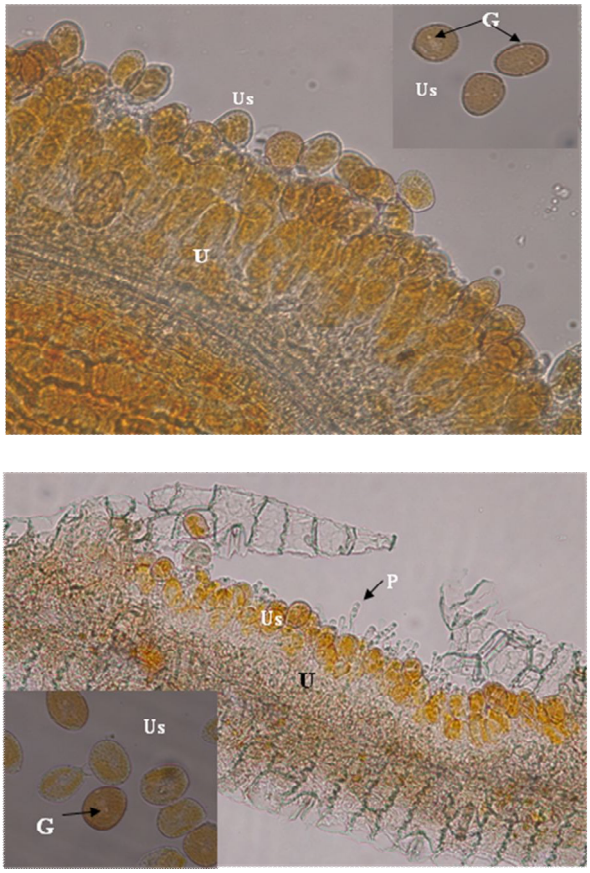

Fig 2. Morphological characters of uredium and urediospore of *Puccinia polysora* . U, uredium; Us, urediospore; G, germ pore; P, paraphysis

ISSR fingerprint analysis

The genetic diversity of corn rust fungi in Thailand has not been reported, although the rust disease is a main problem of corn production in Thailand. The genetic diversity of corn rust was analyzed using PCR amplification of inter simple sequence repeat (ISSR) region. The results showed polymorphic bands and various band patterns among the specimens (Fig. 3). Thirty-eight polymorphic bands were found from the 38 specimens (Table 1), after PCR amplification using five ISSR primers, $GAG(TCG)_5$, $GAG\ (CGA)_5$, $(CGA)_5$,

$(GTC)_5$ and $(CAG)_5$.These bands were used to constructing the UPGMA dendogram, which the UPGMA cluster analysis of 38 markers revealed 13 ISSR phenotypes (excluding outer groups) at 75% Dice's similarity coefficient (Fig. 4). The cophenetic correlation coefficient (r) was 0.826, indicating the dendrogram was a good fit representation of the original data. The isolates from Chiang Mai, Saraburi and Petchabun representing 3 of the groups and isolates from Lop-Buri, Chaiyaphom, Tak and Songkhla representing two groups were observed. While, five isolates from Nakhon Ratchasima were nested in the one group (J). The isolates of each province were clusted in more than one ISSR group, they occured on various type and varieties of corn. On the other hand, the isolates from Nakhon Ratchasima were found on the In-See variety which was introduced to the local farmer. The isolates from different localities were presented in the same groups. The data also indicated that southern rust is a autoecious rust and reproduces only asexual spores (urediospores) on the corn leaves and stems. The urediospores are distributed to others localities by wind and infected to the same or different corn varieties. This assumption can be supported by ISSR marker analysis, which the specimens were distributed in groups of the dendrogram. In the other study, Yu *et al.* (2006) . evaluated genetic population of *Melampsora larici-populina* in China using ISSR marker. The results indicated that this marker used for intraspecies population study because *M. larici-populina* in China can be divided into two populations (Western and Northern population). Ratanacherdchai *et al.* (2010) determined the genetic variability of *Colletotrichum capsici* isolated from three varieties of chilli (Bell pepper, Long cayenne pepper and Bird's eye chilli) using ISSR-PCR. The phylogenetic grouping based on ISSR showed a correlation between genetic diversity and geographical distribution of isolates. Therefore, ISSR markers are a useful method of studying genetic diversity in *Colletotrichum* spp.

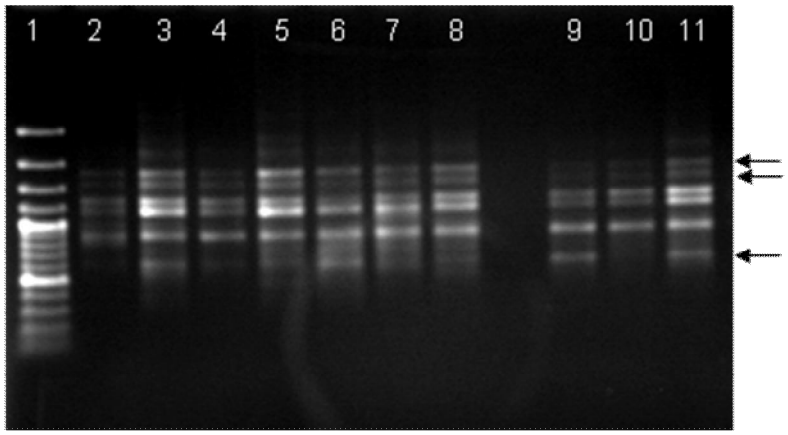

Fig 3. DNA fingerprint of *Puccinia polysora* by ISSR markers using $(GTC)_5$ primer. The polymorphic bands shown by arrows and lane 1 is 100 bp plus DNA Ladder (Fermentas). Number 2-7 (KKFC021,023,025,026,027,030) and 8-11 (KKFC034,038,042,053) represent the isolates collected from Tak and Petchabun province, respectively.

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Table 1. Thirty six specimens of *Puccinia polysora* used for ISSR fingerprint analysis

Voucher Specimens	Host	Localities
KKFC001	*Zea may* Linn.	Saraburi
KKFC002	*Zea may* Linn.	Saraburi
KKFC005	*Zea may* Linn.	Saraburi
KKFC007	*Zea may* Linn.	Saraburi
KKFC012	*Zea may* Linn.	Saraburi
KKFC021	*Zea may* Linn.	Tak
KKFC023	*Zea may* Linn.	Tak
KKFC025	*Zea may* Linn.	Tak
KKFC026	*Zea may* Linn.	Tak
KKFC027	*Zea may* Linn.	Tak
KKFC030	*Zea may* Linn.	Tak
KKFC034	*Zea may* Linn.	Petchabun
KKFC038	*Zea may* Linn.	Petchabun
KKFC042	*Zea may* Linn.	Petchabun
KKFC053	*Zea may* Linn.	Petchabun
KKFC056	*Zea may* Linn.	Songkhla
KKFC058	*Zea may* Linn.	Songkhla
KKFC061	*Zea may* Linn.	Songkhla
KKFC063	*Zea may* Linn.	Songkhla
KKFC066	*Zea may* Linn.	Songkhla
KKFC067	*Zea may* Linn.	Chiang
KKFC068	*Zea may* Linn.	Chiang
KKFC069	*Zea may* Linn.	Chiang
KKFC071	*Zea may* Linn.	Chiang
KKFC081	*Zea may* Linn.	Chaiyaphom
KKFC083	*Zea may* Linn.	Chaiyaphom
KKFC084	*Zea may* Linn.	Chaiyaphom
KKFC085	*Zea may* Linn.	Chaiyaphom
KKFC093	*Zea may* Linn.	Lop-Buri
KKFC114	*Zea may* Linn.	Lop-Buri
KKFC119	*Zea may* Linn.	Lop-Buri
KKFC159	*Zea may* Linn.	Nakhorn Ratchasima
KKFC160	*Zea may* Linn.	Nakhorn Ratchasima
KKFC162	*Zea may* Linn.	Nakhorn Ratchasima
KKFC164	*Zea may* Linn.	Nakhorn Ratchasima
KKFC165	*Zea may* Linn.	Nakhorn Ratchasima
KKFC264	*Brachiaria distachya*	Nakhorn Ratchasima
KKFC265	*Echinochloacrus-galli*	Nakhorn Ratchasima

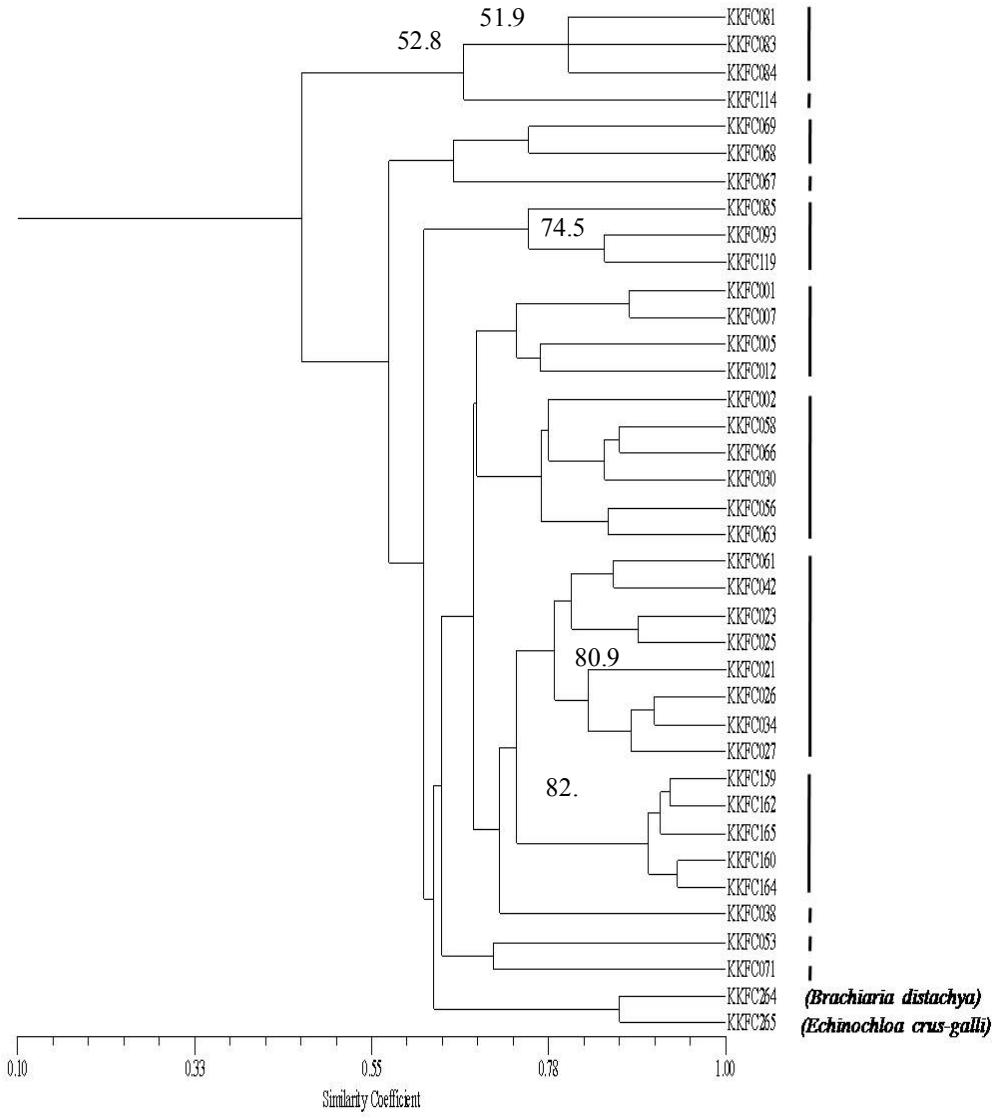

Fig 4. Phenetic dendrogram of *P. polysora* isolates and weed rust fungi based on the binary matrix of polymorphic bands, using the UPGMA algorithm and Dice's similarity coefficient (NTSYS program). Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branch.

The DNA fingerprint-based analysis indicated that ISSR marker would useful tool for analysis of genetic diversity of *P. polysora* (southern corn rust). The isolates from different localities were presented in the same groups, supporting that *P. polysora* from different provinces in Thailand are similar genetic characteristic that resulted from the spore migration.

Acknowledgements

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