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Genetic variation among isolates of *Rigidoporus microporus* causing white root disease of rubber trees in Southern Thailand revealed by ISSR markers and pathogenicity

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The genetic variation among 32 isolates of *Rigidoporus microporus* was determined using pathogenicity tests and molecular marker (intersimple sequence repeats; ISSR). Isolates were collected from six sites of two provinces in the south of Thailand. Based on the pathogenicity results, all isolates could divide into three groups depended on their virulence to rubber trees as follows: low virulent isolates, moder-ately virulent isolates and high virulent isolates. Cluster analysis based on ISSR characters grouped the isolates according to geographical origins. A dendrogram resulting from a cluster analysis showed two main distinct groups, designated as A and B rooting from outgroup. Group A contained isolates col-lected from Surat Thani province and group B those collected from Narathiwat province. There was no clear relationship between degrees of disease and geographical regions.

Key words: ISSR, pathogenicity, *Rigidoporus microporus*, white root disease.

INTRODUCTION

Rubber tree (*Hevea brasilliensis* (Wild.) Muell.-Arg) is an economically important crop in Thailand which their products are exported worldwide and produces significant revenue for the country. The planting areas are mostly in the south which climate suitable for rubber plantation and also suitable for pathogen growth, especially white root disease (Anonymous. 2009). This disease is the most de-structive root in rubber plantation in many countries. The causal agent of this disease is *Rigidoporus microporus* (Fr.) Overeem (Jayasuriya and Thennakoon 2007). This fungus belongs to Basidiomycete. It persists on dead or live root debris for a long time. It forms many white, flattened myce lialstrands with 1 - 2 mm thick which grows and extends rapidly through the soil in the absence of any woody substrate (Nandris et al., 1987). Thus, the root of healthy rubber tree can be infected by contact with a disease source, such as rhizomorphs, infected root, dead stump, or wood debris (Guyot and Flori, 2002). The fruiting body of this fungus form mainly at the collar of the dead infected tree which can produce a large number of basidiospores seems to have a limited role in dissemination of this disease (Nandris et al., 1987).

The molecular techniques were accepted to be made in genomics, and in many areas of applied mycology. The molecular or DNA data has led a major study in development of the systematic, biochemistry and ecology of fungi. (Bridge, 2002). The development of molecular technology has provided approach which is already beingused to identification of unknown species, genetic variability, characterization and relatedness of fungal isolates and species and pathogen detection (Bridge, 2002; Wang et al., 2005; Glen, 2006). Moreover, molecular

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methods are now starting to give function of fungi in the environment, where they are exists, and how they interact with other organisms (Bridge, 2002). During the last years different methods of DNA such as microsatellites (simple sequence repeats; SSR), inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) have become available to detect genetic variation within populations and among individuals. These methods detect genetic variation directly at the DNA level (McDonald, 1997; Hassel and Gunnarsson, 2003) and require different genetic markers due to important features, such as ge-nomic abundance, level of polymorphism, locus-specificity, quantities of DNA required, reproducibility, technical re-quirements and operation costs. Each molecular method has its own advantages and disadvantages (Spooner et al., 2005). The ISSR method was first developed by Ziet-kiewicz et al. (1994) and developed from SSR technique (Yu et al., 2006). Although SSR analysis technique pro-vides a codominant and high reproductivity but develop-ment needs time-consume and laborious (Chadha and Gopalakrishna, 2007). ISSR technique which involves polymerase chain reaction (PCR) amplification of DNA segments using a primer composed of a microsatellite region (Ratnaparkhe et al., 1998). ISSR do not require genome sequence information but provide multi-loci am plification and quickly and easy operates as same as RAPD but give more high reproductivity than those with RAPD (Bornet and Branchard 2001; Spooner et al., 2005). The ISSR is detects a higher level of polymorphism than those detected with RFLP or RAPD (Godwin et al., 1997). ISSR method was first reported as a technique for analysis the genetic variation in plant and animals and later used to obtain DNA markers in fungi (Bornet and Branchard, 2001; Bayraktar et al., 2008) . It is power tool for investigating genetic variation within closely related species (Yu et al., 2008) and also for studying of genetic population on fungi (Menzies et al., 2003; Chadha and Gopalakrishna, 2007). This technique is reliable and suitable for population genet-ics analysis of intraspecies (Yu et al., 2006) and study on intraspecific genetic variation of fungi pathogenic provide useful information for controlling disease (Takatsuka, 2007). ISSR was successfully to study the diversity and molecular relationships of fungi such as *Colletotrichum* isolates from the Iwokrama forest in Guyana (Lu et al. 2004) and study detection of genetic variation and population structure among isolates of *Fusarium oxyaporum* f.sp. *ciceris* on Chickpae in Turkey (Bayraktar et al., 2008).

The genetic diversity and gene flow within or between pathogen populations will lead to an understanding of how the pathogen is likely to adapt or evolve in the environ-mental change such as exposure to abiotic stresses, fungicides and plant resistant (McDonald et al., 2002). Thus, understanding the genetic variation in populations of the causal agent of disease would be useful in the improvement of disease management system or help to develop cultivars with tolerance and/or resistance to disease (Stenglein and Balatti, 2006; Bayraktar et al., 2008). Although white root disease of rubber trees has been an economically important disease in Thailand, but it lacks of information about the genetic variation of pathogen population associated with rubber trees by using molecular technique. The objective of the study was to assess the genetic variability within populations of white root disease pathogen by using ISSR technique and pathogenicity.

MATERAILS AND METHODS

Fungal isolates

All isolates used in this study were obtained from 6 sites in 2 provinces (Surat Thani and Narathiwat) in the south of Thailand (Figure 1). The cultures were maintained in potato dextrose agar (PDA) medium and deposited at Biocontrol Research Unit, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Pathogenicity tests

Inoculum preparation: All tested isolates were separately cultured in the inoculum medium of 200 g mixed substrates in plastic bag. The inoculum medium was consisted of 100 g sawdust, 3 g rice bran, 2 g sugar, and 5 ml water. The inoculum mixed substrates were sterilized in autoclave at 121°C, 15 lbs/inch^2 for 30 min. Then each isolate which cultured on sterile sorghum seed at the age of 7 days was separately transferred onto sterilized inoculum and incubated at room temperature (27 - 30°C) for 30 days. After that these inoculum were taken to test for their pathogenicity.

Inoculation technique: The experiment was done by Completely Randomized Design (CRD) with four replications. The 5 months rubber trees variety RRIM600 were planted in pots containing sterilized mixed soil (soil : sand : compost; 8:8:2). The 30 day-old inoculum mixed substrates was placed into the soil planting pot next to the root system of the rubber tree. Control plants were grown in a sterilized mixed soil without inoculum. The inoculated rubber trees were maintained and recorded for disease incidence at 90 days. The Disease Index (DI) were determined as follows:- level 1 = healthy, green leaves, level 2 = 1 - 25% yellow leaves, level 3 = 26 - 50% yellow leaves, level 4 = 51 - 75% yellow leaves and level 5 = 76 - 100% yellow leaves. The virulent group of the isolates was determined according to DI. The root rot of inocu-lated trees were reisolated to confirm the pathogenic isolate. Treatment means were compared with Duncan's Multiple Range Test (DMRT) at P = 0.05.

ISSR-PCR analysis

DNA extraction: Mycelium of each isolate was grown on Petri dishes (90 mm diameter), containing 20 ml potato dextrose agar (200 g potato, 20 g dextrose, 20 g agar and 1,000 ml distilled water) and incubated for approximately 10 days at 25°C and aerial mycelium were harvested from each isolate under sterile conditions. Genomic DNA was extracted by CTAB buffer as described by Ratanacherdchai et al. (2007) by grinding mycelium of each isolate with liquid nitrogen and transferred to 1.5 ml Eppendorf tube. The 600 µl Cetyltrimethylammonium bromide (CTAB) was added and incubated at 65°C for 30 min and vortexed every 10 min. The tube was cooled for a few minutes after that 600 µl

Figure 1. Collection area in the south of Thailand, Surat Thani and Narathiwat province.

Table 1. Sequence, the amount of $MgCl_2$ and dNTP, annealing time and G+C content for ISSR analysis.

Primer sequence	$MgCl_2$ (µl)	dNTP (µl)	Annealing time (°C)	G+C content (%)
(AG)8C	4	1	52	47.06
(GA)8T	4	1	52	47.06
(TG)8A	3	1	52	44.06
(GA)8YG	3	1	52	52.78
(GT)8YC	4	1	52	52.78
GGGC(GA)8	3	1	52	60.00
(CGA)5	3	1	52	66.67

Y = Pyrimidine.

chloroform : isoamyl alcohol (CIA; 24:1, v/v) was added, gently mixed and centrifuged at 7000 rpm for 5 min at 4°C. The aqueous layer was removed to new tubes and extracted again with CIA. Finally DNA was precipitated by adding 300 µl isopropanol, mixed well and incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was decanted. The DNA pellet was suspended in 40 µl ddH_2O.

Amplification conditions: Ten primers were screening for ISSR analysis and sevens primers yielded polymorphic banding patterns and were selected to analyzed diversity among isolates of *Rigido-porus microporus* as follows: (AG)8C, (GA)8T, (TG)8A, (GA)8YG, (GT)8YC, GGGC(GA)8, and (CGA)5. To optimize reaction condi-tions, the concentration of $MgCl_2$ and the amount of 10 mM dNTP were examined. The annealing temperature was optimized to obtain clear and reproducible patterns. The optimum reaction condition and G+C content of seven primers were shown in Table 1. The amplifi-cations were performed in reaction volumes of 50 µ l containing 5 µl of 10x PCR buffer, 2 µl of 10 µM primer, 3 µl of 25 mM $MgCl_2$ (for (TG)8A, (GA)8YG, GGGC(GA)8 and (CGA)5) and 4 µl of 25 mM

Table 2. Fungal isolates used in this study and their pathogenicity results.

Provinces	Collection sites	Isolates	Disease index (DI)*	Virulent group**
Surat Thani	Muang	Sss 01	1.3^{c}***	L
	Tachana	Sst 01	3.0^{abc}	M
		Sst 02	3.0^{abc}	M
		Sst 04	2.3^{abc}	M
		Sst 05	4.0^{abc}	M
		Sst 06	2.5^{abc}	M
		Sst 07	2.3^{abc}	M
		Sst 08	1.3^{c}	L
		Sst 09	2.0^{bc}	L
		Sst 11	1.3^{c}	L
		Sst 12	2.5^{abc}	M
		Sst 13	2.8^{abc}	M
		Sst 14	2.0^{bc}	L
		Sst 15	3.3^{abc}	M
		Sst 16	4.0^{abc}	M
Narathiwat	Sungai Padi	Sns 01	1.3^{c}	L
		Sns 02	2.0^{bc}	L
		Sns 03	2.5^{abc}	M
		Sns 04	1.3^{c}	L
		Sns 07	2.5^{abc}	M
		Sns 10	1.5^{c}	L
		Sns 11	3.3^{abc}	M
	Kokparimeng	Snk 02	5.0^{a}	H
		Snk 03	5.0^{a}	H
		Snk 05	4.0^{abc}	M
		Snk 06	4.0^{abc}	M
	Parulu	Snp 05	2.0^{bc}	L
		Snp 06	3.0^{abc}	M
		Snp 08	3.5^{abc}	M
	Todeng	Snd 05	2.0^{bc}	L
		Snd 07	2.0^{bc}	L
		Snd 08	4.5^{ab}	H

*DI; level 1 = healthy, green leaves, level 2 = 1 - 25% yellow leaves, level 3 = 26 - 50% yellow leaves, level 4 = 51 - 75% yellow leaves and level 5 = 76 - 100% yellow leaves. **L= low virulence, M= moderately virulence, H = high virulence.
***Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at P = 0.05.

$MgCl_2$ (for (AG)8C, (GA)8T and (GT)8YC), 1 µl of 10 mM dNTP and 0. 3 µ l of 1.5 U Taq polymerase, 1 µl of 10 ng genomic DNA, and 37.7 µl of ddH_2O. PCR was carried out in a MyGene^{TM} Series Peltier Thermal Cycler (Model MG96G) under the following conditions: 2 min initial denaturation at 94°C followed by 35 cycles of 1 min de-naturation at 94°C, annealing for 1 min at 52°C, extension for 1.5 min at 72°C, and final extension for 6 min at 72°C. PCR products were separated by 1.5% agarose gel strained with ethidium bromide which included in the agarose and visualized under UV fluorescence.

Data analysis: The ISSR DNA bands obtained for each isolate were scored based on their presence (1) or absence (0). Only reliable and reproducible bands were considered for score. A similarity matrix was generated from the binary data using DICE similarities coefficient in SIMQUAL program of NTSYS-PC Package (Rohlf, 2000). Dendrograms were constructed by cluster analysis based upon the unweighed pair group method with arithemetical mean (UPGMA).

RESULTS

Fungal isolates

All tested isolated of *Rigidoporus microporus* used in this study which obtained from six sites from two provinces in the south of Thailand were shown in Table 2. Their mycelium on potato dextrose agar (PDA) medium at room

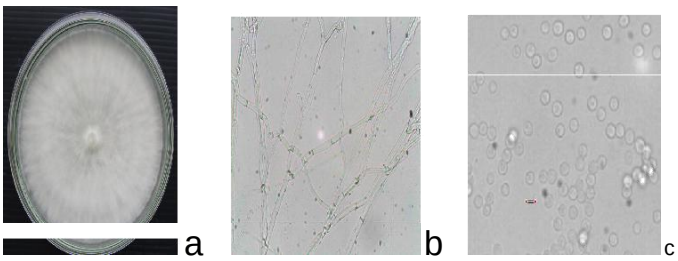

Figure 2. Morphology of *Rigidoporus microporus*: mycelium on PDA medium at 6 days (a), hypha (40x) (b) and basidiospore (40x) (c).

temperature appeared white and flattened mycelium. The hypha of the fungal isolate showed hyaline, septate, and possession many branches but no clamp connection. The width of the hypha varies from 2.8 - 7.2 um. Basidiospores showed globose, colorless, thin-walled, smooth and 3.7 - 4.1 um in size (Figure 2). It also showed that all isolates were similar in morphology.

Pathogenicity test

Pathogenicity tests of all isolates on rubber trees variety RRIM600 showed symptom of yellowing leaves that was significantly different in disease incidence at P = 0.05.
Base on the results, all isolates were grouped into three categories depended on DI as follow: high virulent isolates (DI = 4.1 - 5), moderately virulent isolates (DI = 2.1 - 4) and low virulent isolates (DI = 1 - 2). There were 17 isolates which grouped into moderately virulence, 12 isolates found low virulence and only 3 isolates showed high virulence and these three high virulent isolates were ob-tained from Narathiwat Province (Table 2).

ISSR-PCR analysis

Inter-simple sequence repeat (ISSR) markers were used to investigate genetic diversity among 32 isolates of *Rigidoporus microporus* isolates from different geographical origin and one isolate outgroup of *Ganoderma* spp GM101. Among ten primers, seven ISSR primers showed multi band patterns in each isolate. The primers amplified a total of 32 bands from 33 isolates tested. The average number of bands per primers was 4.6. Band size ranged from 250 - 2000 bp. In this study the G+C content of the primers ranged from 47.06 - 66.67% and annealing temperature was 52°C for all primers. The bands obtained from all isolates were shown in Figure 3.

UPGMA analysis based on total ISSR characters differences was carried out to group the 32 isolates of *R. microporus* with the outgroup *Ganoderma* spp GM101. A dendrogram resulting from a cluster analysis showed two main distinct groups, designated as A and B rooting from outgroup. All of the isolates obtained from Surat Thani province were grouped together as “A” and all of the isolates obtained from Narathiwat province were grouped together as “B”. The results also indicated that there was an association between *R. microporus* isolates and their geographical origin. Within group B, isolates from different site could be separated from each other. The high virulence isolates were found to obtain from Narathiwat province. However, there was no clear relationship between degrees of disease and geographical region (Figure 4).

DISCUSSION

Rigidoporus mciroporus , the causal agent of white root disease of rubber tree, is an important fungal pathogen causing economically important crop loses in rubber plantation in Thailand and many countries. White root disease is being controlled by an integration of cultural and chemical methods such as removal and burning of the infected root, applying the chemical fungicides but sometimes it is too late to control disease. The important control strategies are to reduce the source of inoculum and to inhibit disease spread (Guyot and Flori, 2002). The un-derstanding the genetic variation in populations of the causal agent of disease would be useful in the improve-ment of disease management system (Bayraktar et al., 2008) or help to develop cultivars with tolerance and/or resistance to disease (Stenglein and Balatti, 2006). However, there is no report on determination of genetic variation among pathogen populations of white root dis-ease pathogen in Thailand. In this study, 32 isolates of *R. microporus* were tested with 7 ISSR primers to determi-nate the distribution of genetic diversity among isolates which represents the difference rubber tree planting areas. Base on the results, *R microporus* isolates obtained from different sites of two provinces from the south of Thailand were examined with pathogenicity test and ISSR analysis. The isolates which obtained from two provinces were separated into two groups. All of the isolates obtained from Surat Thani province were grouped together and were found to be a moderately virulent and low virulent isolates. All isolates which obtained from Narathiwat province were also grouped together and found three kinds of virulence isolate. It was clearly that there was a relationship be-tweenthe geographical distributions and clustering of iso-lates. These may be depending on the two areas of sam-ple collection; Surat Thani which located in the middle of the south and Narathiwat which located in the southern-most of Thailand (Figure 1). However, there was no clear relationship between the pathogenicity and clustering of the isolates in this study. This result similar to those by Yu *et al* (2006) studied ISSR marker of *Melamspora lar-rici-populina* and found that ISSR analysis could divide tested isolates in to Northern population and Western

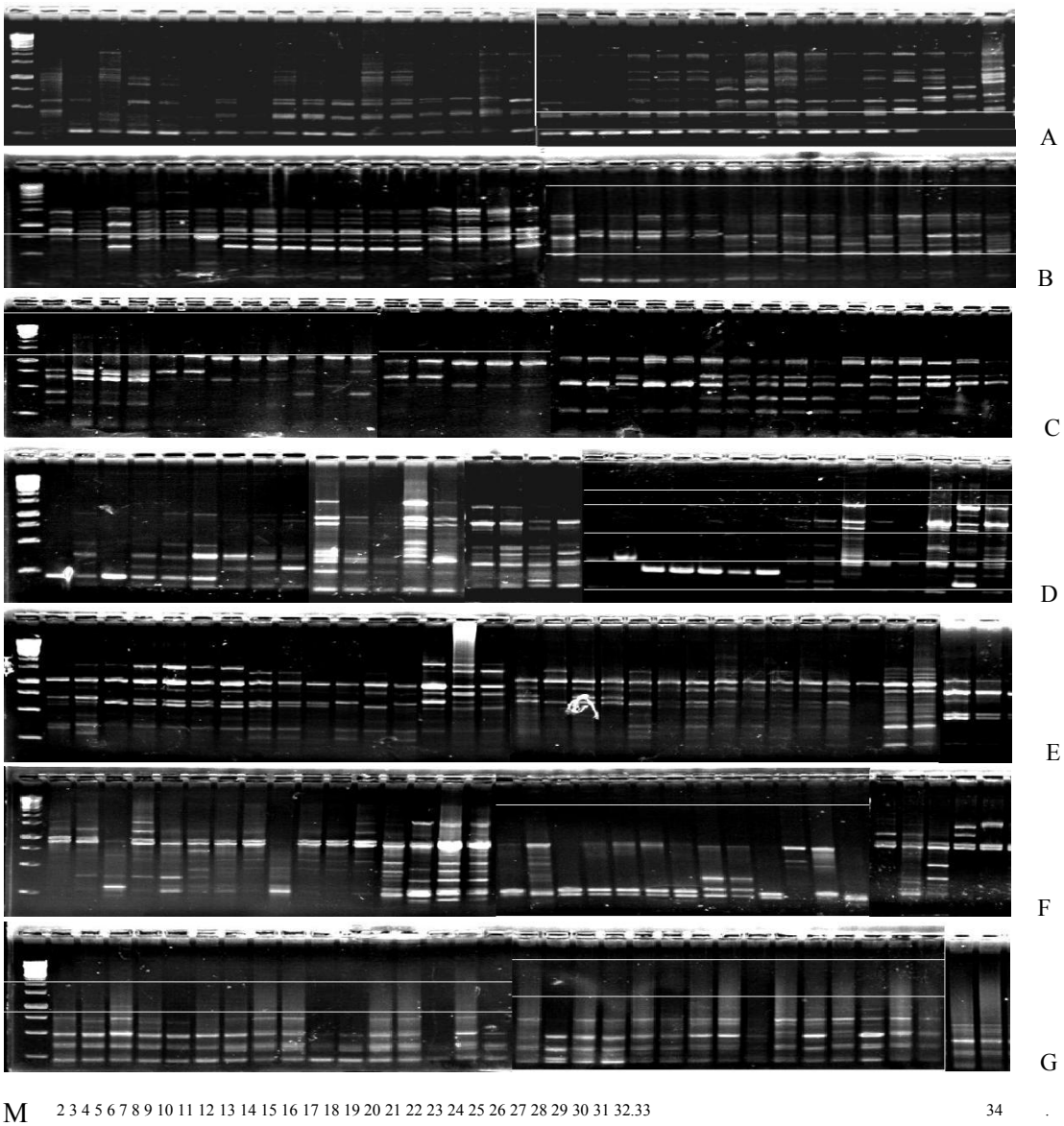

Figure 3. DNA banding profiles generated by ISSR-PCR with the (AG)8C (A), (GA)8T (B), (TG)8A (C), (GA)8YG (D), (GT)8YC (E), GGGC(GA)8 (F), and (CGA)5 (G) primer, M lanes represent 1 kb DNA ladder and lanes 2-33 represent tested isolates as shown in Table 2 and lanes 34 represent Gm101.

population. On the contrary, Rodrigues et al. (2004) found that ISSR- PCR analysis which separated strains of *Guignardia mangiferae* into three groups but not corresponded either to the host or to the geographic origin and Bayraktar *et al* (2008) also found that the genetic variation among isolates of *Fusarium oxysporum* f.sp. *ciceris* had no correlation with the clustering of isolates from different geographical regions. Moreover, the isolates of wood-decay fungus *Fomitopsis rosea* from the different geographical area did not separate in clusters (Kauserud and Schumacher, 2003). However, ISSR-PCR technique is suitable and reliable tool for population structure studies and discrimination among individual fungal isolates (Rodrigues et al., 2004). ISSR markers also are a good choice for DNA fingerprinting because they are quick and easy to handle (Bornet and Branchard, 2001).

In conclusion, the genetic variation among isolates of *Rigidoporus microporus* was determined by pathogenicity test and ISSR. The distinct genetic groups depended on geographical region but no clear relationship in disease virulence and pathogenicity. Moreover, this work provided new information on the ISSR analysis among isolates of *R. microporus* causing white root disease of rubber trees in Thailand.

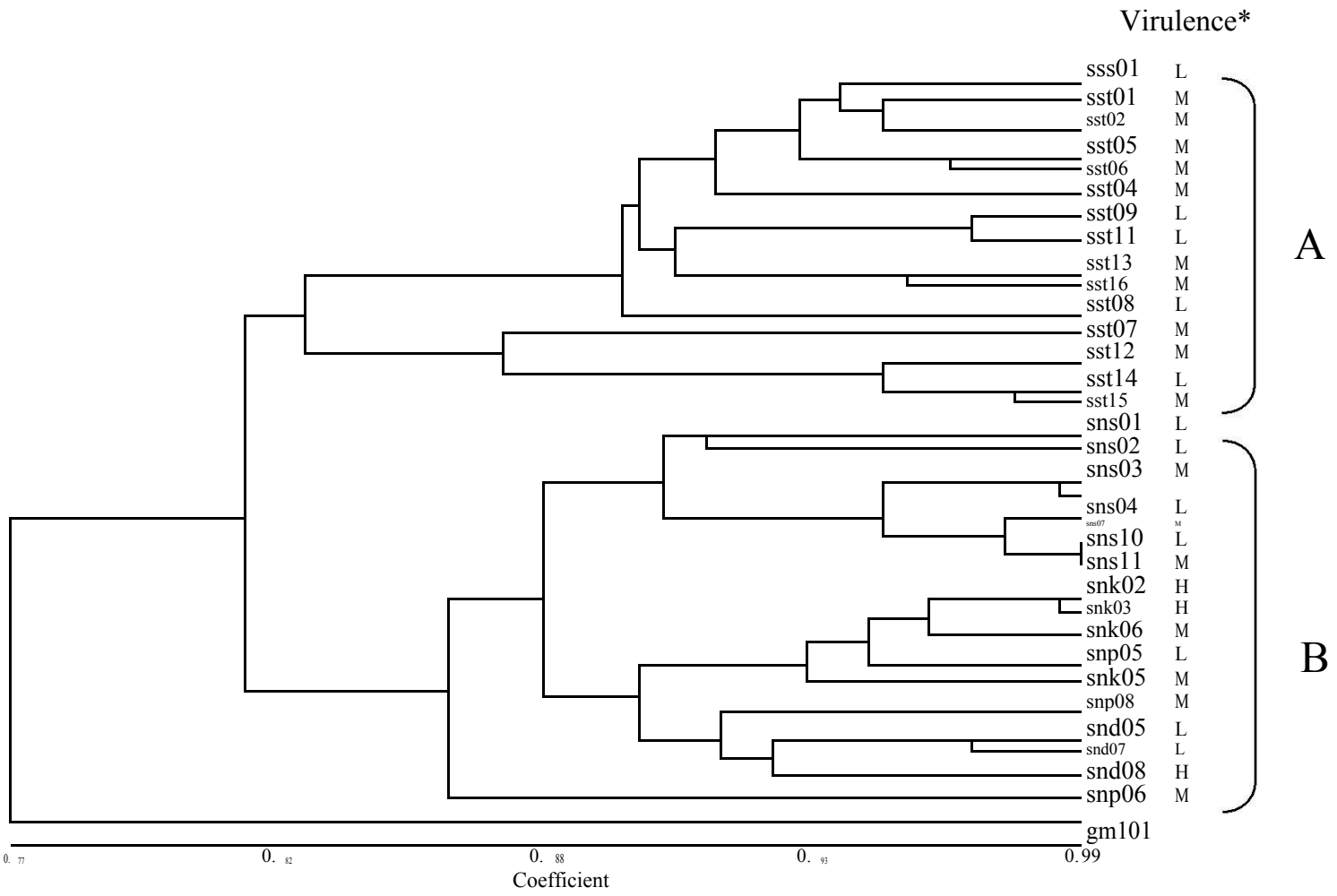

Figure 4. Dendrogram constructed with UPGMA -SHAN clustering of band data using 7 ISSR primers in 32 *isolates of Rigidoporus microporus* with outgroup *Ganoderma* spp GM101 and A = Surat Thani group and B = Narathiwat group.
*L = low virulent isolates, M = moderately virulent isolates, and H = high virulent isolates.

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