
Genetic Relationships of *Myrothecium roridum* Isolated from Water Hyacinth in Thailand Using ISSR Markers and ITS Sequence Analysis

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Abstract Water hyacinth (*Eichhornia crassipes*) is an invasive aquatic weed causing serious threats to irrigation networks and drainage canals in many countries including Thailand. At present, the management focus for this weed has changed from pesticides to biological control using natural enemies of water hyacinth including fungal pathogens. The leaf blight diseased water hyacinths were collected from 19 provinces of Thailand. One hundred thirty-seven fungal isolates were observed for morphological characteristics. According to morphological and molecular data sequenced in the internal transcribed sequence (ITS) region of the rDNA, the fungal isolates were identified as *Myrothecium roridum*. Inter simple sequence repeat (ISSR) markers were used to evaluate genetic variation of forty-two isolates of *M. roridum*. The results showed that forty-one polymorphic bands were generated using three ISSR primers. The cluster analysis indicated that *M. roridum* isolates could be separated into 6 subgroups at cophenetic values (r) = 0.99, supported by a 100% bootstrap value. There was no correlation between locations and the genetic diversity of *M. roridum*.

Keywords: *Myrothecium roridum*, water hyacinth, ISSR markers, ITS rDNA

Introduction

Water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae) is a free-floating aquatic plant and originated in Amazon, Brazil. The weed has spread to Africa, Asia, North America and occurs in at least 62 countries (Aboul-Enein *et al.*, 2011). Water hyacinth is an aquatic vascular plant with rounded, upright and shiny green leaves and lavender

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flowers. It has rapid reproduction and poses difficulty for eradication. The weed obstructs waterways, irrigation and navigation including destroying wildlife resources, reducing outdoor recreation opportunities, and lowering dissolved oxygen levels resulting in reduced available oxygen for animals and other plants. It also increases potential health risks by enabling the breeding of bilharzias, mosquitoes, and other parasites of animals and humans (Dagno *et al.*, 2012).

Many millions of baht are spent each year by the government to control the weed in Thailand. Physical, chemical and biological methods are used for controlling and removing water hyacinth from water resources. Mechanical or chemical methods have not removed this aquatic weed effectively and sustainably. Recently, the use of biological methods has been of increasing interest, since it is a cost-effective and nontoxic to the environment. Several highly effective fungi are known to cause diseases of water hyacinth. There are several species of *Myrothecium*, that can control water hyacinth such as *M. verrucaria* (Okunowo *et al.*, 2010; Hoagland *et al.*, 2011) and *M. roridum* (Liyanaige and Gunasekera, 1989; Piyaboon *et al.*, 2014).

The knowledge of the genetic diversity and intra species relationships are important for understanding how fungal populations may be correlated with geographical areas when used as biological controls (Padmavathi *et al.*, 2003). Therefore, molecular data have been employed to identify and study the diversity of this fungus. Molecular techniques have been developed for a PCR procedure to amplify DNA sequence for identification of fungal pathogen species (Pannecouque *et al.*, 2009; Inami *et al.*, 2010). Sequence analysis of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) could be used to differentiate species of *Myrothecium* (Okunowo *et al.*, 2013).

Several types of molecular markers such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeat markers), and ISSR (Inter Simple Sequence Repeat markers) are successfully used to study the variability in fungal populations (Zhou *et al.*, 2001). ISSR markers are a PCR based method, that amplifies inter microsatellite sequences at multiple loci in genomic DNA. ISSR markers are useful for unknown sequence information, and use a single primer for detection and random amplification (Unartngam *et al.*, 2011).

The purposes of this study were to identify and confirm the species of *M. roridum* from leaf blight disease of water hyacinth and to determine the genetic diversity and variation of *M. roridum* populations isolated from water hyacinth using ISSR markers.

Materials and methods

Fungal isolation

The leaf blight disease of water hyacinth was observed and collected from different geographical areas in nineteen provinces of Thailand including, the northern region including Chiang Mai, Lampang, Lamphun and Uttaradit, north-eastern region including Chaiyaphum and Nakhon Ratchasima, central region including Chai Nat, Nakhon Pathom, Nakhon Sawan, Pathum Thani, Phra Nakhon Si Ayutthaya, Phichit, Phitsanulok, Samut Songkhram, Samut Sakhon and Suphan Buri and western region including Kanchanaburi, Phetchaburi and Ratchaburi. Fungal pathogens were isolated from infected leaf using the tissue transplanting method and single spore isolation was performed on each isolate. All pure culture of isolates were maintained on potato dextrose agar (PDA) (Piyaboon *et al.*, 2014).

DNA extraction

Fungal mycelia of morphologically identified *M. roridum* were prepared from spore suspensions following Piyaboon *et al.* (2014). Freeze-dried mycelium was ground by mortar and pestle using liquid nitrogen, then 50 mg of the ground mycelium was suspended in 500 μ l of extraction buffer (200 mM Tris-HCl, pH 8.0; 250 mM NaCl; 25 mM EDTA and 0.5% SDS) and incubated at 65°C for 30 min. 500 μ l of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was then added. After centrifugation at 13,000 rpm for 10 min, the upper aqueous phase was deproteinized by the addition of 1 vol. of Chloroform: Isoamyl alcohol (24:1) and again centrifuged at 13,000 rpm for 10 min. The upper aqueous phase was transferred to a 1.5 ml microtube containing 2 μ l of 10 mg/ml RNAase and incubated at 37°C for 30 min. After that, 500 μ l of Chloroform: Isoamyl alcohol (24:1) was added and placed in a centrifuge at 13,000 rpm for 10 min, two volumes of absolute ethanol were added to the upper aqueous phase and the solution was stored at -20°C for 1 h. The solution was again centrifuged at 13,000 rpm for 10 min. The precipitated DNA was washed with 200 μ l of 70% ethanol and then was centrifuged at 13,000 rpm two times, each for a duration of 10 min. After drying, the DNA was dissolved with TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

PCR and ITS sequence analysis

Nineteen isolates of morphological identified *M. roridum* were randomly selected to study molecular-based identification (Table 1). DNA samples were amplified in the regions of ITS using the following universal primer; ITS 1(5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4(5' TCCTCCGCTTATTGATA TGC-3') (White *et al.*, 1990). Reactions were performed in a final volume of 40 µl with the following components: 0.2 pmole of each primer, 2.5 mM MgCl₂; 0.2 mM dNTP and 1 unit of *Taq* DNA polymerase. The thermal cycles were as follows: 95 °C for 30 seconds, 35 cycles of 95 °C for 30 seconds, 55 °C for 1 min, and 72 °C for 1 min, and a final step of 72 °C for 10 min. After amplification, 5 µl of the PCR product was resolved by gel electrophoresis on a 1% (W/V) agarose gel and then added to 0.1 µl/ml GelStar (Nucleic acid Gel stain, 1,000 X concentrate in DMSO) in a TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). The PCR products were purified using Illustra™ MicroSpin S-400 HR columns (GE Health care UK Limited). The purified PCR products were sequenced at 1st BASE DNA Sequencing Services, Malaysia. All newly generated sequences of *M. roridum* have been submitted to GenBank.

ITS sequences were aligned together with sequences obtained from the GenBank database, including *M. rodidum* (JF724153.1 and KC478534.1), *M. gramineum* (FJ235084), *M. verrucaria* (AB778924) and *Fusarium oxysporum* (KC292853) by MEGA 5 (Tamura *et al.*, 2001) using ClustalW. Phylogenetic analyses were based on the neighbour-joining (NJ) method (Saitou and Nei, 1987) using the same program. Node support was evaluated by bootstrap analysis using 1,000 replications in the same program (Felsenstein, 1985).

Table 1. Nineteen isolates used for ITS sequences analysis

Species	Isolate number	Localities	Accession number
<i>M. roridum</i>	KKFC390	Pathum Thani	AB823651
<i>M. roridum</i>	KKFC400	Nakhon Pathom	AB823652
<i>M. roridum</i>	KKFC402	Nakhon Pathom	AB823653
<i>M. roridum</i>	KKFC403	Nakhon Pathom	AB823654
<i>M. roridum</i>	KKFC406	Nakhon Pathom	AB857216
<i>M. roridum</i>	KKFC408	Nakhon Pathom	AB823655
<i>M. roridum</i>	KKFC447	Lamphun	AB857217
<i>M. roridum</i>	KKFC448	Chiang Mai	AB857218
<i>M. roridum</i>	KKFC457	Chai Nat	AB857219
<i>M. roridum</i>	KKFC460	Suphan Buri	AB857220
<i>M. roridum</i>	KKFC470	Samut Sakhon	AB857221
<i>M. roridum</i>	KKFC480	Phetchaburi	AB857222
<i>M. roridum</i>	KKFC483	Phetchaburi	AB857223
<i>M. roridum</i>	KKFC492	Uttaradit	AB857224
<i>M. roridum</i>	KKFC496	Phitsanulok	AB857225
<i>M. roridum</i>	KKFC497	Phitsanulok	AB857226
<i>M. roridum</i>	KKFC499	Phitsanulok	AB857227
<i>M. roridum</i>	KKFC509	Nakhon Ratchasima	AB857228
<i>M. roridum</i>	KKFC519	Chaiyaphum	AB857229
<i>M. roridum</i>	-	-	JF724153.1
<i>M. roridum</i>	-	-	KC478534.1
<i>M. gramineum</i>	-	-	FJ235084
<i>M. verrucaria</i>	-	-	AB778924
<i>F. oxysporum</i>	-	-	KC292853

ISSR markers and phylogenetic analysis

DNA samples of *M. roridum* were amplified in the ISSR regions using a 25 µl PCR reaction each containing 5 µl genomic DNA (50 ng), PCR buffer (1x), dNTP (0.2 mM), 10 pmole of each primer, MgCl₂ (2.5 mM) and *Taq* polymerase (2.5unit). The ISSR regions were amplified using three primers as follows: (GTG)₅, GCG (CGA)₅ and (CAG)₅. PCR was carried out using T professional Standard Gradient (Biometra) under the following condition: initial denaturation (94°C/5 min) followed by 37 cycles of denaturing (94°C/1 min), annealing (54, 54 and 58°C) for different primers for 1 min, extension (72°C/1 min) and a final elongation step (72°C/10 min). After amplification, 5 µl of the PCR product was electrophoresed in a 1.5% agarose gel in 0.5x TBE buffer (0.1 M Tris, 0.05 M boric acid and 0.001M Ethylene Diamine Tetra Acetate (EDTA)), at 70 V for 50 min and stained with ethidium bromide at 0.5

mg/ml and the gel image was recorded by using a gel documentation system (SynGene).

All polymorphic bands were manually scored as present (1) or absent (0) in the same molecular weight. The binary data was used for statistical analysis using the computer program NTSYS pc version 2.02 (Rohlf, 1993). An unweighted pair group arithmetic mean method (UPGMA) cluster analysis was performed using the Dice's similarity coefficient. Dendrograms were generated with the tree option (TREE) and a cophenetic value distance matrix derived from the dendrogram with the COPH program in NTSYSpc. The cophenetic value distance matrix was compared for level of correlation with the original matrix using the NTSYS program. Bootstrap values were calculated with 1000 replications by the Winboot program (Yap and Nelson, 1996).

Results

PCR and ITS sequence analysis

Nineteen sequences of morphologically identified *M. roridum* (Fig. 1) were aligned and analyzed together with the sequences obtained from GenBank database (NCBI) such as *M. roridum* (JF724157 and EU927366), *M. gramineum* (FJ235084), *M. verrucaria* (AB778924) and *Fusarium oxysporum* (KC292853). All sequences of *M. roridum* were deposited in the GenBank database as the following isolates KKFC 390 (AB823651), KKFC 400 (AB823652), KKFC 402 (AB823653), KKFC 403 (AB823654), KKFC 406 (AB857216), KKFC 408 (AB823655), KKFC447 (AB857217), KKFC 448 (AB857218), KKFC 457 (AB857219), KKFC 462 (AB857220), KKFC 470 (AB857221), KKFC 480 (AB857222), KKFC 483 (AB857223), KKFC 492 (AB857224), KKFC 496 (AB857225), KKFC 497 (AB857226), KKFC 499 (AB857227), KKFC 509 (AB857228), KKFC 519 (AB857229). The sequence lengths were 509-588 bp when aligned together with other sequences from the database. The similarity coefficient among nineteen sequences of *M. roridum* was 99.99% when compared with the sequence of *M. roridum* obtained from the database. Furthermore, the NJ clustering found that nineteen sequences of *M. roridum* were in same group with other sequences of *M. roridum* recorded in the database; this was supported by a 98% bootstrap value and separated from other species such as *M. gramineum*, *M. verrucaria* and *F. oxysporum*. The result indicated that the nineteen isolates could be identified as *M. roridum* based on ITS sequence which was similar to the morphologically based identification (Fig. 2).

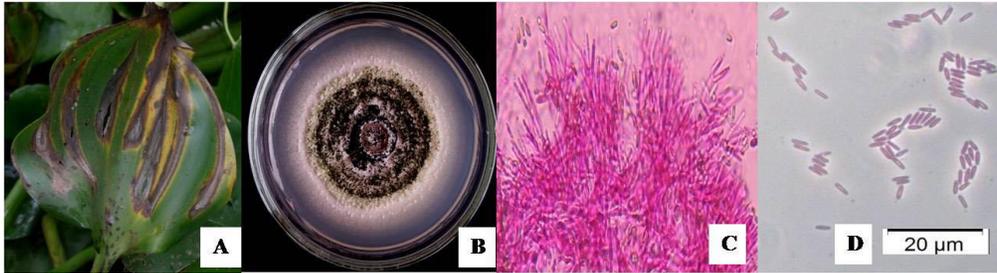


Figure 1. Leaf blight symptom on water hyacinth leaf (A) morphological characteristics of *M. roridum*; concentric zones diffused in sporodochia (B), conidiophores (C, 400X) and conidia (D, 400X)

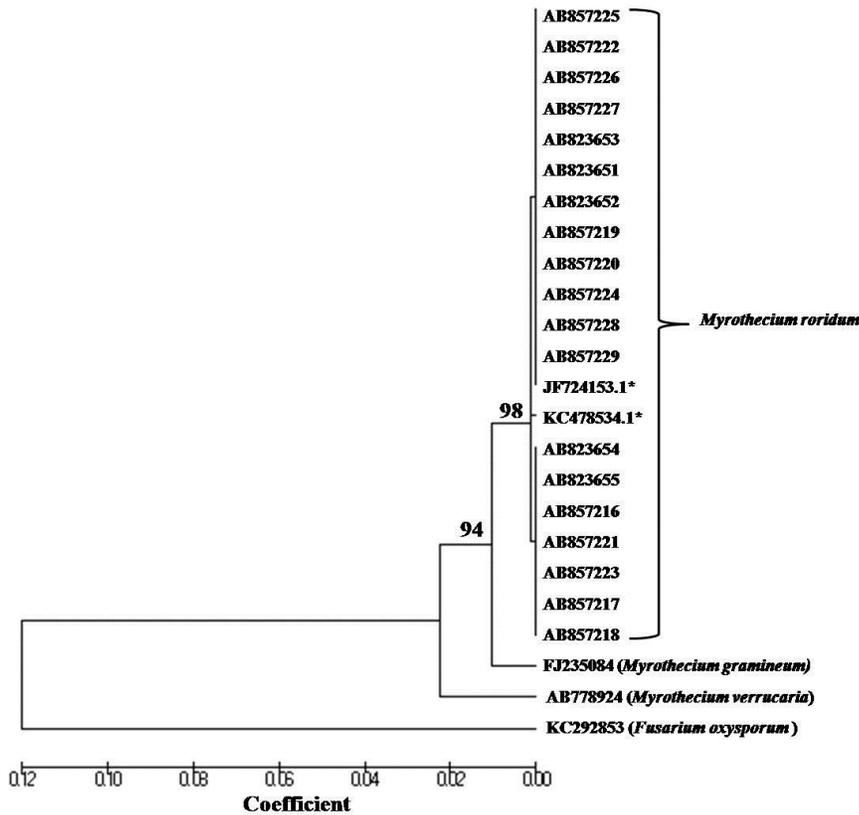


Figure 2. Phylogenetic analysis of the nucleotide sequences of the ITS region including 5.8S rDNA of 19 *M. roridum* isolates, 4 sequences from *Myrothecium* species and 1 sequence from *Fusarium oxysporum*. Percentage bootstrap support (1000 replications; ≥ 94 (%) is shown on branches (*= sequences obtained from GenBank database).

ISSR markers and phylogenetic analysis

The genetic diversity of *M. roridum* was analyzed using PCR amplification of the ISSR region. Forty-one polymorphic bands were generated among 44 isolates of *M. roridum* including *Fusarium moniliforme* and *F. oxysporum* as an outgroup (Figs. 3, 4 and 5). Polymorphic bands were analyzed to construct a dendrogram using the UPGMA method. ISSR cluster analysis showed that all samples showed subgroups at similarity coefficients from 90 to 95% supported by a 100% bootstrap value and cophenetic values (r) = 0.99 (Fig. 6). Forty-two samples of *M. roridum* were divided into 6 subgroups. Subgroup 1 had 5 isolates from the central region of Thailand: KKFC385, KKFC389, KKFC496, KKFC497 and KKFC499. Subgroup 2 had 14 isolates from the central region of Thailand: KKFC457, KKFC458, KKFC459, KKFC501, KKFC502, KKFC 503, KKFC504, KKFC460, KKFC462, KKFC463, KKFC465, KKFC466, KKFC467 and KKFC470. Subgroup 3 had 8 isolates from the northern region of Thailand: KKFC444, KKFC447, KKFC448, KKFC456, KKFC487, KKFC491, KKFC492 and KKFC493. Subgroup 4 had 4 isolates from the central region of Thailand: KKFC390, KKFC414, KKFC408 and KKFC424. Subgroup 5 had 4 isolates from the western regions of Thailand: KKFC430, KKFC442, KKFC434 and KKFC436 and subgroup 6 had 7 isolates from the western and northeastern regions of Thailand: KKFC479, KKFC480, KKFC483, KKFC509, KKFC511, KKFC514 and KKFC519. The results showed that all isolates of *M. roridum* observed by ISSR markers were not correlated to the geographical areas.

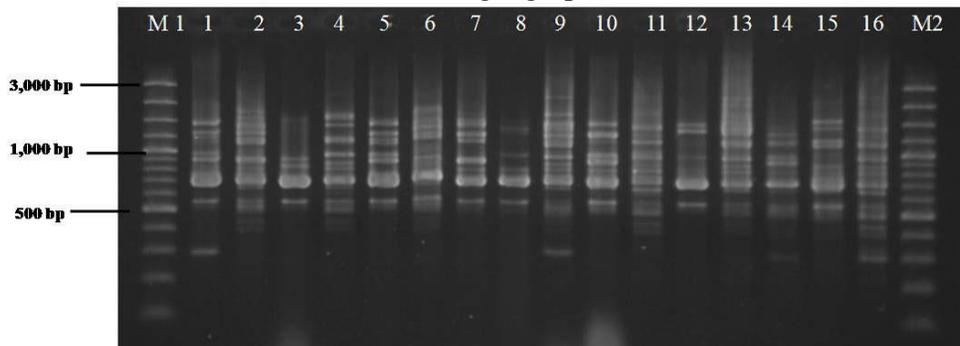


Figure 3. PCR fingerprinting patterns of *M. roridum* isolates using the ISSR primers (GTG)₅ M1 and M2 = 100 bp Plus DNA Leader; 1 = KKFC 385; 2 = KKFC 389 ; 3 = KKFC 390; 4 = KKFC 408; 5 = KKFC 414; 6 = KKFC 424; 7 = KKFC 430; 8 = KKFC 434; 9 = KKFC 436; 10 = KKFC 442; 11 = KKFC 444; 12 = KKFC 447; 13 = KKFC 448; 14 = KKFC 456; 15 = KKFC 457; 16 = KKFC 458

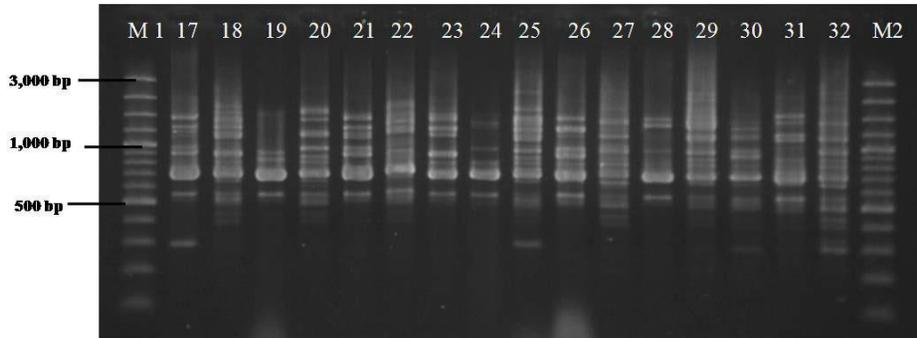


Figure 4. PCR fingerprinting patterns of *M. roridum* isolates using the ISSR primers (GTG)₅. M1 and M2 = 100 bp Plus DNA Leader; 17 = KKFC 459; 18 = KKFC 460; 19 = KKFC 462; 20 = KKFC 463; 21 = KKFC 465; 22 = KKFC 466; 23= KKFC 467; 24= KKFC 470; 25 = KKFC 479; 26 = KKFC 480; 27 = KKFC 483; 28 = KKFC 487; 29 = KKFC 491; 30 = KKFC 492; 31 = KKFC 493; 32 = KKFC 496

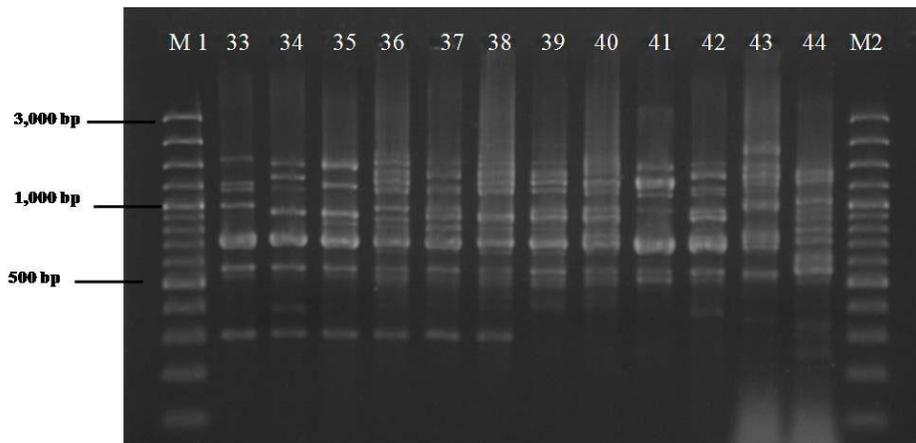


Figure 5. PCR fingerprinting patterns of *M. roridum* isolates using the ISSR primers (GTG)₅. M1 and M2 = 100 bp Plus DNA Leader; 33 = KKFC 497; 34 = KKFC 499; 35 = KKFC 501; 36 = KKFC 502; 37 = KKFC 503; 38 = KKFC 504; 39 = KKFC 509; 40 = KKFC 511; 41= KKFC 514; 42= KKFC 519; 43= *Fusarium solani*; 44 = *Fusarium oxysporum*

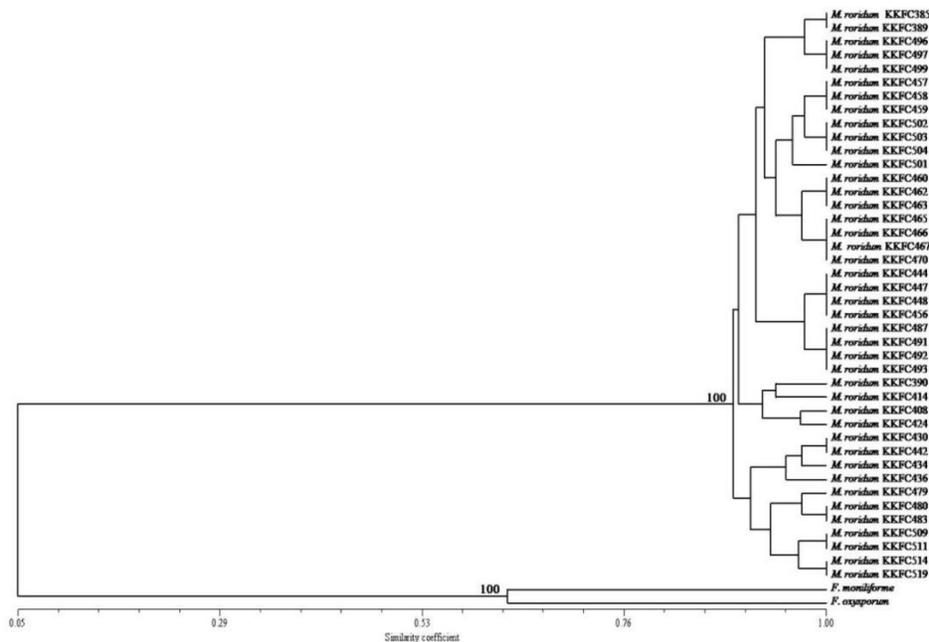


Figure 6. Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 42 *M. roridum* isolates. Genetic similarity was obtained by ISSR marker, using the Dice similarity coefficient.

Discussion

The fungus isolated from infected water hyacinth is an aggressive pathogen which causes leaf blight in water hyacinth in Thailand. Fungal isolates could be used to control water hyacinth. Therefore, fungal pathogen is a candidate biocontrol agent against water hyacinth (Piyaboon *et al.*, 2014). In this study, the fungal isolates were identified to the species level by ITS sequencing and phylogenetic analysis. The result of phylogenetic analysis comparing the ITS sequence to deposited sequences in GenBank showed *M. roridum* as the closest relative at a 99.99% similarity coefficient. The sequences were confirmed that fungal isolates can be identified as the same by both phenotypic and molecular methods. This supports the investigations of Okunowo *et al.* (2013), who studied the sequence of the ITS regions of *M. roridum* isolated from water hyacinth in Nigeria. The results showed that *M. roridum* isolates were in the same group as *M. roridum* recorded in the GenBank database based on sequence similarity. There are many reports showing that ITS regions of rDNA have been used to confirm the species of *M. roridum* (Worapong *et al.*, 2009; Okunowo *et al.*, 2013; Piyaboon *et al.*, 2014).

ISSR regions are inter microsatellite sequences at many loci in eukaryotic genomes, and ISSR regions have also been used to study inter and intra species relationships (Zhou *et al.*, 2001). Use of ISSR markers, and other molecular markers is preferred to morphological and biochemical markers for genetic diversity because molecular markers are not affected by the environment and developmental stage. This study confirmed the usefulness of ISSR markers to analyze genetic variability in *M. roridum* genotypes in Thailand. The ISSR analysis, indicated that nearly 80% of the bands generated were polymorphic bands. This shows a high level of genetic variation that exists among the different isolates. The fungus has parasexual reproduction, which normally offers high genetic variability (Estrada *et al.*, 2007). ISSR markers have the capacity to reveal variation in special regions of the genome (Mohammadi *et al.*, 2011). Similarly, Piyaboon *et al.* (2014) evaluated the genetic variation of *M. roridum* population from Thailand using AFLP markers. The results indicated that about 70% of bands generated were polymorphic among the fifteen isolates of *M. roridum* which reflects high a level of genetic variation that exists among the different isolates. While, the high similarity coefficient (90-95%) among six subgroups indicated the low genetic variability between the six groups of isolates. Subgroup 1, 2 and 4 contained isolates from the central regions and subgroup 3 contained isolates from the northern region. On the other hand, subgroup 5 contained the isolates from the western regions and subgroup 6 contained the isolates from the western and northeastern regions. Therefore, the results showed that phylogroups were not correlated to the geographical areas. Moreover, this result indicated asexual spore or conidia was presumably dispersed across geographical areas.

Conclusion

The results of the present investigation demonstrate that fungal isolates could be identified as the species of *Myrothecium* based on ITS sequence which was related to the morphologically based identification. Genetic variations among isolates of *M. roridum* based on ISSR markers were observed. The phylogroups were not related to geographical area indicate that *M. roridum* can be used for controlling water hyacinth in every area of Thailand.

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