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Identification of antagonist molds against *Pyricularia oryzae* using internal transcribed spacers (ITS)

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Abstract

There are several troubles that found in rice production of national program, for example is blast disease that caused by *Pyricularia oryzae*. One of problem solving to control this disease is using biocontrol agent which more ecofriendly and specific target, such antagonist molds. Identification of antagonist molds using ITS5 and ITS4 universal primary was to completing identification using phenotype characteristics. This primary will amplify *ribosomal cassette* (partial sequence of 18S rDNA, complete sequence of *5.8S rDNA* and partial sequence of 28S rDNA also complete sequence of ITS1 and ITS2). Molecular analysis using ITS5 and ITS4 was applied on every antagonist molds, including DNA isolation, amplification, purification, sequencing and alignment. The result showed that five isolates TBd 11, TBd 9, TG 2.1, TG 4.1 and TG 6 same as *Trichoderma* Genus. Isolates TBd 11, TBd 9 dan TG 2.1 same as *Trichoderma asperellum* with similarity value 100 %, isolate TG 4.1 same as *Trichoderma hamatum* with similarity value 94,96 % and TG 6 same as*Trichoderma viride* with similarity value 98,42 %. In contrast, isolate TBd 6 formed another clade with Genus *Rhizomucor, Rhizomucor variabilis* species with similarity value 100 %.

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Introduction

Rice is one of crussial food material in some countries, no exception Indonesia. In Indonesia rice production is imbalance with population growth rate reached 1.3 % annually. It need increasing of rice production national program. But, on the way to make it happen, there were several troubles that inhibit it. One of trouble maker is rice plant diseases. There are a lot of types of rice plant diseases that caused by pest and infection of microbial pathogen, for example was blast diseases. Blast disease was caused by pathogenic mold *Pyricularia oryzae* which could infected from the vegetative until reproductive stage. Santika and Sunaryo (2008) point out that the heaviest infections is at the stage of generative, so it can make thwart of harvest.

Utilization of antagonist molds as biocontrol agents is necessary because more effective (Seshu *et al.*, 1985), specific target and environmental welfare (Yasmin and D'Souza, 2010). Determination of antagonists mold against *P. oryzae* in Indonesia have not been much done and the extent of identification based on phenotype characters only. Thus, need identification in molecular to completing the identification based on morphological characteristics, for instance by using universal primary ITS5 and ITS4. This primary will amplified ribosommal cassette site which conclude partial sequence of *18S rRNA*, complete sequence of *5.8S rRNA*, partial sequence of *28S rRNA* and complete sequence of ITS1 and ITS2 (White *et al.*, 1990). In present study, there were six of 25 molds which examined as potentially antagonist molds against *P. oryzae*. Five of potential antagonist molds such as TBd 9, TBd 11, TG 2.1, TG 6 and TBd 4.1 were predicted as *Trichoderma* sp., while isolate TBd 6 was predicted as *Rhizomucor* sp. Morphological identification is not enough to determine spesies of the molds. Hopefully, identification based on morphological characteristics that conducted before would be better with these advanced study.

Materials and methods

Isolation of antagonist molds

Antagonist molds was obtained from isolation of soil in agricultures land which infected by blast disease in Gembongan, Bacem and Bendo Village, Ponggok, Blitar, Indonesia. Isolation and screening of antagonist molds was using (Nugrahani, 2011) and (Shyamala and Sivakumaar, 2012) methods. Based on screening result,there were six antagonist molds which inhibit pathogen growth more than 50 % were used in this study. There were (TBd9, TBd11, TG2.1, TG6, TG4.1 and TBd6).

DNA extraction of antagonist molds

Six antagonist molds (TBd9, TBd11, TG2.1, TG6, TG4.1 and TBd6) were subcultured into Potato Dextrose Broth (PDB) for 3-4 days. Incubation was conducted in a shaker incubator, with speed condition 120 rpm, 30 ^{o}C. Whole genome extraction of antagonist molds was conducted based on modified method of (Doyle and Doyle, 1990).

DNA Amplification using Polymerase Chain Reaction (PCR), Purification and Sequencing

Ribosommal cassette was amplified using ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') (White *et al.*, 1990). It also using PCR solutions (Intron Biotechnology, 2011) and PCR program as Table 1 (Suharjono *et al.*, 2010; Schoch *et al.*, 2012). Product of amplification than separated by electrophoresis modified method of (Suharjono *et al.*, 2010) and using *GeneRuler*™ DNA *LadderMix* 100 as DNA marker. Purification and sequencing were conduted in Macrogen, Korea.

Results and discussions

Electrophoresis of amplicon showed that all of amplicons had single band about 900 bp (Fig. 1). Those result according to amplification target and same as (Cooke *et al.*, 2000) result which amplification using ITS5 and ITS4 as primary always resulting single band about 900 bp.

Constructions of evolutionary history, evolutionary distance and the relationship between anchestor and modern species was conducted in phylogenetics analysis. The comparison of ITS was based on the

similarity among characters. Evolutionary history could be identified through out the differences of characteristics among species. It always presented as phylogeny tree.

Table 1. PCR Reaction Program to amplified DNA of pathogen mold *P. oryzae* 1a Ras 073 and antagonist molds.

No.	Reaction	Temperature (°C)	Time (menit)
1	Pre-Denaturation	95	10
2	35 cycles : Denaturation	94	0,25
	Annealing	55	0,5
	Extension	72	0,25
3	Extension	72	7

Maximum Likelihood methods was used to construct sequence data of each isolates. Calculation in this method was used to find possible tree which had best calculated of sequence variation. Beside that, this method considers the number of sequence changes or mutations, it can be used to explore this untapped relationships between sequence that more diverse, where these conditions cannot be done properly if using the other method like Maximum Parsimony.

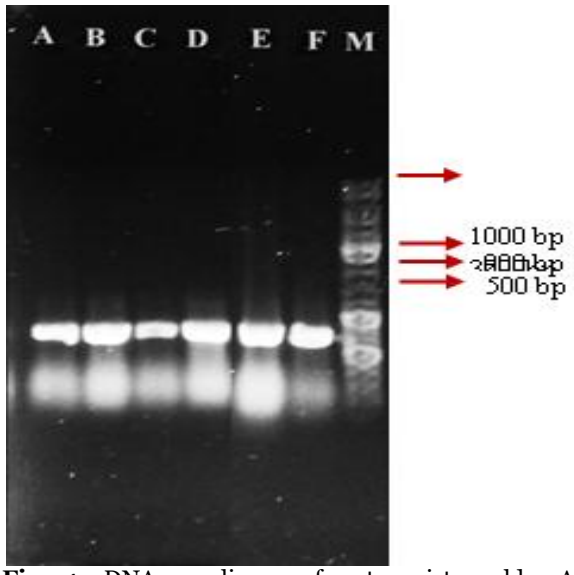

Fig. 1. DNA amplicons of antagonist molds. A (TBd11), B (TG6), C (TBd9), D (TG2.1), E (TBd6), F (4.1) and M is marker (*GeneRuler*™ DNA *LadderMix* 100).

The phylogenetics analysis showed that Isolates TG2.1, TBd11 and TBd9 had similarity value 100 % with *Trichoderma asperellum* (bootstrap value 63 %) (Fig. 2). Bootstrap value more than 50 % showed the significancy of phylogeny tree topology. Dharmayanti (2011) point out that prediction of phylogeny branches which conducted repeatedly will produce more significant topology. The perfect similarity of three species indicates that all of them were belong to same strain. Similarity value of 100 % indicates single strain. If similarity value arround 99 % indicates include similar species, while if similarity value arround 89–99 %, it were classified as one Genus (Henry *et al.*, 2000).

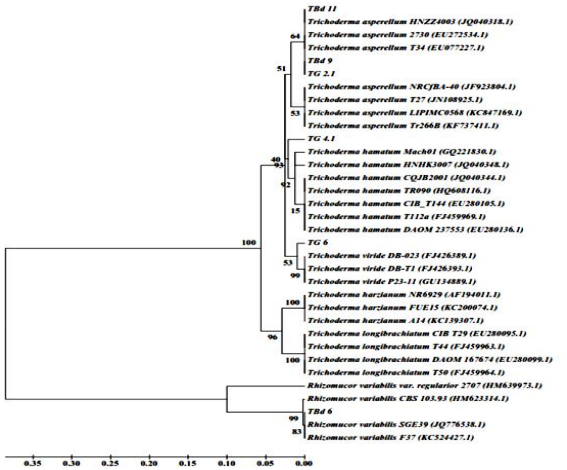

Fig. 2. Phylogeny tree which showed phylogenetics of antagonist molds and refferences isolates from Gen Bank based on ITS sequence which construsted with *Maximum Likelihood* algorithm and *Tamura-Nei* method.

Isolate TG4.1 was formed sisterclade and belong to same Genus with *T. hamatum* because it had similarity value 94.69 % (evolution distance at 0.053). Isolate TG6 was formed sisterclade and belong to same Genus with *Trichoderma* too, *T. viride* Species, because it had similarity value 98.42 % (evolution distance at 0.016) (Fig. 2). Based on present study, five isolates which includes on *Trichoderma* sp. had some specific characteristics such as growth in room temperature (25-30 °C), dark green pigmentation with radial conture of colony. In microscopics observation on magnification 400x showed that *Trichoderma* sp. has short hypha with septum. It also has conidiofor that brances out with globuse conidia in the end of conidiofor (Fig. 3a and 3b) (Castle *et al.*, 1998; Funder, 1953; Samuels *et al.*, 1999; Shah *et al.*, 2012).

Isolate TBd6 with similarity value 100 % involves as single strain with *Rhizomucor variabilis*. One of characteristics which owned by both TBd6 and *R. variabilis* is it has long branched hypha with sporangium in the end of hypha (Fig. 3c, 3d, 3e and 3f). Determination of all isolates based on amplification using Internal Transcribed Spacers (ITS) compared to other primaries as a barcode for fungi, ITS had inter- and intraspecies barcode gap about 0.372664 and 0.025013 respectively (Schoch *et al.*, 2012). It also had higher sequence variation than other genic regions of DNA (SSU and LSU), so it is functional to understanding variaton among sequences and among individual rDNA repeats.

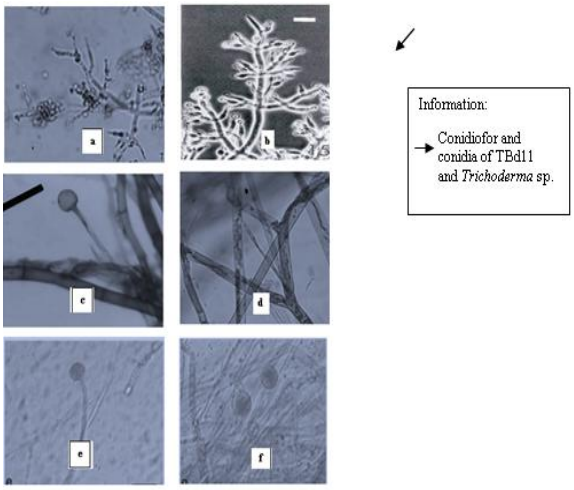

Fig. 3. Morphology of antagonist molds isolate TBd11 (a); *Trichoderma* sp. (b) (Samuels dkk., 1999); isolates TBd6: (sporangium (c) and branch (d)); the branches and sporangium of *Mucor irregularis* (e and f) (Lu dkk., 2013). Observation on magnification 400x.

In identification of taxas, it is necessary to combine ITS primer with the other primers such as 18S rDNA and RNA Polymerase II (RPB1). RPB1 primer may play role in transcription process. It also had high descrimination among species than ITS primer. Combination both ITS and RPB1 showed phylogenetics analysis more informative than single utilization (Schoch *et al.*, 2009; Urbina and Blackwell, 2012).

Conclusions

Based on result can conclude that Isolates TBd11, TBd9 dan TG2.1 belong to same *Trichoderma asperellum* with similarity value 100 %, isolate TG4.1 same as *Trichoderma hamatum* with similarity value 94.96 % and TG6 similar with *Trichoderma viride* (similarity value 98.42 %). In contrast, isolate TBd 6 was formed another clade with Genus *Rhizomucor, Rhizomucor variabilis* species with similarity value 100 %.

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