Exploration of entomopathogenic fungi as potential biocontrol of corn earworm (*Helicoverpa armigera* (Hübner))

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Abstract. In Indonesia, *H. armigera* caused loss in corn production until 40% and almost attack all region. Therefore, this pest is a concern especially in corn plantations in Indonesia. In the development of *H. armigera* pest control, the use of entomopathogenic fungi increases after the potential risks of using chemical pesticides in various aspects. Biological control using entomopathogen agents is an alternative control method that has been studied widely and considerably more effective and environmentally friendly. This study aims explore entomopathogen that attack *H. armigera* which will later be formulated and projected as candidates for biopesticides controlling *H. armigera*. This research sited in East Java, South Sulawesi, and North Sulawesi in 2011. This research was divided into several stages within a year which were carried out including surveys and samples collection, isolation, characterization, morphological identification and molecular identification (genotyping phylogenetic tree). The research found several pathogens from several location such as *Rhizopus* sp., *B. bassiana*, *Fusarium* sp., *A. flavus*, and *Gliocladium* sp. The character of each Entomopathogenic fungus showed that every isolate has different color and shapes. Based on the results of DNA amplification from eleven fungal isolates using BOX primers, various fragments were produced with sizes ranging from 225 to 1250 bp. The results also showed that all the isolates tested had polymorphic bands with a polymorphic information Content (PIC) value of 0.84. This indicates that these isolates have a low level of polymorphism.

1. Introduction

Of the several controls implemented, pesticide pollution is one of the great concerns in the world. Based on reviews from conducted by [1], the increase in climate change is in line with the increase in pesticide use that also has an impact on human health. Therefore, it is necessary to regulate and limit the use of pesticides to avoid the occurrence of pollution that results in human health, the environment, and other non-targeted organisms. Research on biological agents has provided a lot of information about the effectiveness of some agents such as parasitoids, predators and pathogens [2, 3].

As one of the biological agents, entomopathogen was developed as an environmentally friendly pest controller. In an integrated pest control program, the correct combination of various population control programs is essential if one is to maintain a pest population below a certain economic threshold. Entomopathogen fungi can be an important tool in the context of control strategies. This can reduce the application of synthetic products and have a low negative environmental impact and reduce the risk of killing non-target insects [4]. Based on reviews of [5] control using entomopathogen mold is able to induce a high level of susceptibility to insecticides in target pests by suppressing enzyme activity and influencing in fungal infection.

Abundant research on these pest control agents has been published such as the *metharizium* and *beauveria* strains for controlling eucalyptus snout beetles in Brazil [6], the entomopathogen agent...
Spodotera litura [7], utilizing Aspergillus nomius to control outbreaks of D. Thoracicus [8], and so on. In the midst of high research on the use of these biological agents, there is still a lot of distrust of the use of these agents in pest control. Therefore, more applicable research and practical work should be improved to promote the use of these pest control agents. Entomopathogen infects hosts in some processes that were initially through direct penetration. Pathogenesis also requires several agents such as adhesin, enzymes, and secondary metabolites. This high effectiveness is also accompanied by other advantages such as removing toxic contaminants and antimicrobial agents [9].

Various studies on entomopathogens in lepidopteran types have been conducted with results showing the successful use of these bio agents [10-13]. The purpose of the study was to obtain what pathogenic character attacks the larvae of corn cob borers (H. armigera).

2. Materials and Methods

2.1 Collection of samples from the field and propagation of pathogen isolates

In this study was conducted in 2011 and took samples from 3 provinces namely South Sulawesi (Malino and Bajeng), North Sulawesi (Modoinding, Rurukan), and East Java (Batu Malang, Pujon Malang, Mount Bromo Probolinggo). The collection is carried out by collecting the larvae of corn cob borers (H. armigera) on corn plants suspected of being attacked by pathogenic microorganisms with body features that have weakened or who have died infected. Larvae suspected of being infected with pathogens are then collected for further isolation and propagation of pathogens. The propagation of isolates is done through potato Dextrose Agar (PDA) media as a medium of entomopathogen breeding.

2.2 Pathogen breeding/pathogen isolation and morphological identification

Larvae collected from the field are then reared to see the attack of pathogens on the larvae. Larvae affected by pathogens with symptoms are stiff, foul smelling and encased by fungal hyphae then separated for later propagation of pathogens. To make the pathogen suspension, as many as 10 larvae of the affected larvae are discarded and the surface sterilized in 1% sodium hypochlorite for 3 minutes, then washed three times in distilled water after which identification of the isolate is carried out using standard procedures with the help of several key taxonomic fungal isolates [14]. The observed characteristics/morphology include colony morphology, hyphae structure and conidia. Colonies are observed after the isolate is grown for ± 7 days. For microscopic structures are observed with a light microscope with a magnification of 400x. Before being observed under a light microscope, preparations are made by scanning mycelium from hyphae and then dripped with Lactophenol Cotton Blue (LCB) as a hyphae dye, then mycelium is decomposed using a needle. Then the isolate is preserved on PDA media coupled with 75 mg/l of Rifampicin in a 30 ml tube, stored in the refrigerator at 8-100°C, and sub cultured every month.

2.3 Extraction and Purification of DNA

DNA extraction is done using the method [15]. Pathogen cultures that are 9 days old in Czapek-dox + yeast are filtered using a filter then frozen at -20°C for 3 days. Mycelia, which has been frozen, is eroded using mortars previously added liquid nitrogen. Mycelia powder is then inserted into the micro centrifuge tube as much as 1/2 of the contents of the tube and added 500 μl TES buffer (Tris-EDTA-SDS) then in the vortex. Into the suspense added 5 μl proteinase K and then put on the water bath there is a temperature of 55°C for 1 hour. Supernatant is further added with 140 μl NaCl and 65 μl CTAB (which had previously been incubated on water heaters at 65°C) and incubated for 10 minutes at 65 °C. Chloroform isoamilalkohol was further added as much as 710 μl and incubated at 0°C Selma 30 minutes. The supernatant is then concentrated at 10,000 rpm for 10 minutes, the supernatant is taken and moved at 1.5 ml of micro centrifuge tube and added 225 μl NH₄ acetate and homogenized slowly overnight. Centrifugation is re-performed at 10,000 rpm for 10 minutes. The pellets are washed twice with 500 μl of 70% cold ethanol and dried with a heater. Pellets are then taken and compensated with 50 μl buffer TE plus 3 μl RNAse for 1 hour at a temperature of 37°C
The quality of extractable DNA can be known after the electrophoresis process. If the quality of DNA obtained is not optimal, then the purification stage is carried out. The DNA solution is added with *isoamylalcohol phenol chloroform* (25:24:1 v/v) of 940 μl and incubated for 5 minutes on ice. The DNA suspension is then centralized at 10,000 rpm for 10 minutes. Supernatant is taken and transferred on a new micro centrifuge tube. In the next supplement added *isoamylalcohol chloroform* (24:1 v / v) as much as 940 μl centrifugation is done at 10,000 rpm for 10 minutes. The supernatant is transferred on the new micro centrifuge tube then added 0.55% of the volume of cold *isopropanol* and recentrifuged. The supernatant is discarded and the pellets are washed 2 times with 500 μl of 70% cold ethanol. The obtained pellets are then denmed to the heater block. The pellet is then suspended with a 50 μl TE buffer.

DNA quality checking is done through horizontal electrophoresis at 1.8%. Agarose with TBE buffer system (Tris-Borat-EDTA). DNA visualization colored with *ethidium bromide* (1 μg/μl) for 15 minutes and washed with distilled water for 5 minutes and detected using UV light.

The primer used in this DNA analysis is the primary box (BOXAIR: 5'-CTACGGCAAGGCGACGCT GACG-3'). A total of 8 μl of PCR products are separated through horizontal electrophoresis with 2% agarose gel and TBE buffer at 90-volt voltage for 30 minutes. Visualization of the DNA profile stained with *ethidium bromide* (1 μg/mul) for 15 minutes, followed by distilled water for 5 minutes, then detected using UV light. Gel documentation is then done using a Polaroid camera.

To calculate the genetic distance used the formula:

\[ d_{ab} = 1 - F_{ab} \]

Where \( d \) is the genetic distance between A and B, \( F \) is the similarity calculated with the formula, A and B were the comparable isolates.

The result of DNA shooting in the form of DNA band patterns is translated into the form of values with the provision of value 0 (zero) for the absence of bands and a value of 1 (one) for the presence of bands in the same position of each isolate compared to using the formula Nei and Li (1979) as follows:

\[ F = \frac{n_{ab}}{n_a + n_b} \]

Data is analyzed using analysis [17]

### 3. Result and Discussion

The results of a study of 11 pathogenic were successfully isolated attacked corn cob borer pests (*H. armigera*) from various locations in South Sulawesi, North Sulawesi, and East Java. Isolates collected from South Sulawesi amounted to five (Bajeng and Malino), North Sulawesi amounted to two isolates (Modoinding and Rurukan) and East Java amounted to three isolates (Malang, Pujon Malang, and Mount Bromo Probolinggo)(Table 1).

| No | Province       | Location            | Pathogen               |
|----|----------------|---------------------|------------------------|
| 1  | Jawa Timur     | Batu, Malang        | *Rhizopus* sp          |
|    |                | Pujon, Malang       | *Rhizopus* sp          |
|    |                | G.bromo, Probolinggo| *Rhizopus* sp          |
|    |                | Bajeng 1            | *Beauveria bassiana*   |
|    |                |                     | *Rhizophs* sp          |
| 2  | Sulawesi Selatan| Bajeng 2            | *Fusarium* sp          |
|    |                | Malino              | *Rhizopus* sp          |
|    |                |                     | *Aspergilus flavus*    |
| 3  | Sulawesi Utara | Midoinding (Minsel)| *Gliocladium* sp       |
|    |                | Rurukan (Tomohon)   | *Rhizopus* sp          |
The results of a collection of pathogenic isolates originating from three provinces there are eleven isolates that successfully isolated *Beauveria bassiana*, *Aspergillus flavus*, *Fusarium*, *Rhizopus*, *Gliocladium* then isolated on PDA media and then ready to see the character of each pathogen isolate.

![Morphology of Beauveria bassiana, Fusarium, Gliocladium, Aspergillus, Rhizopus](image)

**Figure 1.** Morphology of *Beauveria bassiana*, *Fusarium*, *Gliocladium*, *Aspergillus*, *Rhizopus*

### 3.1 DNA Characterization

To complete morphological characterization, DNA characteristics are carried out by understand the diversity of DNA profiles. Based on [17], the genetic variability of a population can be observed at the isoenzyme level and the DNA level. One of the detections that can be used for DNA detection is Repetitive PCR (Rep-PCR).
Based on the DNA amplification results of eleven fungi isolates using the BOX primer produced fragments that vary in size between 225 to 1250 bp (Figure 2). The results also showed that all isolates tested had bands that were polymorphism with a polymorphic information content (PIC) value of 0.84. This indicates that the isolate is a low level of polymorphism.

3.2 Analysis of genetic similarities

Similarity analysis was used to find a genetic relationship among the 11 pathogen isolates. The relationship is in the form of dendrogram visualization as in 20 images and 3-dimensional visualizations in 3D images through analysis of NTSYS pc 2.1 derived from the matrix of genetic similarities. Dendrogram and 3-dimensional visualization are constructed through UPGMA (Unweighted Pairs Group Method Using Arithmetic Average).

Based on dendrograms isolates are at similar levels of genetic similarity ranging from 0.0-1.00. From the results of the dendrogram cutting is formed 3 groups or clusters (Figures 3 and 4). The genetic similarity value for group I of 0.47.47 for group II has a genetic similarity of 0.24 while the stand-alone group III has a genetic similarity value of 0.0. According to[18] the determination of groups or clusters is done based on the average linkage method by choosing the largest distance difference and supported by the population of genotype origin, in addition also supported by the results of bootstrapping analysis then drawn dendrogram cutting lines on the scale of genetic similarity.

The value of the similarity coefficient from left to right indicates the closer the level of kinship between isolates, where the value 0.0 indicates the smallest similarity value and means the value of kinship between distant isolates, while the value of the similarity coefficient 1.00 indicates the value of similarity or kinship that is closest or identical. The value of the range of cophenetic coefficients obtained indicates a considerable degree of similarity between the isolates. According to [19] the level of genetic similarity ranging from 0.23 to 0.45 indicates that the kinship between one inbred and another is quite far away. Of these similar coefficients, isolates in one location have the highest genetic similarity of
1.00. The highest level of genetic similarity is 1= the most similar. This value indicates that the isolate used is identical.

Figure 3. Coefficient of Genetic Similarity

Figure 4. Three-dimensional appearance of 11 pathogen isolates

3.3 Genetic distance
From the profile of the band pattern and using the Jaccard coefficient among 11 ampel pathogens obtained a matrix of genetic similarity (Genetic similarity). From the matrix of genetic similarities obtained the value of genetic distance from each pair between 11 samples (Table 2). The largest genetic distance is 1 in the pair of isolates from several locations and the smallest genetic distance is 0 in some isolates from
several locations. This shows the kinship relationship between the isolates from Gg Bromo and Batu Malang is the most distant compared to the kinship relationship between the isolates from Pujon Malang and Bajeng 1, and the isolates from Pujon Malang and Bajeng 1 have the closest kinship. So the greater the value of the genetic distance, the more distant the kinship relationship. This statement is in accordance with [20] that closely related individuals will have a close genetic distance, while a close-related individual will have a long genetic distance.
**Table 2.** Genetic distance of pathogen isolates based on the appearance of DNA bands

| Location           | Batu Malang | Modoinding/Minsel | Pujon Malang | Malino | BajengI | BajengII | G.Bromo | Rurukan/Tomohon | BajengI | BajengII |
|--------------------|-------------|-------------------|--------------|--------|---------|---------|---------|-----------------|---------|---------|
| Batu Malang        | 0.00        |                   |              |        |         |         |         |                 |         |         |
| Madoinding/Minsel  | 0.67        | 0.00              |              |        |         |         |         |                 |         |         |
| Pujon Malang       | 0.50        | 0.33              | 0.00         |        |         |         |         |                 |         |         |
| Malino             | 0.83        | 0.50              | 0.67         | 0.00   |         |         |         |                 |         |         |
| Malino             | 0.50        | 0.33              | 0.00         | 0.67   | 0.00    |         |         |                 |         |         |
| BajengI            | 0.50        | 0.33              | 0.00         | 0.67   | 0.00    | 0.00    |         |                 |         |         |
| BajengII           | 0.75        | 0.60              | 0.80         | 0.57   | 0.80    | 0.80    | 0.00    |                 |         |         |
| G.Bromo/Probolinggo| 1.00        | 1.00              | 1.00         | 1.00   | 1.00    | 1.00    | 0.00    |                 |         |         |
| Rurukan/Tomohon    | 1.00        | 0.80              | 1.00         | 0.88   | 1.00    | 0.83    | 1.00    | 0.00            |         |         |
| BajengI            | 1.00        | 0.67              | 0.83         | 0.43   | 0.83    | 0.71    | 1.00    | 0.67            | 0.00    |         |
| BajengII           | 1.00        | 0.67              | 0.83         | 0.43   | 0.83    | 0.71    | 1.00    | 0.67            | 0.00    | 0.00    |
4. Conclusion
Based on the results of DNA isolation obtained dna with varying quality because it has a varying thickness, analysis of gene diversity with UPGMA obtained genetic diversity by forming 3 groups, one group that has a high kinship value consisting of three locations, the greater the value of the genetic distance then the relationship of kinship is further.

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