Molecular identification of three entomopathogenic fungi infecting the brown plant hopper pest in Indonesia

Endang Warih Minarni *, Loekas Soesanto, Agus Suyanto and Rostaman

Abstract

Background: Brown plant hopper (Nilaparvata lugens Stal.) is a very damaging pest to rice crops. One of the efforts to control it is the use of entomopathogenic fungi (EPF). Three fungal local isolates found in Indonesia were effective in controlling the brown plant hopper pest. This study aimed to molecularly identify the 3 fungal isolates. Molecular identification is very important to get the exact identity of these fungi. The accuracy of EPF identification will greatly determine the success of control. Molecular identification is based on a partial genetic analysis of the internal transcribed spacer (ITS) locus of ribosomal fungal DNA.

Result: Morphology of the local isolates named J22 and J60 were identified as Paecilomyces sp., while the isolate J34 was identified as Beauveria sp. The results of molecular identification of the isolates J22 and J60 were identified as the fungi Lecanicillium saksenae and Simplicillium sp., while isolate J34 was identified as Myrothecium sp. The results of literature search showed that the 3 fungi have never been previously reported to infect the brown plant hopper.

Conclusion: In Indonesia, 3 types of EPF, namely L. saksenae, Simplicillium sp., and Myrothecium sp., were found having the potential to control the brown plant hopper pest.

Keywords: Entomopathogenic fungus, Lecanicillium saksenae, Molecular identification, Myrothecium sp., Nilaparvata lugens, Simplicillium sp., Brown plant hopper

Background

Brown planthopper (BPH) Nilaparvata lugens is a major insect pest of rice that causes 20–80% yield loss through direct and indirect damage. The typical damage caused by BPH is drying of plants as if burning (hopperburn) (Balachiranjeevi et al. 2019). BPH can also transmit grassy stunt and ragged stunt viruses (Helina et al. 2019).

The frequency of BPH infestation is increasing frequently in developing Asian countries due to the killing of its natural enemies because of the use of synthetic chemical insecticides (Minarni et al. 2018). Entomopathogenic fungi (EPF) are fungi that can infect and kill insects (Litwin et al. 2020). The EPF that have been widely researched and known to be effective for controlling BPH pests are B. bassiana (Sumikarsih et al. 2019) and Metarhizium sp. (Chinniah et al. 2016). However, in their implementation in the field, the use of EPF to control BPH pests still has many weaknesses. After application in the field, insect pathogens are exposed to various abiotic stresses such as temperature and humidity (Hsia et al. 2014), UV radiation (Shafighi et al. 2014), and edaphic factors (Klingen et al. 2015).

In addition to biotic stress, the effectiveness of EPF in controlling insect pests is influenced by the diversity of varieties or strains or types of them. EPF have large genetic variations among different isolates. The pathogenicity, virulence, enzymatic characteristics, and DNA also varied among different isolates of different insects. The origin of the isolate affects the virulence diversity of the
fungus against the host insect, due to the type or race or strain of the fungus (Chen et al. 2017a, b).

The results of previous studies have reported 3 effective fungal isolates to control the brown plant hopper pest. The 3 isolates caused 70–80% mortality within 3.43–4.87 days. The 3 isolates were Pasir Kulon (J22), Cipete (J34), and Papringan (J60). According to morphological characteristics, isolates J22 (Pasir Kulon) and J60 (Papringan) were identified as Paecilomyces sp., while J34 (Cipete) isolate was identified as Beauveria sp. (Minarni et al. 2020).

Accuracy of identification is very important in the use of EPF for insect pest control. Identification based on morphological characters cannot be used to distinguish fungi to the species level so it is necessary to identify them molecularly (Imoulan et al. 2017). This research aimed to precisely identify the 3 previously mentioned EPF isolates that attack the brown plant hoppers.

Methods

Identification process

Fungal isolates J22 (Pasir Kulon), J34 (Cipete), and J60 (Papringan) were identified molecularly based on a partial genetic analysis on the internal transcribed spacer (ITS) locus of ribosomal DNA of fungi. Fungal isolates that will be identified previously were grown in potato dextrose broth (PDB) liquid media. After being incubated for 72 h, the fungal mycelia were harvested, using sterile filter paper and washed with sterile distilled water. The fungal mycelia were crushed in a sterile mortar by a sterile grinder and liquid nitrogen was added. Half a gram of dry fungal biomass was transferred to a 1.5-ml micro-tube containing 600 μl of cetyl trimethylammonium bromide (CTAB) buffer solution. Afterwards, the tube was shaken out and incubated at 65 °C for 30 min, then incubated in ice for 5 min. A mixture of chloroform and isoamyl alcohol with a ratio of 24:1 of 600 μl was added to the tube. The tubes were then centrifuged at 4 °C for 10 min at a speed of 25,000×g. The supernatant was transferred to a new tube and added with 0.1× volume of 2M NaOAc pH 5.2 and 3× volume of ethanol then incubated at −20 °C for 2 h.

Fungal DNA pellets were obtained by centrifugation at 25,000×g at 4 °C for 5 min. The fungal DNA pellets were dried in an airtight chamber for 5 min, then dissolved in 0.2× volume of RNase and 30 μl of sterile TE (TrisHCl 10 mM, pH 7.4, EDTA 1 mM) buffer and then incubated at 37 °C for 10 min and 70 °C for 10 min.

Extraction of fungal DNA was done using Nucleon PhytoPure reagent kit (Amersham LIFE SCIENCE, USA). PCR amplification was at ITS, using ITS Primer 4: 5′-TCC TCC GCT TAT TGA TAT GC-3′ and ITS Primer 5: 5′-GGA AGT AAA AGT CGT AAC AAG G-3′ (White et al. 1990). DNA amplification was carried out by making a volume of 30 μl containing 10.5 μl of alkaline free water, 15 μl 2× PCR mastermix (Promega), 0.75 μl and 10 pmol respectively of primer ITS 4 and ITS 5 and 3 μl (about 250 ng/μl) DNA template. The amplification reaction was carried out in 35 cycles as follows: pre-denaturation at 95 °C for 15 min, denaturation at 95 °C for 30 min, heating (annealing) at 55 °C for 30 s, lengthening at 72 °C for 1.5 min, re-extension at 72 °C for 5 min. and lastly stored at 25 °C for 10 min.

Purification of PCR products was carried out by using Polyethylen Glycol (PEG) precipitation method (Hiraishi et al. 1995) and continued with a sequencing cycle. The results of sequencing cycle were purified again, using the ethanol purification method. Analysis of nitrogen base sequence readings was done using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The raw data resulting from the sequencing was then trimmed and assembled, using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence data that was assembled was then carried out in BLAST with genomic data that was registered at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to determine taxon or species that have the greatest homology/similarity and molecularly.

Results

Morphological identification

Fungal isolates, isolated from brown plant hoppers, were infected by EPF. Fungi were purified and cultured on potato dextrose agar (PDA) media. The results of the observation on morphological characteristics, the isolates J22 (Pasir Kulon) and J60 (Papringan) were identified as

| Isolate | Color and shape of the colony | Conidial form | Conidial color | Genus | References |
|---------|-------------------------------|---------------|----------------|-------|------------|
| J22 and J60 | Round, flat, white which then turns to be creamy in old age | fusiform, sometimes cylindrical, and smooth walled | Hyaline | Paecilomyces (Figs 1 and 3) | Dong et al. (2016), Nguyen et al. (2017) |
| J34 | White, the edges are pale yellow and the base color is white, round shape, and widened growth | oval slightly rounded, stick to the ends and sides of the conidiophores (branches), have long crossed hyphae, and conidial growth clustered. | Hyaline | Beauveria (Fig. 2) | Rosmini and Lasmini (2010), Nuraida and Hasyim (2009) |
Paecilomyces sp. while J34 (Cipete) isolate was identified as Beauveria sp. (Minarni et al. 2020). The morphological characters of each EPF isolate (J22, J34 and J60) are presented in (Table 1 and Figs. 1, 2, and 3).

Molecular identification
The results of the ITS rDNA sequencing of fungal isolates J22, J34, and J60 are as follows:

1. ITS rDNA isolate sequence
   (a) Pasir Kulon_ITS4

   |   |   |   |   |   |   |
|---|---|---|---|---|---|
| 1 | TCAGT | CCAGG | CCG | CACGTC | GATGGG |
| 51 | GGTGCGAG | GCTAG | GCT | GTTAGC | GCCAC |
| 101 | GATTTTGA | CAGCCGGA | GTAAGTCC | GGAGTAC | ATTC |
| 151 | AGGAAATC | AGGGAAGT | AGG | ACAAGC | CTAAGA |
| 201 | GTGGCGGG | GCAGGTTG | AAC | ACCACG | CAGAG |
| 251 | AATCCACA | ACTATAG | CCAG | CAAGCC | TAGCC |
| 301 | CAAGAAGA | GTGTTGTA | AAAG | AACTCA | GTCAA |
| 351 | GATTCCA | GATCCTA | CCG | CCGCTT | CAGGA |
| 401 | GCTCGAGT | CCGCGG | TAGG | CCAAGA | CCAAG |
| 451 | GTCGCC | CGAAA | GCA | ATCATC | CTTG |
| 501 | AAACCTCT | TAAGATCCC | GTG | TGTCGTTT | CAG |

(b) Pasir Kulon_ITS5

|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 1 | GTTGC | CTGG | GCCGATC | CAGCTG | CCAG |
| 51 | GGCAGG | CGGCCG | CGAAG | CAGTCG | GAC |
| 101 | AGTATCTT | CTGGG | GTTTCGG | GAG | CAGCCAA |
| 151 | AAACAGG | TAAGAAG | AAAG | ACTCAG | CTC |
| 201 | TAAGAAAT | GCTTAG | GAAG | CAGTGGA | GGCT |
| 251 | CTTGTCG | CCAG | GAG | CAGTGGA | GCC |
| 501 | AAACCTCT | TAAGATCCC | GTG | TGTCGTTT | CAG |

(c) Contig-PasirKulon

|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 1 | GTAACAG | GTAG | GACCA | GAGGTAC | TT |
| 51 | CAAACT | CCAA | GAG | ACATACAT | TA |
| 101 | GGCAGG | CGGCCG | CGAAG | CAGTCG | GAC |
| 151 | CAGCAG | GCAG | AAAA | ATCAG | TA |
| 201 | GCCCAAG | GCAG | AAAA | ATCAG | TT |
| 251 | TGTGGCA | TCAG | GATGAAG | ATC | GAGAA |
| 301 | CAGAAT | TCAG | GAG | ATCAG | TA |
| 351 | ATCCGTC | GGGG | GTGGG | CAG | CAG |
| 401 | TTTGGGAA | TCAAAG | AGT | TAAACAA | CT |
| 451 | TTGGGA | TCG | GAAGT | CTG | GAG |
| 501 | CCGGAACC | AT | AAACA | AAACAA | TA |
| 551 | GACCTCG | CCAG | GTGAAGT | CTG | GAG |

(d) Cipete_ITS4

|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 1 | CTGGGCGG | GCAG | CACAA | ATC | CTG |
| 51 | AGGGAGG | GAAG | CGCCGAC | TAG | GCC |
| 101 | CGGCCGAC | GAG | CACAA | ATC | CTG |
| 151 | CCGGG | CGG | AAAA | AAACAA | TA |
| 201 | CTTGGG | CTA | GAG | ATCAG | CTG |
| 251 | ACTTAA | TTCG | GAAGT | CTG | GAG |
| 501 | GACCTCG | CCAG | GTGAAGT | CTG | GAG |

(Molecular identification (Continued))
### Molecular identification (Continued)

| 151 | AAATGACC | CAGACAGG | TGCCCGG | GATATGG | GCCGCAAT |
|-----|----------|----------|---------|---------|----------|
| 201 | GCGTCAAG | ATTCGATG | TCACGTAAT | CTTGCAATT | CATTACTT |
| 251 | CGCATTCGC | TGCGTCTCT | ATCGATGCC | GAACCAAG | ATTCGATGC |
| 301 | GAAAGTTG | ATTATTTT | AAAAAGCA | CATGAGAT | ATGAGAAT |
| 351 | AAGAGTTG | CTGCCCG | GCCTGCCG | GAAAGTG | ATCAGTAAG |
| 401 | CGGGGGGCC | ATGCCGAGA | GTCTCGCA | ATCGAGCA | AGGTGAGA |
| 451 | GTTGTTAAA | CTGGTATG | TGCTCCGA | ATCAGTAAG | AGGTGAGA |

(e) Cipete_ITS5

| 1 | TCGTGTGC | TGGCGGATC | CGGCGCCGC | CAGAATCC | CCGGATCC |
|----|----------|------------|-----------|----------|----------|
| 51 | GGGCGGC | GGGCGGC | CTGGCGGC | CAGAATCC | CCGGATCC |
| 101 | CTTTTTA | CAAT | AATAA | TAAA | AATAA |
| 151 | CATCGA | GAACGCA | ATGG | CTGG | CTGG |
| 201 | TGATGAA | ATC | GAATC | AATC | AATC |
| 251 | CGGGCGAT | GTCTCGA | CGGCGG | CTGG | CTGG |
| 301 | GGTGTTG | GC | CCACTG | CCACTG | CCACTG |
| 351 | AGTGACCG | GT | GTAGAT | GTAGAT | GTAGAT |
| 401 | GGAGGAGG | GC | CAGCTCG | CAGCTCG | CAGCTCG |
| 451 | TGAGATC | GAAG | TAGAATC | TAGAATC | TAGAATC |

(f) Contig-Cipete

| 1 | TCCGTGTC | AACCAG | GGGATCAT | CCGATTT | AACTCC |
|----|----------|--------|----------|---------|--------|
| 51 | CCCATG | CAGACCTTCA | GGGATCATG | CCGACGC | GGGACCTTCA |
| 101 | TCGGTGCG | GGATCAG | GGGCGGC | GGACCTTA | TCTGTTCGG |

(h) Papringan_ITS5

| 1 | TAGTTGGG | TTTTACGG | TGGCGCGT | GATTTCC | GTGCAGGG |
|----|----------|------------|----------|---------|----------|
| 51 | AGTTACGA | CAGAGGGC | ATCGAAGG | CGCAAGC | ATCGAAGG |
| 101 | GGGCGGCC | GGGCGGCC | GGGCGGCC | GGGCGGCC | GGGCGGCC |
| 151 | GGGTCGTT | CC | TGGCAGA | TGGCAGA | TGGCAGA |
| 201 | CAGAGCGG | TA | GAGAGTTG | GAGAGTTG | GAGAGTTG |
| 251 | ATTACGCT | TG | ATCGATT | ATCGATT | ATCGATT |
| 301 | TACGACG | CA | AGTTGAG | AGTTGAG | AGTTGAG |
| 351 | AGGGACG | GG | CTAGCCGT | CTAGCCGT | CTAGCCGT |
| 401 | TACTAGAC | AC | GCTGGG | GCTGGG | GCTGGG |
| 451 | TCACCAGG | ACGG | TGGG | TGGG | TGGG |

| 501 | TCCGCCAACC | TT | TGTACG | TGTACG | TGTACG |

| 551 | CTGAA |
**(i) Contig-Papringan**

|     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|
| 1   | TCGGTTG | GACGCT | TGGCATC | TCAGTGT | TCAAATC | CCAGC |
| 251 | CGGGGTTA | GTCGGT | GTCGGGT | GTCGGG | GTCGGG | GTCGGG |
| 301 | TGGGGGCA | GTTGGT | GTCGGG | GTCGGG | GTCGGG | GTCGGG |
| 351 | GAAATTC | ACAATC | ACAATC | ACAATC | ACAATC | ACAATC |
| 401 | GCCGACGG | GACGCG | GACGCG | GACGCG | GACGCG | GACGCG |
| 451 | GACCTTCA | CTCATC | CTCATC | CTCATC | CTCATC | CTCATC |

**Discussion**

Based on the results of the sequences, isolate J22 showed (99.83%) similarity to the *L. saksenae* strains GFRS14 and *L. saksenae* isolate Ecu121. Isolate J35 had a similarity with the sequences *Myrothecium* sp. F129 and *Myrothecium* sp. 1 TMS-2011 amounted to 98.82 and 98.93%, while isolate J60 had 99.10% similarities to the sequence *Simplicillium* sp. LCM 845.01 and 98.92% with *Simplicillium* sp. KYK00024 sequence (Table 2).

EPF isolates that showed high phylogenetic relationship and had a similarity value of 28S rDNA sequence of more than 99% with the reference species that could be expressed as one species. Ribosomal DNA sequences are used to identify and determine the phylogenetic relationships of organisms to taxa species (Bich et al. 2021). Based on the concept of phylogenetic species, it is stated that an organism is in one species when the difference in DNA sequences is between 0.2 and 1% (Shenoy et al. 2007). According to Henry et al. (2000) isolates, which have a similarity value of 100% can be stated as the same strain and a similarity value of 99% is stated as the same species, while the similarity value of 89–99% belongs to the same genus.

The similarity between 99 and 100% indicated that isolates J22, J34, and J60 each had the same chromosome number, genome size, and gene function as *L. saksenae* strain GFRS14 and *L. saksenae* strains isolate Ecu121, *Myrothecium* sp. F129, and *Myrothecium* sp. 1 TMS-2011 and *Simplicillium* sp. LCM 845.01 and *Simplicillium* sp. KYK00024, respectively.

The identification results based on morphological characters turned out to be different from molecular identification. Accuracy of identification is very important in the use of EPF for insect pest control. Identification based on morphological characters cannot be used as a definite reference. The genera *Lecanicillium, Simplicillium, Beauveria,* and *Isaria* have similar morphological characters, so that molecular identification is needed to determine the species certainty of EPF found in Banyumas Regency, Central Java Province, Indonesia. According to Lim et al. (2014) of the genus *Lecanicillium, Simplicillium* (both previously *Verticillium* spp.), *Beauveria* and *Isaria* belong to family Cordycipitaceae. According to Chen et al. (2016), the genus *Myrothecium* belongs to family Stachybotryaceae and has a worldwide distribution. Species in this genus were previously classified based on the asexual morphology, especially the characters of conidia and conidiophores. Morphology-based identification alone is imprecise because there are few characters to distinguish between species in the genus and, therefore, molecular sequence data are important in species identification.
Fig. 1  

a  Colony of 8 days old Pasir Kulon (J22) isolate.  
b  Paecilomyces sp. conidia (Minarni et al. 2020).  
c  Paecilomyces lilacinus conidia (Dong et al. 2016)

Fig. 2  

a  Colony of 8 days old Cipete (J34) isolate.  
b  Beauveria sp. conidia (Minarni et al. 2020).  
c  Beauveria bassiana conidia (Nuraida and Hasyim 2009)

Fig. 3  

a  Pure cultures of 8 days old Papringan isolate (J60).  
b  Paecilomyces sp. conidia (Minarni et al. 2020).  
c  Paecilomyces javanicus conidia (Dong et al. 2016)
Table 2 Results of the nearest fungi taxon BLAST homology ITS1, 5.8S, and ITS2 of rDNA in NCBI (https://www.ncbi.nlm.nih.gov/)

| Isolate                  | Type                              | No accession / host | DNAsize (bp) | Similarity percentage (%) | Query coverage (%) | Totalscore |
|--------------------------|-----------------------------------|---------------------|--------------|---------------------------|--------------------|------------|
| J22 (Pasir Kujon); No. accession/host/size, MWS31463/Nilaparvata lugens Stal/585 bp | Lecanicillium saksenae strain GFRS14 | MT447482/Lycium barbarum L | 585          | 99.83                     | 100                | 1075       |
|                          | Lecanicillium saksenae isolate Ecu121 | KF472156/Coccoloba uvifera | 585          | 99.83                     | 100                | 1073       |
| J34 (Cipete); No. accession/host/size, MWS31464/Nilaparvata lugens Stal/555 bp | Myrothecium sp. F129 | KM979797/Glycine max cultivar Monarca | 549          | 98.82                     | 100                | 549        |
|                          | Myrothecium sp. 1 TMS-2011 | HQ631058/Saccharum officinarum | 549          | 98.92                     | 100                | 549        |
| J60 (Papringan); No. accession/host/size, MWS31465/Nilaparvata lugens Stal/558 bp | Simplicillium sp. LCM 845.01 | MF495400/Terminalia sp. | 552          | 99.10                     | 100                | 1002       |
|                          | Simplicillium sp.KYK00024 | AB378539/Acari | 551          | 98.92                     | 100                | 996        |

Simplicillium sp. is one of the dominant genera of symbiont fungi in unfertilized brown planthopper eggs. The other 3 genera are Microdochium, Fusarium, and Cladosporium (Shentu et al. 2020). One of the species of the genus Simplicillium is S. lanosoniveum. The fungi belong to this genus are known as mycoparasites. However, silkworms (Bombyx mori) inoculated with the fungus isolate S. Lanosoniveum, died during the larval or pupal stage, as shown by the EPF, B. bassiana. The first report on the entomopathogenicity of S. lanosoniveum and demonstrated its potential for use in insect biological control was recorded by Lim et al. (2014). The fungus S. lanosoniveum was able to cause mortality of Hysteroneura setariae ticks on Plum plants by 86.33% (Chen et al. 2017a, b). Chen et al. (2019) found 3 new species, namely Simplicillium cicadellidae, S. formicidae, and S. lepidopterorum. So far, there are limited reports of the fungus Simplicillium sp. being isolated from insects infected with the fungus.

The fungus L. lecanii effectively controlled brown plant hoppers with a density of 10^{10} conidia/ml, where the mortality value of (78.33%) and a time of death at 5.81 day after treatment occurred (Khoiroh et al. 2014). L. lecanii can cause more than 50% of brown planthopper mortality within 14 days after treatment (Atta et al. 2020), whereas according to Shaikh and Pandurang (2015), this fungus is less effective in controlling this pest. Sankar and Rani (2018) have found a new Lecanicillium isolate, namely L. saksenae, which can control stink bug (Leptocorisa acuta). This fungus can kill 100% of L. acuta nymphs and imago at 72 h after treatment at conidia densities 10^{7} and 10^{8}.

Myrothecium verrucaria has a high activity against extracellular insect cuticles and produces chitinase, proteinase, and lipase (Vidhate et al. 2015).

Based on the literature search, the 3 fungi Simplicillium sp., L. saksenae, and Myrothecium sp. have never been reported to infect brown plant hopper. Data obtained from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), also showed that these 3 fungi were not obtained from insect pests (Table 2). The results of this study revealed 3 types of new EPF that had the potential to be developed as control agents for brown plant hopper pests.

Conclusion
The results of molecular identification showed that the isolates J22, J34, and J 60 were fungi from L. saksenae, Myrothecium sp., and Simplicillium sp., respectively. The results of literature search showed that these 3 fungi had never been reported to infect brown plant hopper. So that the results of this study can be considered new finding of EPF as biological agents of the control brown plant hopper pests.

Abbreviations
BPH: Brown plant hopper; BLAST: Basic Local Alignment Search Tool; CTAB: Cetyl trimethylammonium bromide; DNA: Deoxyribonucleic acid; ITS: Internal transcribed spacer; PCR: Polymerase chain reaction; PDA: Potato dextrose agar; PDB: Potato dextrose broth; PEG: Polyethilen Glycol; UV: Ultraviolet

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Authors’ contributions
EWM performed the experiments on bioassay and analyzed the data. The manuscript was prepared by EWM, LS, AS, and R. All the authors read and approved the manuscript.

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**Availability of data and materials**

All data are available in the article and the materials used in this work are of high quality and grade.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Competing interests**

The authors declare that they have no competing interests.

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