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Genetic diversity of *Metarhizium* sp. isolated from various hosts in East Dumoga, North Sulawesi, Indonesia

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Abstract. *Siahaan P, Mangais RER, Kolondam B, Tangapo A, Mambu S. 2023. Genetic diversity of* Metarhizium *sp. isolated from various hosts in East Dumoga, North Sulawesi, Indonesia. Biodiversitas 24: 6888-6896. Metarhizium* fungus is an entomopathogenic fungus known to kill many insect pests. This means that *Metarhizium* sp. has an important ecological role in the ecosystem, especially in controlling insect populations and recycling nutrients. Studying the genetic diversity of *Metarhizium* species and their relationships with insect hosts provides insight into pest management and the study of their taxonomy. The study aimed to investigate the genetic variability among *Metarhizium* fungi by examining their respective host types, which can be used as basic data for taxonomic studies, germplasm conservation efforts, and pest management. Exploration results showed that three insect species were infected with the *Metarhizium* sp fungus, namely *Scotinophara coarctata*, *Nilaparvata lugens*, and *Recilia dorsalis*. The results of a phylogenetic analysis has shown that the *S. coarctata* isolate was in the same group as four comparable *Metarhizium anisopliae* isolates from GenBank with a similarity level of 100%, while the *N. lugens* and *R. dorsalis* isolates were in the same group as four *Metarhizium huainamdangense* isolates with a similarity level. 99% and three isolates of *Metarhizium flavoviride* with 98% similarity. Molecular analysis confirmed that the *Metarhizium* isolated from *S. coarctata* was the *M. anisopliae* species, while those isolated from *N. lugens* and *R. dorsalis* were the M. *huainamdangense* species. Differences in host insects have been proven to provide genetic variation to the *Metarhizium* sp. fungus.

Keywords: Fungi, insect, *M. anisopliae*, *M. huainamdangense*, molecular analysis

INTRODUCTION

Metarhizium fungus is a type of entomopathogenic fungus known as "green muscardine fungi" because almost all species of this fungus produce green conidia (Nishi and Sato 2017). This fungus is a generalist, so it has geographic diversity and a wide host range with various morphological characteristics (Luangsa-ard et al. 2017). This fungus is known to kill many insects and has been found to infect more than 200 species in 17 different insect families (Nishi and Sato 2017; St. Leger and Wang 2020). It implies a significant role in the ecosystem for *Metarhizium*, particularly as regards insect control and nutrient recycling. (Fernández-Bravo et al. 2021). Therefore, looking at its important role, many studies have been done against these fungi, one of which is related to diversity at the genetic level.

Studying the genetic diversity of *Metarhizium* species and their relationship with host insects provided an understanding of pest management and taxonomic studies. Apart from that, studying the genetic diversity of a species is one of the conservation efforts at the genetic level because genetic diversity is variation in a population that occurs with individual genetic diversity. Through genetic conservation, the genetic variation within the population of *Metarhizium* fungus can be preserved. This is crucial for maintaining the fungus's ability to adapt to the environment and its success in controlling various types of pests. Genetic diversity can be used to observe relationships between species by constructing a phylogenetic tree (Kolondam 2015). So far, the identification has been conducted based on traditional methods by looking at the morphological characteristics, both macroscopic and microscopic. However, morphological characters are considered inadequate, because information about species diversity within one genus is incomplete (Imoulan et al. 2017). In response to these problems, molecular analysis is important in species identification efforts. Lately, molecular techniques have been employed in the examination of the fungus *Metarhizium*, with several studies focused on genetic diversity at a local scale (Iwanicki et al. 2019), genetic diversity of isolates in distant locations (Francis 2019), fungal isolates originating from of closely related species (Luan et al. 2013), and isolates originating from agricultural areas (Hernández-Domínguez and Guzmán-Franco 2017). This research focuses on the genetic diversity of fungi isolates of the same agricultural land and different host insects.

Research has been conducted on the diversity of *Metarhizium* genes, such as the genetic diversity of fungi in several biomes in Brazil (Botelho et al. 2019), isolated from insects in Japan (Nishi and Sato 2017), genetic diversity in New Zealand (Glare et al. 2021), isolated from agricultural and natural habitat subsites (Inglis et al. 2019) Isolated from the rice fields of Thua Thien Hue, Vietnam

(Cuong et al. 2022). In Indonesia, research on the genetics of *Metarhizium* spp. has been conducted, including genetic comparisons of fungi from Indonesia and the Philippines (Sanjaya et al. 2022) and genetics of new entomopathogenic fungi from soil (Herlinda et al. 2020). This research provides an overview and comparison of the related genetic diversity in a region of origin of the *Metarhizium* fungus isolate.

East Dumoga District, one of the rice-producing areas in North Sulawesi (Diansari et al. 2023), has experienced several types of rice plant pest attacks; this pest has entomopathogenic natural enemies (Siahaan et al. 2020). This makes the *Metarhizium* fungus one of the entomopathogenic fungi that control insect populations and can also be found in this region. Identification of the *Metarhizum* fungus isolated from the insect *N. lugens* at this location was conducted by Wongkar et al. (2022) previously. Genetic diversity of *Metarhizium* fungi from different insect hosts has been widely carried out, however, the genetic diversity of *Metarhizium* spp. isolated from different insect hosts in the Dumoga area have never been researched or studied scientifically. Therefore, this research was conducted to study genetic diversity based on host type, which can be used as basic data for taxonomic studies, germplasm conservation efforts, and pest management.

MATERIALS AND METHODS

Study area

This research was conducted from May to July 2023. The fungus of *Metarhizium* sp. was explored in rice plantations in East Dumoga Sub-district, Bolaang Mongondow District, North Sulawesi Province, Indonesia (Figure 1). Isolation stages and genetic analysis were conducted at the Laboratory Advanced in the Department of Biology, Universitas Sam Ratulangi, Manado, Indonesia.

Procedure

Metarhizium fungus exploration

Exploration was conducted in the rice fields of the East Dumoga area, Bolaang Mongondow Regency and three observation stations were selected. At each station there are 10 quadrants measuring 1x1 meter and placed randomly. Sampling was conducted by hand on all types of insects infected with the fungus *Metarhizium* sp. Infected insects were characterized by bodies covered with greenish mycelium and conidia or fungal spores. Each infected insect is put into a one test tube previously sterilized for each of the same species and then covered with cotton. The collected specimens are then taken to the laboratory for isolation and molecular analysis.

Isolation of fungi on PDA media

The fungi isolation process was initiated by sterilizing the insects (infected with fungus) using 70% ethanol for 3 minutes. Following that, the insect specimens were washed with sterile water and then dried on sterile filter paper. Next, the insect specimen was placed in a petri dish with wet tissue (to maintain humidity) and then incubated to trigger the growth of the fungus. After the fungus grew on the body of the insect sample, it was inoculated and cultured on Potato dextrose agar (PDA) media using the quadrant method. Incubation is conducted for approximately 7 days at 23-26° C (Wang et al. 2020).

DNA extraction

Total DNA extraction from fungus isolates was conducted by taking 0.5 g of fungal mycelium to be processed using a Plant Genomic DNA Mini Kit (Geneaid). All isolates were extracted using standard procedure according to the manual. The total DNA in the elution buffer was stored at -10° C before the Polymerase Chain Reaction (PCR) process.

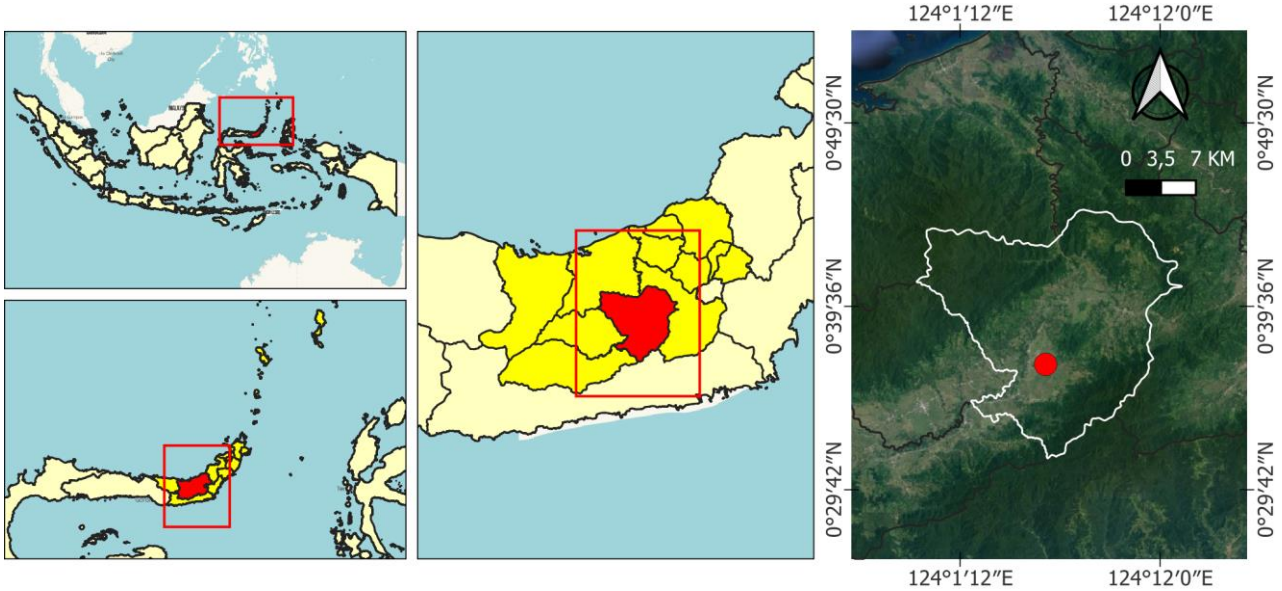

Figure 1. *Metarhizium* sp. fungi exploration in East Dumoga Sub-district, Bolaang Mongondow District, North Sulawesi Province, Indonesia (red dot: 0° 36'29.5"N 124° 05'49.3"E)

DNA amplification using Internal Transcribed Spacer (ITS) primers

The DNA amplification was conducted by a T-Personal (Biometra) thermocycler using MyTaq HS Red Mix (Bioline) and universal ITS primers (Integrated DNA Technologies, Singapore). Primers used for the amplification were based on Ashraf et al. (2022), such as ITS1 as forward primer and ITS4 as reverse primer. In each 40 µL PCR mix contained 1.5 pmol of each primer. The thermocycler setting was started by initial denaturation (3 minutes) continued with 35 cycles of denaturation, primer annealing, and DNA elongation for 20 seconds.

Electrophoresis and visualization of amplification results

The DNA from PCR amplification was separated using 0.8% agarose gel electrophoresis. The 10 µL of the PCR product were added to each well. The gel was electrified by 100 Volts for 30 minutes. Furthermore, this gen was stained in ethidium bromide and visualized with a UV-Transilluminator. The existence of single DNA bands in the around 600 bp (base pairs) indicated the success of the PCR process.

DNA sequencing and molecular identification

The sequencing process was conducted by First Base CO (Malaysia) using the PCR product and primers. The chromatograms from the sequencing were visualized and edited using Geneious v5.6.4 software. The DNA sequences for DNA editing were analyzed using Basic Local Alignment Search Tool (BLAST) available at the NCBI database.

Genetic distance analysis and phylogenetic tree constructed

Genetic distance analysis this was done to see the genetic distance between species using MEGA X software. Phylogenetic tree constructed using MEGA X software to see the level of relationship.

RESULTS AND DISCUSSION

Exploration of *Metarhizium* isolates

Explorations conducted in the rice fields of East Dumoga District obtained three species of insect pests infected with the entomopathogenic fungus *Metarhizium* sp., each infecting 29 individuals *Recilia dorsalis*, 21 individuals *Nilaparvata lugens* and 16 individuals *Scotinophara coarctata*. The host insects that were found were infected with the fungus *Metarhizium* sp., namely *Scotinophara coarctata* coded RMSC, *Nilaparvata lugens* coded RMNL and *Recilia dorsalis* coded RMRD (Figure 2.A), so there were three isolates of the fungus *Metarhizium* sp.

Metarhizium sp. RMSC isolates grown on PDA media had a round colony shape, filamentous edges, raised elevations, and gray-green upper surface color. The colonies of the RMNL and the RMRD isolates have similarities, where macroscopically, both isolates have round colonies with rhizoid-shaped colony edges, have raised colony elevations, with the color of the colonies seen from the top surface is dark green (Figure 2.B). RMSC conidia are cylindrical in shape and hyaline in color, while RMNL and RMRD conidia have an ellipsoid shape with a hyaline color (Figure 2.C). From the results of observing the colonies on the growth media and the shape of the conidia, it can be concluded that the three isolates found were the fungus *Metarhizium* sp. after being compared with mushroom identification books (Barnett and Hunter 1972).

Molecular analysis

DNA amplification results

PCR results on the three isolates using universal primers ITS1 and ITS4 showed very good results; the amplified DNA bands matched the target in the 500-700 bp (Figure 3). The research results of Sepúlveda et al. (2016) revealed six isolates of *Metarhizium* spp. shows one amplified DNA fragment band with a length of around 550 bp using primers ITS1 and ITS4. This is supported by Beeck et al. (2014) who stated that usually the length of the ITS region is in the range of 500 to 600 base pairs.

The resulting electrophorogram results (Figure 3) showed that the three isolates were successfully amplified, and two of the three isolates (RMRD, RMSC) were strongly amplified, as indicated by clearly visible DNA bands. This indicates that the ITS primer could bind effectively at locations homologous to the ITS primer. The RMNL isolate is not clearly implicated but can be used for DNA sequencing.

DNA sequencing results

The edited DNA sequencing results were then converted into FASTA form (Table 1), followed by sequence matching in GenBank using BLAST. The three isolates were compared with 12 sequences taken from GenBank (Table 2).

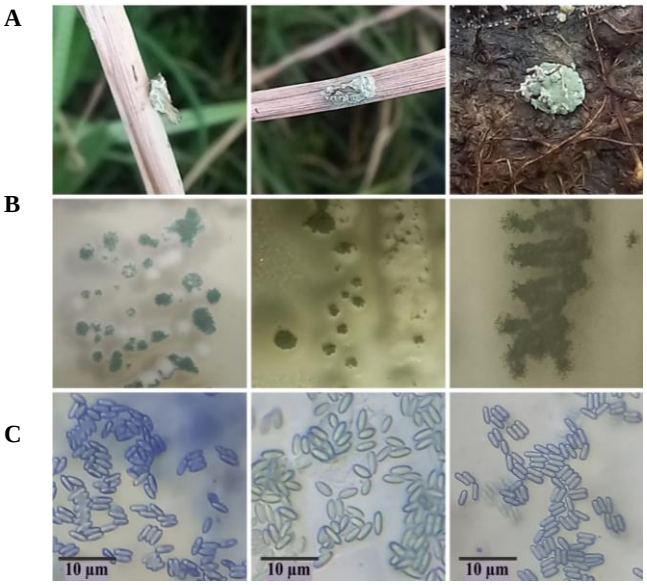

Figure 2. *Metarhizium* Fungus Exploration Results RMNL) Isolate *N. lugens*; RMRD). *R. Dorsalis* isolate; RMSC). *S. coarctata* isolate. A. Insects attacked by the *Metarhizium* fungus; B. Fungal colonies on PDA media; C. Conidia

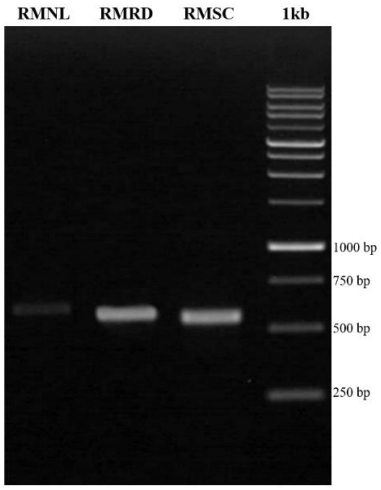

Figure 3. Electrophoregram Amplification of the Three Isolates Using ITS 1 and ITS 4

Table 2. Reference sequence information derived from GenBank

Scientific name	Location	Access number in NCBI
M. huainamdangense	Thailand	MN781898.1
M. huainamdangense	Thailand	MN781900.1
M. huainamdangense	Thailand	MN781899.1
M. flavoviride	China	AY646377.1
M. huainamdangense	Thailand	MN781901.1
M. flavoviride	Thailand	AY624203.1
M. flavoviride	China	AY646379.1
M. flavoviride	China	AY646390.1
M. anisopliae	India	JX122737.1
M. anisopliae	India	JQ766113.1
M. anisopliae	Vietnam	EU530681.1
M. anisopliae	Turkey	OP802632.1

The results of BLAST analysis and matching the sequences of the three existing isolates with the sequences of 12 isolates from Genbank (Table 2) showed that the RMSC isolates had similarities to *Metarhizium anisopliae* (JX122737.1; JQ766113.1; EU530681.1; OP802632.1) with similarity is 100% and query cover is 100% so that the RMSC isolate is identified as *M. anisopliae*. Different results were obtained for the RMNL and RMRD isolates, which were similar to four *Metarhizium huainamdangense* isolates (MN781898.1; MN781900.1; MN781899.1; MN781901.1) with 99% similarity and 100% query cover. Apart from that, these two isolates have similarities with three *Metarhizium flavoviride* isolates (AY646377.1; AY624203.1; AY646379.1) with a similarity of 98% and query cover of 91, 96 and 100% respectively. This means that the RMNL and RMRD isolates cannot be conclusively identified as *M. huainamdangense*, so they need to be confirmed through the construction of a phylogenetic tree to see the level of relationship and a comparison of the macroscopic and microscopic morphological characteristics of the two isolates (RMNL; RMRD) with the fungi *M. huainamdangense* and *M. flavoviride*.

Alignment and genetic distance of sequences for each isolate and 12 comparative sequences

The results of the alignment of the fifteen sequences (Figure 4) showed the presence of nucleotide base substitutions. Nucleotide base substitution occurs due to mutations; the mutations found were synonymous point mutations. According to Pawlak et al. (2023), this mutation did not cause a change in the amino acid it codes for. This happened because a type of amino acid had several variations in the codon code. Hence, the possibility of a mutation occurring at the third position of the codon was very low to change the amino acid it represents.

Table 1. Nucleotide base sequence results from the three samples

No	Sample	Nucleotide base sequence
1	RMNL	>RMNL TTCGGCGGGTTTCGCCCCGGGACAGGCTCGTTAGGGCCTGCCCGGAACCAGGCGCCCGCCGGAGGACCCCGATAAACTCTGTATTTTT TTTTTACTTTATGGCATGTCTGAGTGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAAC GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCTGTGGTTTGGTGTTGGGGACCGGCGATAGTGCTCTGCTCCGGCAGGCG CACGCCGCCCCCGAAATGAATTGGCGGTCTCGTCGCGGCCTCCTCTGCGTAGTAGCACAAAACCTCGCAACAGGAGCGCGGCGCGGCC ACTGCCGTAAAACGCCCCAACTTCTCTTAGAGT
2	RMRD	>RMRD GAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACTTATACCATTTTTACCGTTGCTTCGGCGGGTTTCGCCCCGGGACA GGCTCGTTAGGGCCTGCCCGGAACCAGGCGCCCGCCGGAGGACCCCGATAAACTCTGTATTTTTTTTTTACTTTATGGCATGTCTGAG TGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATT TCAACCCTCAAGCCCCTGTGGTTTGGTGTTGGGGACCGGCGATAGTGCTCTGCTCCGGCAGGCGCACGCCGCCCCCGAAATGAATTGG CGGTCTCGTCGCGGCCTCCTCTGCGTAGTAGCACAAAACCTCGCAACAGGAGCGCGGCGCGGCCACTGCCGTAAAACGCCCCAACTTC TCTTAGAGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAA
3	RMSC	>RMSC CCCAACCCCTGTGAATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTTAATA AGTATCTTCTGAGTGGTTTAAAAAAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGCGGGCATGC CTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTT AAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAA CCCCCCAACTTTTTATAGT

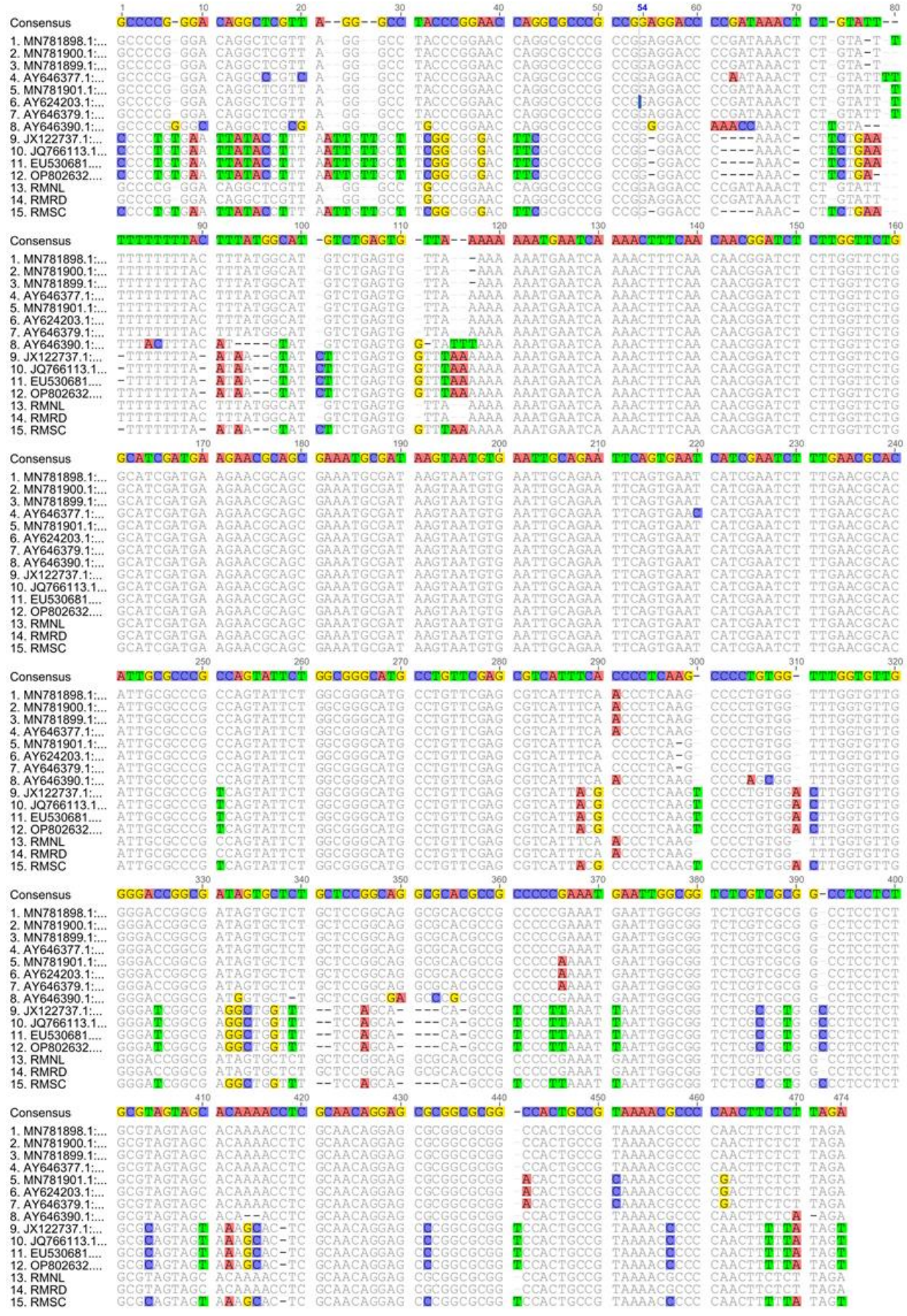

Figure 4. Sequence Alignment of Each Isolate and 12 Sequences from GenBank

Nucleotide base substitution estimates are shown in Table 3, with transition substitution rates in bold and transversion substitution rates in italics. The highest transition substitution occurred in Cytosine and Thymine bases (14.4701%), while the lowest was in substituting adenine with guanine. The highest transversion substitutions were for cytosine and adenine and cytosine and guanine at 6.6049% each, while the lowest was for substitutions for adenine and thymine and adenine and cytosine at 5.4783% each.

The results of the genetic distance calculations (Table 4) show that the genetic distance was 0.000 to 0.229. Three isolates of *M. huainamdangense* (MN781898.1; MN781900.1; MN781899.1) have a genetic distance of 0.000, which indicated that the 3 isolates were still closely related. The RMSC isolate had a genetic distance of 0.000 to 3 for *M. anisopliae* specimens (JX122737.1; JQ766113.1; EU530681.1), so they were still closely related. The RMNL and RMRD isolate themselves had the lowest genetic distance, namely 0.002, with three *M. huainamdangense* specimens (MN781898.1; MN781900.1; MN781899.1), which indicated that these 5 isolates were still closely related. The highest genetic distance was found in the *M. flavoviride* isolate (AY646377.1) to 4 specimens (JX122737.1; JQ766113.1; EU530681.1; RMSC), namely 0.229. A low genetic distance value indicated a small genetic diversity value in that group; conversely, the higher the genetic distance value, the greater the genetic diversity in that group. The high or low genetic distance showed the level of relatedness of each species; the lower the genetic distance, the more closely related each species is to other species.

Internal factors in fungi could also influence their genetic diversity. According to Gnanam (2013), two factors could influence fungal diversity, namely the process of heterokaryosis, which involves the fusion of cell nuclei originating from different individual fungi, but complete cell fusion has not yet occurred. This results in cells having several nuclei with different genetic properties. Apart from that, there were parasexual cycles, which also contributed to fungal diversity. The parasexual cycle involves genetic recombination without any clear stage of sexual reproduction. DNA fragments could be transferred between different fungal individuals in this cycle, producing new genetic variations. These cycles could produce combinations of genes different from those in the original population, enriching genetic diversity. This genetic diversity could help the *Metarhizium* fungus adapt to changing environments and provide new potential in interacting with its host.

Phylogenetic construction

A phylogenetic analysis was performed utilizing the Neighbor-Joining method. This method was applied to 3 isolates, which were then analyzed using 12 sequences taken from the GenBank database as a comparison reference, shown in Figure 5. The position of RMSC is in the same clade as 4 *M. anisopliae* isolates originating from India and Turkey; this indicates that the 4 species are closely related and monophyletic. Although RMNL and RMRD have different branches from the 7 comparison species, they are still in the same clade, and this shows that the 8 species were closely related and are monophyletic. According to Slobodian and Prastana (2020), a monophyletic group is a group of taxa with a common origin from a single ancestor. This group includes all taxa from that ancestor, including the ancestor itself. The 2 taxon groups formed in the cladogram originate from the same ancestor. The species *M. flavoviride* (AY646390.1) was in a different branch; this shows that this species was distantly related to the 2 existing taxon groups.

The RMSC isolate was in the same taxon group as the four *M. anisopliae* species with a distance of 0%, indicating a very close relationship, so it can be confirmed that the RMSC isolate is the *M. anisopliae* species. This differed from the RMNL and RMRD isolates in the same taxon group with two different species, namely *M. huainamdangense* and *M. flavoviride*. These results allow for an error in naming *M. flavoviride* to the species found because the naming of the species with access numbers AY646377.1, AY646379.1, and AY624203.1 (Table 2) is only based on the similarity of nitrogen base sequences without analysis of morphological characteristics. Genetic identification was indeed an important approach in naming species when morphological identification experiences problems; however, morphological characteristics have continued to play a decisive role in the identification of species. This follows the statement of Galan et al. (2018) that introducing morphological characteristics and genetic information was an inseparable element in all studies in the fields of biology and ecology. Therefore, these two approaches were needed for fast, accurate, and effective species identification.

The *M. huainamdangense* species is a newly discovered species and was described by Mongkolsamrit et al. (2020) described it in Thailand, which was isolated from planthopper insects. The identification conducted included morphological characteristics, both macroscopic and microscopic, and genetic analysis and obtained characteristics that differed from all *Metarhizium* species previously described. The species identified by Kwong et al. (2004) and Luangsa-Ard et al. (2005) may actually be a different species from *M. flavoviride*, but because its nitrogen base sequence was similar to *M. flavoviride*, it was given the name *M. flavoviride*.

Table 3. Maximum probability estimate of the substitution matrix

From\To	A	T	C	G
A	-	5.8149	6.6049	**13.0615**
T	5.4783	-	**14.4701**	6.1309
C	5.4783	**12.7394**	-	6.1309
G	**11.6712**	5.8149	6.6049	-

Note. Every item represents the likelihood (r) of replacing one base (row) with another base (column). The Tamura-Nei (1993) model was employed to gauge patterns and speeds of substitutions. Bold denotes the rates for specific transition substitutions, while not bold values indicate rates for transversion substitutions. The nucleotide frequencies are A = 22.80%, T/U = 24.20%, C = 27.49%, and G = 25.51%

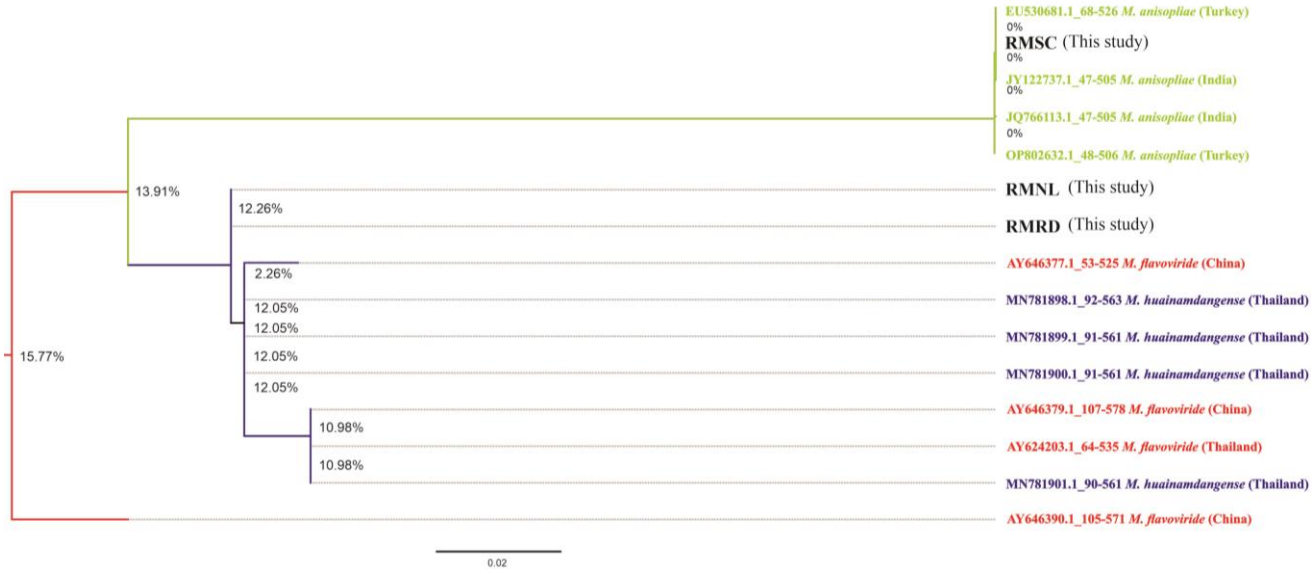

Figure 5. Cladogram of each isolate with 12 sequences from GenBank

Table 4. Calculation of evolutionary divergence values between sequences

No	Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	MN781898.1:92-563_*M. huainamdangense*														
2	MN781900.1:91-561_*M. huainamdangense*	0.00													
3	MN781899.1:91-561_*M. huainamdangense*	0.00	0.00												
4	AY646377.1:53-525_*M. flavoviride*	0.09	0.09	0.09											
5	MN781901.1:90-561_*M. huainamdangense*	0.01	0.01	0.01	0.02										
6	AY624203.1:64-535_*M. flavoviride*	0.01	0.01	0.01	0.02	0.00									
7	AY646379.1:107-578_*M. flavoviride*	0.01	0.01	0.01	0.02	0.00	0.00								
8	AY646390.1:105-571_*M. flavoviride*	0.06	0.06	0.06	0.06	0.07	0.07	0.07							
9	JX122737.1:47-505_*M. anisopliae*	0.21	0.21	0.21	0.29	0.27	0.27	0.27	0.22						
10	JQ766113.1:47-505_*M. anisopliae*	0.21	0.21	0.21	0.29	0.27	0.27	0.27	0.22	0.00					
11	EU530681.1:68-526_*M. anisopliae*	0.21	0.21	0.21	0.29	0.27	0.27	0.27	0.22	0.00	0.00				
12	OP802632.1:48-506_*M. anisopliae*	0.21	0.21	0.21	0.22	0.23	0.23	0.23	0.26	0.02	0.02	0.02			
13	RMNL	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.05	0.21	0.21	0.21	0.21		
14	RMRD	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.05	0.21	0.21	0.21	0.21	0.00	
15	RMSC	0.21	0.21	0.21	0.29	0.27	0.27	0.27	0.22	0.00	0.00	0.00	0.02	0.21	0.21

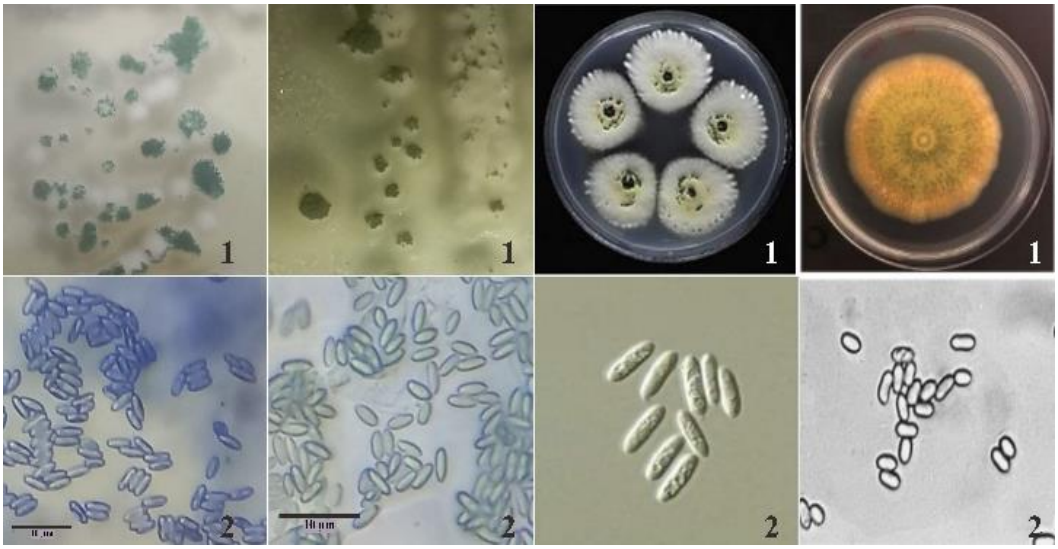

Figure 6. Comparison of the characteristics of three species of *Metarhizium* fungi. A). RMNL isolate; B) RMRD isolate; C). *M. huainamdangense* (Mongkolsamrit et al. 2020); D) *M. flavoviride* (Brunner-Mendoza et al. 2019). 1) Colony morphology on PDA media; 2) Conidia

A comparison of the morphological characteristics of the isolates RMNL, RMRD, *M. flavoviride*, and *M. huainamdangense* is shown in Figure 6. The appearance or characteristics of the colonies grown on PDA media and the shape of the conidia of the isolates RMNL and RMRD are similar to *M. huainamdangense* compared to *M. flavoviride*. Based on the *Metarhizium* identification key created by Mongkolsamrit et al. (2020) showed that *M. huainamdangense* has pale yellow to dark green colonies on PDA media and conidia are cylindrical to ellipsoid in shape, while *M. flavoviride* has yellowish-white colonies and conidia are pyriform, reniform and ovoid. Apart from morphological characteristics, the origin of the host insect is one of the factors indicating that the RMNL and RMRD isolates are *M. huainamdangense* fungi. As described by Mongkolsamrit et al. (2020), the fungus *M. huainamdangense* belongs to the *Metarhizium* fungus group, which only infects planthopper insects (Cicadellidae, Cercopidae, Delphacidae). This follows the results obtained by isolating RMNL from brown planthoppers and RMRD from zig-zag planthoppers. Comparative analysis of morphological and genetic characteristics showed that the two isolates were *M. huainamdangense* species. However, the two species found in the Dumoga area were in different branches from *M. huainamdangense* found in Thailand, but these two species came from the same ancestor based on the construction phylogenetic tree.

Molecular analysis confirmed that the *Metarhizium* fungus isolated from *S. coarctata* was the *M. anisopliae* species, while that from *N. lugens* and *R. dorsalis* was the *M. huainamdangense* species. Based on variations in sequence analysis and differences in alignment of the third isolate with 12 comparative sequences from GenBank, it showed quite high genetics with the discovery of nucleotide base substitutions. The results of the phylogenetic construction showed that the *S. coarctata* isolate was in the same group as four comparable *M. anisopliae* isolates from GenBank with a similarity level of 100%, while the *N. lugens* and *R. dorsalis* isolates were in the same group as four *M. huainamdangense* isolates with a similarity level 99%. Differences in host insects have been proven to provide genetic variation to the *Metarhizium* sp. fungus, where two species of *Metarhizium* spp fungus were found isolated from three host insects.

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