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ARTICLE

Studying, Analyzing, and Interpreting the Gut Microbiome of the Earthworm *M. peguana* (Rosa, 1890) Using Next-Generation Sequencing

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ABSTRACT

This study investigates the diversity of gut microbiota in Metaphire peguana, an earthworm species commonly found in agricultural areas of Thailand. Earthworms play a critical role in soil ecosystems by supporting nutrient cycling and breaking down organic matter. Understanding the microbial diversity in their gut is essential for exploring their ecological contributions. Using Next Generation Sequencing (NGS), we analyzed the mycobiome in the gut of *M. peguana*.

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Our findings revealed a high diversity of fungal species, primarily belonging to two major phyla: Ascomycota and Basidiomycota. Ascomycota was the most abundant phylum, comprising 40.1% of the total fungal species identified. A total of 33 distinct fungal species were identified, which underscores the richness of microbial life within the earthworm gut. This study successfully created the first genetic database of the microbial community in *M. peguana*, providing a foundation for future research in agricultural applications. The microbial species identified, particularly siderophore-producing fungi, could have significant implications for improving soil fertility and promoting sustainable agricultural practices. The use of NGS technology has enabled comprehensive profiling of microbial communities, allowing for precise identification of fungi that may play essential roles in soil health. Furthermore, the study paves the way for future studies on the potential applications of earthworm gut microbiomes in biotechnology, especially in enhancing soil nutrient availability and plant growth. The findings of this research contribute to the broader understanding of the ecological roles of earthworms and their microbiomes in soil ecosystems.

Keywords: Gut Microbiome; *Metaphire peguana*; Fungi; Earthworm; Interpreting the Gut Microbiome; Next-Generation Sequencing

1. Introduction

The scientific name for one type of annelid found in nature is *Metaphire peguana*. This species, along with other annelids, is widely distributed around the world. Metaphire peguana is typically found in environments with similar humid conditions, such as forests, parks, orchards, and other plant-rich areas. *Metaphire peguana* has the physical appearance of a large annelid, typically measuring between 15–25 centimeters in length. Its color can vary depending on the environment, but it is usually brown or black. The body is elongated, resembling a rod, with the number of rings varying depending on age and environment. To properly care for *Metaphire peguana*, it is important to maintain the appropriate environment and humidity for the annelid. This includes providing adequate watering, ensuring soil moisture, and preventing direct sunlight by using shaded areas or planting sun-blocking vegetation [1,2]. These measures help to maintain suitable conditions for *Metaphire peguana* to reproduce and thrive [3,4]. *Metaphire peguana* is a type of annelid that is widely found in Thailand. It can be found in areas with high humidity, such as forests, parks, orchards, and other vegetated areas. It thrives in Thailand's favorable climate. *Metaphire peguana*, commonly found in Thailand, typically inhabits areas with high humidity. *Metaphire peguana* may play a crucial ecological role by facilitating natural processes, such as annual soil production through the decomposition of organic material and the mobilization of soil in active areas. *Metaphire peguana* is a cosmopolitan terrestrial earthworm species that is widely spread in agricultural areas of Thailand. This species was identified in 1890 by Rosa (Rosa, 1890). It generally promoted plant growth. *M. peguana* was found in a diverse variety of microhabitats, namely dipterocarp forest, deciduous forest, anthropogenic areas, and even in wastewater-saturated soil from households [5,6]. *M. peguana* has been transported as an alien species by direct or indirect human activities [7].

In nature, *M. peguana* will move and excrete cast throughout the soil mass that makes perfect soil. The report revealed that some of the bacterial species in the earthworm gut can produce indole-3-acetic acid in high concentrations, particularly those belonging to the genus *Bacillus*. However, the culture technique cannot detect and identify all microbial species. Next-Generation Sequencing (NGS) is a biological technology used to rapidly and efficiently create DNA or RNA sequences. This technology has revolutionized sequencing by employing a more automated and effective method than the previously used Sanger sequencing technology. In this research, the next-generation sequencing was used as the primary tool for a fungal diversity study [8,9]. Next-generation sequencing is a powerful tool for classifying and analyzing microorganisms, particularly in providing information about the DNA or RNA of different microorganisms. This aids in comprehending the genetic diversity of microorganisms and analyzing various properties to gain a better understanding of the microorganism. For this reason, this technique is used to sequence all metagenomics in a short time and wildly used in many research fields, especially the study of the whole genetic

structure of individual organisms, or at the metagenome level, or environmental contamination [10]. According to a study, Wang *et al.* found that Actinobacteria, Firmicuts and Proteobacteria, these are the three dominant groups of bacteria in arsenic contaminated soil [11,12], which is absorbed throughout the gut of *Metaphire sieboldi*. Moreover, it is studied all taxonomic abundant microorganisms in central gulf of Thailand passed metagenomics analysis [13,14]. Furthermore, Koo *et al*. demonstrated that metagenomics study and gene expression could apply for studying gene expression level from microbiome in different cold ecosystem [15]. After analyzing the information provided, it can be concluded that the entire fungal structure is present in the gut of *M. peguana*.

Next-Generation Sequencing (NGS) technology is a crucial tool in scientific and medical research, enabling the rapid and detailed analysis of DNA and RNA sequences. The NGS process begins with the preparation of a DNA or RNA sample, followed by the creation of a library through the addition of adapters to the samples. This prepares the samples for sequencing in the machine. The data obtained from sequencing is then analyzed with specialized software, allowing for the detection of genetic differences, mutations, and gene expression. NGS has led to significant discoveries in genetics, disease, and developmental biology, making it a valuable tool for precision medicine and disease diagnosis in the modern era. Therefore, the main goal of this study was to utilize NGS technology to estimate the total fungal genus and species within the microbiome of *M. peguana* gut. This research will be beneficial for further investigation and identification.

2. Methodology

2.1. Collecting Earthworms and Species Identification

Earthworms were collected from the agricultural farm area of Sing Su Wan farm in Mae Tha District, Lamphun Province, Thailand (**Figure 1**). The earthworms were gathered from various locations within the farm, which had diverse agricultural plants, to obtain a sample that reflects the biodiversity of soil organisms. The earthworm samples were collected by digging into the soil at different depths and carefully separating the earthworms from the soil. The collected earthworms were then placed in containers with soil from the original site to minimize contamination by other microorganisms before further analysis. After collection, the earthworms were identified using morphological methods based on the key provided by Gates (1939) [16]. The identification process involved examining the earthworms' morphological features, such as body length, clitellum position, male and female pore locations, spermathecal pore, genital markings, prostate gland, copulatory pouch, and body color. These characteristics were observed under stereomicroscopy, which allowed for detailed and accurate analysis of the earthworm's anatomical traits. Stereomicroscopy enhances the precision of morphological analysis, making it easier to identify species accurately based on their distinctive features.

Figure 1. The Position and Collected Earthworm Area (Sing Su Wan Farm).

2.2. Metagenomic Extraction

Earthworms were collected from the soil around the agricultural areas of Sing Su Wan farm, Mae Tha District, Lamphun Province, Thailand (**Figures 2** and **3**). A total of 90 earthworm samples were collected, with 10 earthworms collected from each of 9 different agricultural areas, to capture the diversity of soil organisms across the farm. The guts of the earthworms were carefully removed and placed into sterile collection tubes. Each sample consisted of 10

mg of earthworm gut, which was then used for metagenomic DNA extraction using the Stool Microbiome DNA Kit (Invitrogen). After extraction, the quality and quantity of the isolated DNA were determined using a Nanodrop (Allsheng) spectrophotometer and by running a 1% agarose gel electrophoresis. This ensured that the DNA was of sufficient quality for further analysis. Once the DNA was confirmed to be of good quality, it was stored at −20°C to prevent degradation or contamination prior to further analysis and sequencing.

Figure 2. Earthworms.

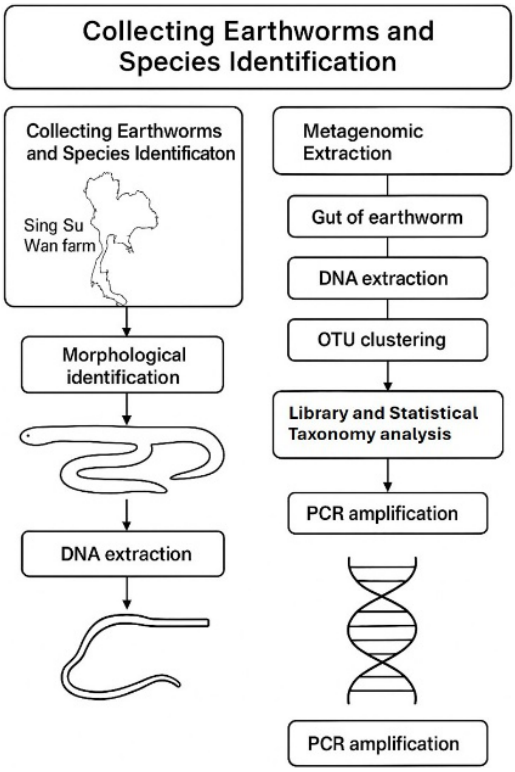


Figure 3. Collecting Earthworms and Species Identification.

2.3. Operational Taxonomic Unit (OTU) Analysis and Statistical Taxonomic Analysis

The DNA fragments were analyzed for nucleotide sequencing using the Illumina Miseq (Macrogen) with a quality control check at Q30 (99.9%) to ensure the high accuracy and reliability of the sequencing data. A Q30 score indicates that the error rate of the sequencing data is less than 1 in 1,000 bases, ensuring the quality of the results. The aligned reads were processed using the Fast Length Adjustment of Short Reads (FLASH) program, which adjusts and merges short sequencing reads to improve the quality and completeness of the alignment. The aligned raw data were then used for taxonomic clustering analysis through the Python program. The OTU output data was generated using the rDnaTool (Python) program, which processed the data to determine the operational taxonomic units (OTUs) and assess the diversity and species richness of the sample. To further explore the taxonomic diversity, the raw data were blasted against the Greengene database using the BLAST algorithm (Basic Local Alignment Search Tool). This allowed for the identification of taxonomic diversity and species richness, enabling comparisons with known reference databases. This process is essential for determining the microbial composition and diversity within the gut microbiome of the earthworms.

2.4. Library Preparation

The results from the study of the gut microbiome of *M. peguana* (Rosa, 1890) using Next-Generation Sequencing (NGS) were obtained by digesting metagenomic samples with the transposase enzyme (Macrogen) into DNA fragments (reads) and duplicating them with different adapters. For fungal species, P5 adapter (5′ TTGGTCATTTAGAGGAAGTAA 3′) and P7 adapter (5′ CGTTCTTCATCGATGC 3′) were applied to the fungal metagenome before amplifying the targeted DNA segments by PCR to generate sequence reads, as shown in **Table 1**.

Table 1. Amplicon Primer (Forward and Reverse) Sequence of *ITS*1.

	Amplicon Primer Sequence for *ITS*1
F	5′ TTGGTCATTTAGAGGAAGTAA 3′
R	5′ CGTTCTTCATCGATGC 3′

* F = forward primer, R = reverse primer.

In this study, the forward primer (F) and reverse primer (R) were used to amplify the *ITS*1 region, which is a specific sequence used to study fungi in the microbiome. The *ITS*1 sequence is crucial for differentiating various fungal species in the samples. Additionally, PCR (Polymerase Chain Reaction) was used to amplify the DNA segments of interest, which were then analyzed to obtain gene sequences for further understanding of the gut microbiome in *M. peguana* (Rosa, 1890).

2.5. PCR Conditions

The PCR conditions should be fully described, including the temperature and time settings for denaturation (94 °C for 3 min), annealing (55 °C for 30 s), and extension (72 °C for 1 min) with a typical cycle number of 30–40 rounds. The primers used for amplifying the *ITS*1 region should also be mentioned, such as the forward primer (5′ TTGGTCATTTAGAGGAAGTAA 3′) and the reverse primer (5′ CGTTCTTCATCGATGC 3′) used in this study.

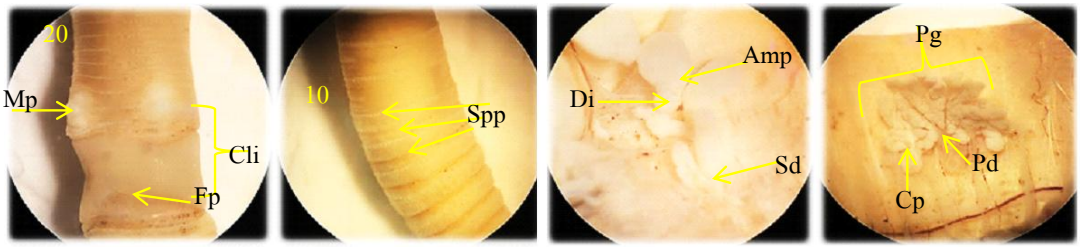
3. Results and Discussion

3.1. Classifying Earthworms and Analyzing the Microbiome Using NGS

They were all identified using morphological methods based on the key provided by Gate (1939). This research explores earthworm morphology using stereomicroscopy, including measurements of body length, clitellum position, male pore, female pore, spermathecal pore, genital marking, prostate gland, copulatory pouch, and body color. The important characteristics of the earthworms are shown in **Table 2**. This table presents the key morphological characteristics used to classify *M. peguana* earthworms based on observations under stereomicroscopy. The earthworms were examined for various traits, including body length, position of the clitellum, presence of male and female pores, spermathecal pore, genital marking, prostate gland, copulatory pouch, and body color. The abbreviations in the table represent anatomical structures, including diverticulum (Di), ampulla (Amp), spermatheca duct (Sd), and prostate duct (Pd). These characteristics were observed in a sample of 10 earthworms (N = 10), which helped in identifying the species.

Table 2. *M. peguana* Morphological Examination.

Morphological Examination	Examination Result
Body length (mm)	141.07±12.85
Clitellum position (Cli)	14-16
Male pore (Mp)	18
Female pore (Fp)	14
Spermathecal pore (Spp)	6/7/8/9
Genital Marking (GM)	17/18, 18/19
Prostate gland (Pg)	Racemose type
Copulatory pouch (Cp)	Found
Body color	Dark grey



N = 10, Di = diverticulum, Amp = ampulla, Sd = spermatheca duct, Pd = prostate duct.

The metagenomics from the gut of *M. peguana* was amplified using *ITS*1. A total of 80,421 reads were managed by FLASH program and passed at Q30 as 99.43%. The total bases for this study were 23,196,360, representing the fungal metagenomics data (**Table 3**). This table summarizes the metagenomic data analysis from the gut of *M. peguana* using the FLASH program. The total number of bases in the fungal metagenome was 23,196,360, with 80,421 reads obtained, and a Q30 score of 99.43%. The Q30 score indicates the quality of sequencing data, with Q30 representing the percentage of bases in the reads that have a probability of error less than 1 in 1000, indicating high-quality sequencing results. The use of the FLASH program ensures that the reads are properly paired and aligned for further analysis.

Table 3. Fast Length Adjustment of Short Reads (FLASH) Program Analysis Showed the Paired Score (Q30) and Read Count in *M. peguana* Metagenomics Based on the ITS1 Region Gene for Fungi.

Sample Name	Total Bases	Read Count	Q30 (%)
Gut of *M. peguana*	23,196,360	80,421	99.43

Diversity identification was conducted using Illumina MiSeq, and the statistical diversity was analyzed with the alpha_diversity.py program. Three statistical parameters were estimated (see **Table 4**), which evaluate the diversity of fungal communities in the gut of *M. peguana*. The parameters presented include: 1. OTUs (Operational Taxonomic Units): A total of 224 OTUs were identified, representing different species or genera of fungi. 2. Shannon Index: The value of 5.99815 reflects the overall diversity within the fungal community, considering both the number of different species and their even distribution. 3. Inverse Simpson Index: With a value of 0.9469, this index indicates the evenness of the fungal community; higher values signify a more even distribution of species. These results suggest a relatively high diversity and evenness of fungal populations within the gut.

Table 4. The Statistics Analysis Using alpha_diversity.py Program for Diversity Richness.

Sample Name	OTUs	Shannon	Inverse Simpson
Gut of *M. peguana*	224	5.99815	0.9469

The result indicated that the *ITS*1 region could detect the most fungal phylum in phylum Ascomycota (approximately 40.1%), followed by Basidiomycota (approximately 6.2%). However, this DNA region could not identify other eukaryotes as approximately 38.8%. These findings are displayed in **Table 5**. This table shows the relative abundance of different fungal phyla identified in the gut of *M. peguana*. The Ascomycota phylum was the most abundant, representing approximately 40.1% of the fungal community. The Basidiomycota phylum accounted for about 6.2%. However, a significant proportion of the sequences (38.8%) could not be classified into any known eukaryotic phylum, referred to as "unidentified eukaryotes" or "dark matter". This suggests the presence of unknown or unclassified organisms within the gut microbiome. Additionally, Arthropoda was detected at 1.2%, and unclassified taxa (unclassified organisms) were detected at 13.6%, showing the complexity and unexplored nature of the gut microbiome.

Table 5. The Percentage of Fungal Phylum Abundance in *M. peguana* Gut.

Phyla	*M. peguana* (%)
Unidentified eukaryote	38.8
Arthropoda	1.2
Ascomycota	40.1
Basidiomycota	6.2
Unclassified taxa	13.6

In this study, we utilized the *ITS*1 region to investigate the gut microbiome of *M. peguana* through Next-Generation Sequencing (NGS). The earthworms were identified based on morphological characteristics such as body length, clitellum position, and the location of genital pores, which were consistent with the classification by Gate (1939). The metagenomic data, comprising 80,421 reads, were processed using the FLASH program, yielding high-quality results with a Q30 score of 99.43%. The metagenomics data revealed that the dominant fungal phylum in the gut of *M. peguana* was Ascomycota, accounting for approximately 40.1%, followed by Basidiomycota at 6.2%. Interestingly, around 38.8% of the sequences did not correspond to any identifiable eukaryote, indicating the presence of unidentified or unclassified organisms, often referred to as "dark matter". This underscores the complexity and diversity of the gut microbiome, which is largely unexplored. In terms of diversity, the analysis using alpha_diversity.py showed a Shannon diversity index of 5.99815 and an Inverse Simpson index of 0.9469, indicating a relatively high richness and evenness of the fungal

communities. The identification of fungal phyla and other eukaryotic species helps in understanding the ecological role of these organisms in the gut ecosystem of *M. peguana.* These findings contribute to a deeper understanding of the fungal community composition in earthworms and the potential role of these microorganisms in the digestion and ecological functioning of the host.

The fungal species abundance from metagenomics of *Metaphire peguana* gut was analyzed using NGS technology. A total of 33 fungal species were identified. The highest frequency of fungal species was found in Fusarium equiseti, at 20.54% (**Table 6**).

Table 6. Fungal Species Analysis in *Metaphire Peguana* Gut.

Genus	**Species**	***Frequency***
Coprinopsis	*Coprinopsis sclerocystidiosus*	0.81%
Coprinopsis	*Coprinopsis subdisisseminatus*	3.49%
Saitozyma	*Saitozyma flava*	0.08%
Hortaea	*Hortaea werneckii*	0.16%
Juxtiphoma	*Juxtiphoma eupyrena*	0.38%
Aspergillus	*Aspergillus chevalieri*	0.50%
Aspergillus	*Aspergillus keratitidis*	0.06%
Aspergillus	*Aspergillus penicillioides*	0.07%
Aspergillus	*Aspergillus versicolor*	0.78%
Aspergillus	*Aspergillus violaceofuscus*	0.06%
Aspergillus	*Aspergillus tamarii*	1.29%
Aspergillus	*Aspergillus flavus*	0.08%
Penicillium	*Penicillium oxalicum*	0.10%
Penicillium	*Penicillium steckii*	0.14%
Talaromyces	*Talaromyces minioluteus*	0.09%
Candida	*Candida tropicalis*	1.01%
Meyerozyma	*Meyerozyma caribbica*	0.09%
Beauveria	*Beauveria feline*	0.09%
Cosmospora	*Cosmospora viridescens*	0.20%
Fusarium	*Fusarium delphinoides*	0.81%
Fusarium	*Fusarium equiseti*	20.54%
Fusarium	*Fusarium solani*	1.93%
Paramyrothecium	*Paramyrothecium roridum*	0.08%
Microascus	*Microascus chartarus*	0.09%
Microascus	*Microascus croci*	0.04%
Microascus	*Microascus longicollis*	0.06%
Chaetomium	*Chaetomium globosum*	0.11%
Chaetomium	*Chaetomium subglobosum*	0.05%
Ovatospora	*Ovatospora pseudomollicella*	0.41%
Thermothielavioides	*Thermothielavioides terrestris*	0.02%
Triangularia	*Triangularia longicaudata*	0.07%
Cercophora	*Cercophora striata*	0.15%
Nigrospora	*Nigrospora oryzae*	0.22%

According to fungal taxonomy, identifying species using cultural methods based on media may not show all fungal species due to a lack of suitable media. This study is the first to investigate the fungal population in the microbiome of Metaphire peguana, which is found in the soil of an agricultural area, specifically the Sing Su Wan farm. Using modern high-throughput sequencing technology (Next Generation Sequencing), we found that it is a powerful tool for estimating the complete structure of the fungal population. *Metaphire peguana* is a terrestrial earthworm belonging to the Megascolecidae family, and it can also be found in Thailand and several other countries in Asia [6,16–20]. Therefore, we surveyed and collected only *M. peguana* to study the fungal microbiome. It is the dominant species, ensuring that the soil remains lively.

The raw data indicated that the region of *ITS*1 gene can amplify the DNA segments in metagenome from *M. peguana* gut in 80,421 reads. Each of the DNA fragments can be tested against the paired score at Q30 (99.43 %). The result showed quality read passed 99.9 % at excellent accuracy. The FLASH program could also order all nucleotide and revealed the raw data through the test in 3 statistics (OTUs, Shannon, Inverse Simpson). The Inverse Simpson showed the high value as 0.9469 (maximum in 1.0). It refers to regular in each fungal species. The OTUs result estimated the fungi taxonomic abundance were high, 33 fungal species was identified (*Coprinopsis sclerocystidiosus, C. subdisisseminatus*, *Cladosporium tenuissimum, Hortaea werneckii, Juxtiphoma eupyrena, Aspergillus chevalieri*, *A. keratitidis*, *A. penicillioides*, *A. versicolor, A. violaceofuscus, A. tamari*, *A. flavus*, *Penicillium oxalicum*, *P. steckii*, *Talaromyces minioluteus*, *Candida tropicalis*, *Meyerozyma caribbica*, *Beauveria feline*, *Cosmospora viridescens*, *Fusarium delphinoides*, *F. equiseti*, *F. solani*, *Paramyrothecium roridum, Microascus chartarus*, *M. croci*, *M. longicollis, Chaetomium globosum*, *C. subglobosum*, *Ovatospora pseudomollicella*, *Thermothielavioides terrestris*, *Triangularia longicaudata*, *Cercophora striata*, *Nigrospora oryzae)*.

*ITS*1 spacer region gene is a region of general fungal gene and sometimes can amplify other eukaryotic microorganisms. For instance, an arthropod in this report was screened in a specimen at 1.2 %, which makes it hard to protect them from some insect DNA contamination, such as house fly (order Diptera). In addition, the previous studies have revealed a role for midgut microbiota during pathogen infection in mosquitoes which the microbiome in the insect midgut involved in microorganism in the fertile soil [21]. The recent study in Thailand, *Aedes albopictus* mosquitoes showed the diversity of midgut microorganism which involve the soil in each area [22]. The diversity of soil microorganisms may be influenced by the presence of different populations of *M. peguana* in each area, which is beneficial to insects and other living things that live in that ecosystem. However, it was successfully done in reaching the goal by using NGS technology for Mycobiome screening fungi community from *M. peguana*. Therefore, it is the first study showing the genetic database of fungi from *M. peguana* gut. This research indicates that fertile soil consists of many microorganisms, most of which can produce secondary metabolites such as IAA. This is the reason why soil containing terrestrial earthworms is fertile. The study explained that some fungi cultured in media could detect IAA in various species. Hence, we have summarized the total mycobiome found in the gut of *M. peguana*. The collected data is crucial for establishing a genetic database to support Thai agricultural efforts related to *M. peguana*.

3.2. Isolating Fungi from the Same Worm Sample, Culturing Them, Identifying the Species, and Creating a Phylogenetic Tree

After isolating the fungi from the intestine samples of *M. peguana*, the next step is to cultivate them in an appropriate environment to allow them to grow and expand. This can be done by culturing them on culture media. Once the fungi are fully grown, the samples can be collected for fungal species analysis using molecular techniques, which are important tools for identifying specific fungal species. After obtaining the base sequence, we can compare it with online databases, such as GenBank, to identify the fungus. Once we know the fungal species, we can create a phylogenetic tree to see the relationship between the fungi isolated from the intestine samples of *M. peguana* and other fungi using genetic analysis programs of PhyML. The phylogenetic tree showed genetic relationship of fungi isolated from *M. peguana* gut (code Mpe 1–11) using UPGMA method (**Figure 4**). This will help us understand the

evolution of this group of fungi and see the closeness of different species. From the experiment, 12 fungal groups were collected from the gut of M. peguana and identified using the *ITS*1 region, as shown in **Table 7**.

Table 7. The 12 Fungal Colonies Collected from *M. peguana* Gut and was Identified by Using *ITS*1 Region.

Codes	Phylum	Identify Species	% Similarity	Accession Number
Mpe1	Ascomycota	*Fusarium solani*	99	KX064991.1
Mpe2	Ascomycota	*Talaromyces trachyspermus*	100	KP055603.1
Mpe3	Ascomycota	*Aspergillus fumigatus*	99	JN2269656.1
Mpe4	Ascomycota	*Gonoderma* sp.	100	KR346906.1
Mpe5	Ascomycota	*Mucor irregularis*	93	MN629208.1
Mpe6	Ascomycota	*Fungal* sp.	95	MF920436.1
Mpe7	Ascomycota	*Fusarium solani*	99	KX064991.1
Mpe8	**Ascomycota**	***Asperigillus terreus***	**99**	**KM924436.1**
Mpe9	Ascomycota	*Fusarium oxysporum*	97	JN400678.1
Mpe10	Ascomycota	*Humicola fuscoatra*	98	GU183113.1
Mpe11	Ascomycota	*Asperigillus japonicum*	100	KY199566.1

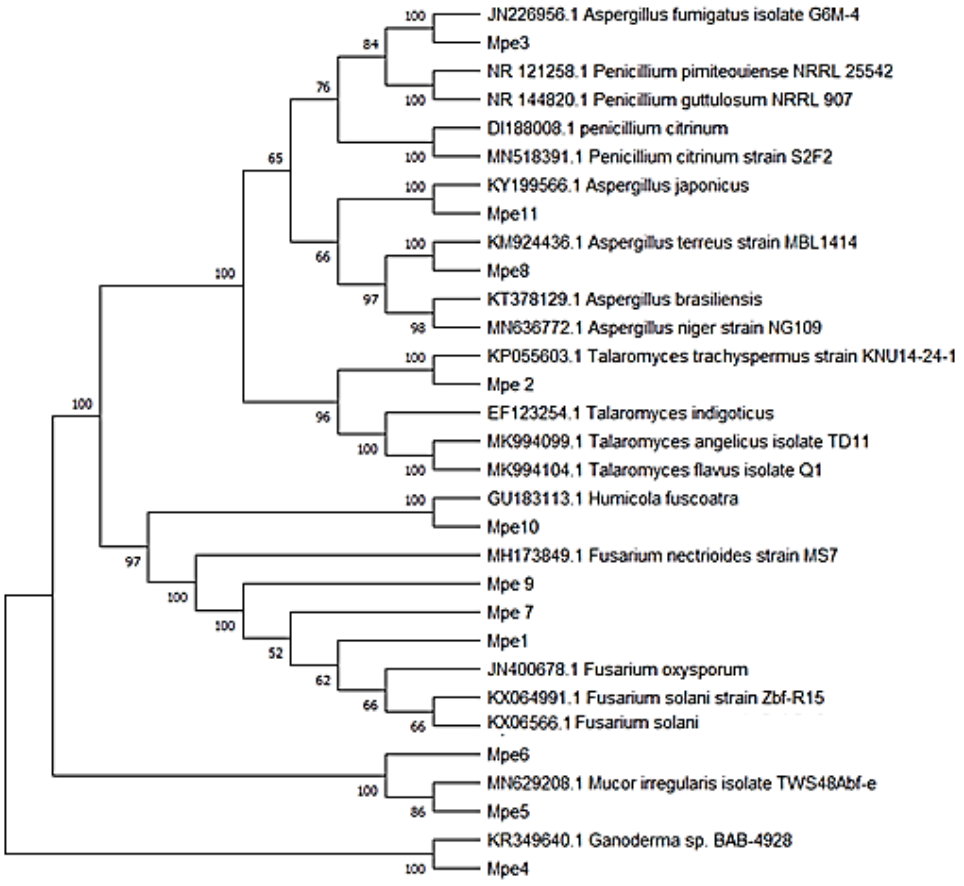


Figure 4. The Phylogenetic Tree Showing Genetic Relationship of Fungi Isolated from *M. peguana* Gut (Code Mpe 1–11) Using UPGMA Method.

Siderophore-producing fungi play a crucial role in absorbing iron, a vital nutrient for the growth of organisms. Fungi isolated from the gut of *M. peguana* may have a high potential for producing siderophores, which benefits both the fungi and the organisms in their ecosystems. The Chrome Azurol S test (CAS assay) is typically used to measure the amount of siderophore production by fungi. Chrome Azurol S is an indicator that is highly responsive to iron (Fe^{3+}) and binds iron strongly when heated [23,24]. When the fungus produces siderophores, these molecules compete with Chrome Azurol S for iron binding, causing a color change from blue to yellow-orange in the presence of iron plaques. The assay involves cultivating fungal cultures in a medium that contains the CAS reagent Chrome Azurol S, iron, and hexadecyltrimethylammonium bromide (HDTMA). As the fungi start to produce siderophores, the medium undergoes various color changes, which are often measured using a spectrophotometer [24–26]. These color changes result from the production of fungal siderophores. Subsequently, the siderophores undergo a color change. The efficiency of siderophore-producing fungi isolated from the gut of *M. peguana* and the qualitative assay of siderophores are presented in **Table 8**. **Figure 5** illustrates the qualitative test for siderophore production, which is indicated by a color change in the solution. The results indicate that Mpe 8 exhibited the highest efficiency in siderophore production, confirmed by the change in color of the solution during the CAS assay process.

Table 8. The Efficiency of *Fungi* Producing Siderophore Isolated from *M.peguana* Gut.

Fungal Isolates	Supernatant Color	Siderophore Unit (% SU) ± SD
Mpe 1	dark green	less than 50%
Mpe 2	dark green	less than 50%
Mpe 3	blue green	less than 50%
Mpe 4	green	less than 50%
Mpe 5	green	less than 50%
Mpe 6	green	less than 50%
Mpe 7	red	82.05 ± 0.79
Mpe 8	red	95.73 ± 0.56
Mpe 9	yellow	85.29 ± 0.57
Mpe 10	red	80.01 ± 0.78
Mpe 11	red	82.54 ± 0.41

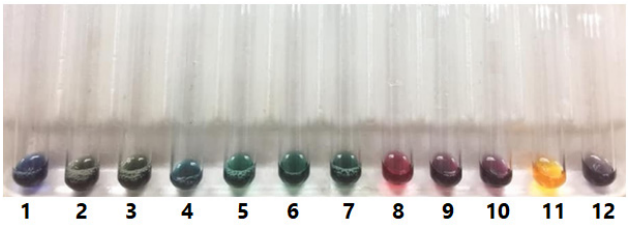


Figure 5. The Qualitative Test of Siderophore; 1 = Negative Control, 2 = Mpe 1, 3 = Mpe 2, 4 = Mpe 3, 5 = Mpe 4, 6 = Mpe 5, 7 = Mpe 6, 8 = Mpe 7, 9 = Mpe 8, 10 = Mpe 9, 11 = Mpe 10 and 12 = Mpe 11.

Table 8 presents the efficiency of siderophore production by fungi isolated from the gut of *M. peguana*. The fungal isolates were assessed for their ability to produce siderophores, and the results indicated that most isolates exhibited low siderophore production. However, five isolates Mpe 7, Mpe 8, Mpe 9, Mpe 10, and Mpe 11 showed significantly higher levels of siderophore units. Notably, Mpe 8 produced the highest siderophore units at 95.73 ± 0.56%, demonstrating its remarkable ability to sequester iron from the environment. Siderophore production is crucial for fungi as it aids in the acquisition of iron, which is often scarce in environments such as soil and the digestive system. The efficiency of the siderophore-producing fungi isolated from the gut of *M. peguana* highlights the potential of Mpe 8, which exhibited the highest siderophore unit content. The percentage of siderophore units (%SU) is calculated based on the change in absorbance of the solution during the CAS assay process. This production results in a color change in the solution; a higher %SU value indicates that the fungus has a greater capacity to extract iron from the solution and produce siderophores, which is essential for retrieving iron from iron-deficient environments [24].

Culturing the fungus named Mpe8 from the results of experiment 3.2, testing the siderophore substance, and comparing the amount of siderophore with the amount of iron**.** They were tested for %SU when cultured in different iron concentrations. The results showed that a high iron concentration (0.3%) inhibited mycelial growth, while 0.003% of Mpe8 also exhibited some inhibitory effect, which was more pronounced than 0.03% (**Table 9**). For species identification, morphology and the *ITS*1 region were used, revealing that Mpe8 belonged to *Aspergillus niger* and RC5 belonged to *Talaromyces angelicus* (**Table 10**).

Table 9. The Siderophore Production Induced by Iron Concentrations between Mpe8 Fungal Isolate.

	Siderophore Unit (%SU)				
Mpe8	**SW**	**Gauze No. 2**	**0.3% $FeCl_3$ + Gauze No. 2**	**0.03% $FeCl_3$ + Gauze No. 2**	**0.003% $FeCl_3$ + Gauze No. 2**
	0.00	60.18	not growth	8.09	23.47

Table 10. Morphology and *ITS*1 Identify Mpe8.

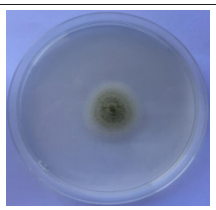
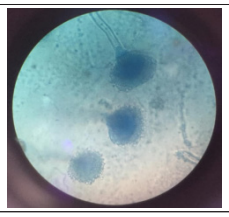
Isolates	Morphological Examination		Identity	Accession	Identity
Mpe8			100 %	KM924436.1	*Aspergillus terreus* strain MBL1414

Table 9 illustrates the impact of various iron concentrations on the production of siderophores by Mpe 8. Higher concentrations of iron (0.3% $FeCl_3$) inhibited fungal growth, while lower concentrations (0.003% $FeCl_3$) had a significant effect on siderophore production. This indicates that Mpe 8 can produce siderophores in response to low iron availability, which is essential for its survival in environments where iron is scarce.

The identification of Mpe 8 was conducted through morphological analysis and examination of the *ITS*1 region, which confirmed that the fungus is *Aspergillus terreus*, showing a 100% identity to the reference strain KM924436. This indicates that Mpe 8 belongs to a well-known species known for producing high levels of siderophores, as demonstrated in previous studies. These results underscore the importance of siderophore-producing fungi in iron acquisition, particularly for organisms in environments where iron availability is limited. Furthermore, the findings suggest that Mpe 8 has potential as a valuable strain for further research on microbial iron acquisition and its applications in enhancing soil fertility and health.

4. Conclusions

M. peguana is a soil-improving earthworm that plays a crucial role in soil health by breaking down organic matter and enhancing soil fertility. Within its gut, a diverse array of microorganisms, particularly fungi, contribute to this process. In this study, we utilized Next-Generation Sequencing (NGS) to identify fungi through metagenomics, and we concluded that this technology is the most effective tool for studying taxonomic species diversity in the gut microbiome of *M. peguana*. The sequencing data underwent rigorous quality control, achieving a Q30 score of 99.9%, which ensured the accuracy and reliability of the results. The taxonomic analysis revealed a high Shannon index of 5.99815, indicating significant species diversity, and a high Inverse Simpson index of 0.9469, showing a balanced distribution of species within the fungal community. The study identified 33 fungal species across 20 genera using the *ITS*1 region, including species from genera such as *Coprinopsis, Aspergillus, Penicillium*, and *Fusarium*. Notably, *Fusarium equiseti* was the most abundant fungal species, representing 20.54% of all fungi in the gut. This research provides valuable insights into the fungal diversity within *M. peguana's* gut and its potential applications in agriculture. The ability of Mpe8, a fungal isolate from the earthworm gut, to produce siderophores was also demonstrated, with the highest siderophore unit content observed. This ability to extract iron from the environment is crucial for enhancing soil health and fertility, particularly in soils that are iron-limited. Overall, this research highlights the ecological role of fungi in the gut of *M. peguana* and its potential applications in agriculture, particularly in enhancing soil fertility and supporting sustainable farming practices.

Author Contributions

Investigation, R.K., A.T. and R.T.; formal analysis, R.K., A.T. and R.T.; validation, P.P., T.M., P.K., N.T. and L.H.; validation, T.M., R.T. and A.T.; supervision, R.T. and

R.K.; project administration, R.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Bangkokthonburi University (protocol code BTU.MD.001/2024 and date of approval was 15 January 2024.).

Informed Consent Statement

Not applicable.

Data Availability Statement

We encourage all authors of articles published in our journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required.

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Conflicts of Interest

The authors declare no conflict of interest.

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