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Identification of Insect Larvae Associated with Pachouli (*Pogostemon cablin*) Leaves Using DNA Barcoding

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ABSTRACT

Patchouli (*Pogostemon cablin*) is an essential oil-producing plant with high economic value widely used in the perfume, cosmetic, and aromatherapy industries. The presence of insect larvae on patchouli plants may cause leaf tissue damage that affects vegetative growth and essential oil production. Morphological identification of insect larvae is often difficult because larval body structures are relatively uniform and possess limited diagnostic characters. This study aimed to identify insect larvae associated with patchouli leaves using a DNA barcoding approach based on the *Cytochrome c Oxidase subunit I* (COI) gene. The research was conducted in Inomunga Village, Kaidipang District, North Bolaang Mongondow Regency, North Sulawesi Province, from January to February 2026. Methods included larval sampling, DNA extraction, PCR amplification using universal primers, gel electrophoresis, DNA sequencing, BLAST analysis, Multiple Sequence Alignment (MSA), sequence similarity analysis, genetic distance analysis, and phylogenetic reconstruction using the Maximum Likelihood method. The results showed that COI gene amplification successfully produced DNA fragments of approximately 709 bp in all samples. BLAST analysis identified the specimens as *Chrysodeixis eriosoma*, *Spodoptera litura*, *Herpetogramma stultalis*, and *Microgastrinae* sp. with 100% query cover and more than 95% percent identity values. Phylogenetic analysis showed that each sample formed a distinct clade according to its species identity, while *Drosophila melanogaster* was used as the outgroup. This study demonstrates that COI-based DNA barcoding is effective for identifying insect larvae associated with patchouli plants and can support biodiversity studies and ecologically based pest management.

Keywords: *DNA barcoding, COI gene, insect larvae, Pogostemon cablin, phylogenetics.*

INTRODUCTION

Patchouli (*Pogostemon cablin*) is a high-value essential oil commodity widely used in the perfume, cosmetic, and aromatherapy industries due to its dominant patchouli alcohol content. Indonesia has long been the world's largest producer and exporter of patchouli oil, contributing more than 80% of global annual production. The productivity of patchouli is strongly influenced by the vegetative health of the plant, where biotic disturbances can directly affect essential oil quality. Larval herbivory has been reported as one of the main constraints in patchouli

cultivation, as larvae can damage leaves, inhibit growth, and reduce secondary metabolite biosynthesis.[1]

Understanding the relationship between patchouli plants and insect pests is important because this interaction directly determines the degree of plant damage and cultivation success. In essential oil-producing plants like patchouli, leaf tissue damage not only reduces biomass but may also affect the biosynthesis and composition of secondary metabolites, including patchouli alcohol.[10,15] Furthermore, understanding pest-plant interactions forms an essential basis for developing effective, environmentally friendly, and sustainable Integrated Pest Management (IPM) strategies.

The larval stage is the most physiologically active phase in terms of plant tissue consumption, contributing most significantly to vegetative damage compared to adult stages.[2] However, larval identification is often hampered by limited morphological characters due to relatively uniform body forms, inter-instar morphological changes, and lack of species-level identification keys for larvae.[12] DNA barcoding, based on the mitochondrial gene *Cytochrome c Oxidase subunit I* (COI), has been widely applied as a more accurate and reliable identification method, especially for larval specimens.[7,8]

Patchouli cultivation in North Bolaang Mongondow Regency is mainly conducted by smallholder farmers using traditional systems, and scientific information regarding pest insects in this area is very limited. Therefore, this study aims to identify insect larvae associated with patchouli leaves using a DNA barcoding approach, to provide taxonomic data that can support ecologically based pest management strategies for patchouli cultivation.

MATERIALS & METHODS

Study Area and Sampling

This study was conducted from January to February 2026. Larval samples were collected from patchouli plantations (*Pogostemon cablin*) in Inomunga Village, Kaidipang District, North Bolaang Mongondow Regency, North Sulawesi Province. Molecular analyses were performed at the Advanced Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University, Manado. Larval specimens were collected using descriptive survey methods through systematic visual inspection of patchouli plants. Leaves showing signs of damage such as rolling, bite marks, necrotic spots, or frass were carefully opened to locate concealed larvae. Collected larvae were preserved in vials containing 70-90% ethanol and labeled with sample code, collection date, and location. Geographic coordinates were recorded using GPS.

DNA Extraction

Genomic DNA was extracted using a Tissue Genomic DNA Mini Kit (Geneaid) with modifications for animal tissue. Approximately 10-25 mg of larval tissue was homogenized in a 1.5 mL microtube using a micropestle in 200 µL Buffer GT, followed by addition of Proteinase K and incubation at 60°C for 10-30 minutes. After cell lysis, 200 µL Buffer GBT was added and incubated again at 60°C for 10 minutes. The supernatant was transferred to a GS Column for DNA binding, followed by washing steps with Buffer W1 and Wash Buffer. DNA was eluted in 150 µL Elution Buffer and stored at -20°C until PCR amplification.

PCR Amplification

Amplification of the COI gene was carried out using universal Folmer primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3').[4] PCR reactions were performed in a total volume of 40 µL containing 20 µL MyTaq HS Red Mix (Bioline), 1.5 µL each primer, 15 µL nuclease-free water, and 2 µL

DNA template. Cycling conditions: initial denaturation at 94°C for 2 min; 35 cycles of 94°C/30 s, 50°C/40 s, 72°C/1 min; final extension at 72°C for 10 min.

Gel Electrophoresis

PCR products were visualized by 0.8% agarose gel electrophoresis in TBE buffer, stained with ethidium bromide, and examined under UV transilluminator. Successful amplification was confirmed by the presence of a single clear band at approximately ±650 bp corresponding to the COI target fragment.[4]

DNA Sequencing and Bioinformatics Analysis

PCR products with clear, specific bands were sent to First Base (Malaysia) for Sanger sequencing. Resulting sequences were edited and analyzed using Geneious Prime v2025.1.1. Species identification was performed by BLAST analysis against the NCBI GenBank database. Multiple Sequence Alignment (MSA) was conducted using the MUSCLE algorithm in Geneious Prime.[3] Pairwise sequence similarity and genetic distance were calculated, and phylogenetic reconstruction was performed using the Maximum Likelihood method in PhyML v3.3.20180621 with *Drosophila melanogaster* (accession number PP764101) as the outgroup.[5]

RESULT

Larval Specimens on Patchouli Leaves

Field observations on patchouli (*Pogostemon cablin*) leaves revealed insect specimens at various developmental stages, including eggs, larvae, and pupae (Figure 1). Egg clusters were pale yellowish-white and deposited in groups on leaf surfaces (Figure 1a), a reproductive strategy common in herbivorous insects that facilitates immediate food access upon hatching. Two larval morphotypes were observed: a brown-grey larva with longitudinal stripe patterns (Figure 1b), and a bright green larva with a soft, relatively homogeneous body (Figure 1c). The brown coloration likely represents mimicry towards dry leaves or stems, while the green coloration reflects cryptic adaptation to the host plant leaf color. Pupae were dark brown, oval-shaped, and inactive (Figure 1d), representing the transition stage before metamorphosis to adults.

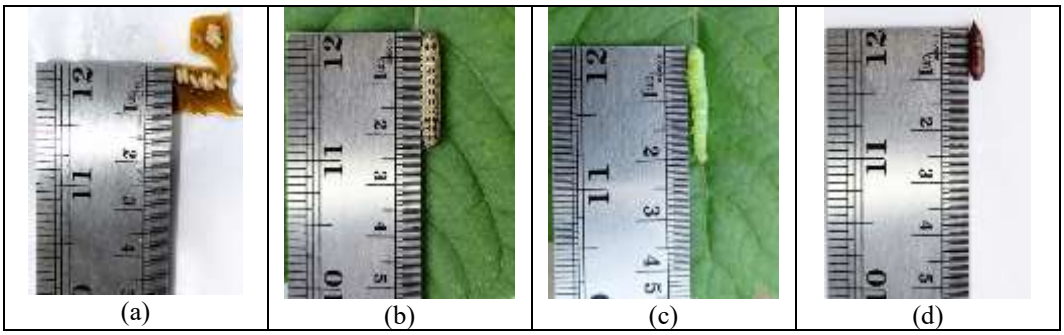

Figure 1. Insect specimens found on patchouli plants: (a) egg mass, (b) brown larva, (c) green larva, and (d) pupa.

PCR Amplification and Gel Electrophoresis

COI gene amplification was successful in all four samples, producing clear, specific DNA bands at approximately 709 bp (Figure 2). The absence of smear or non-specific bands indicates high-quality DNA template, appropriate primer annealing, and optimal PCR conditions. The universal primers LCO1490 and HCO2198 successfully amplified the COI target region across all larval samples, consistent with their broad utility in insect DNA barcoding.[13],[4]

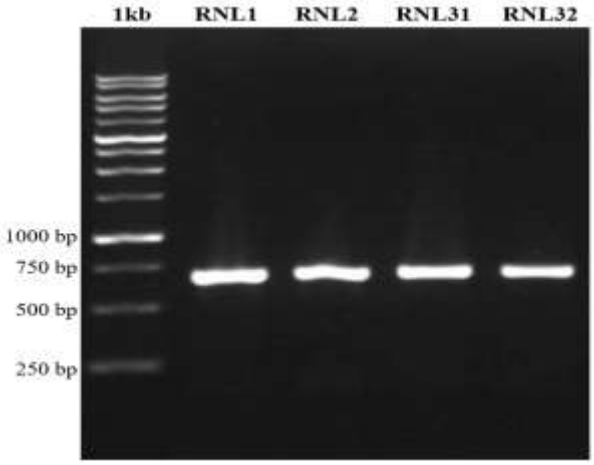

Figure 2. Agarose gel electrophoresis (0.8%) of PCR products showing DNA bands at ±658 bp for all samples (RNL1, RNL2, RNL31, RNL32).

Chromatogram Quality

DNA sequencing chromatograms of all four samples showed clear, well-separated nucleotide peaks with no overlapping signals, indicating high-quality sequence reads suitable for downstream bioinformatic analysis.[9]

BLAST Analysis

Molecular identification was performed using BLAST against the NCBI GenBank database. All samples showed 100% query cover. The results for each specimen are presented in Tables 1-4.

Table 1. BLAST results for specimen RNL1.

Scientific Name	Query Cover (%)	Percent Identity (%)	Accession
Chrysodeixis eriosoma	100%	100.00%	HM877176.1
Chrysodeixis eriosoma	100%	100.00%	HQ951262.1
Chrysodeixis eriosoma	100%	99.9%	HQ991180.1
Chrysodeixis eriosoma	100%	99.8%	HQ990830.1
Chrysodeixis chalcites	100%	99.5%	MW305827.1
Chrysodeixis chalcites	100%	99.5%	MK610322.1

Table 2. BLAST results for specimen RNL2.

Scientific Name	Query Cover (%)	Percent Identity (%)	Accession
Spodoptera litura	100%	100.00%	PV590019.1
Spodoptera litura	100%	100.00%	PQ482506.1
Spodoptera litura	100%	100.00%	OP002859.1
Spodoptera litura	100%	100.00%	OP776686.1
Spodoptera litura	100%	100.00%	OL539320.1
Spodoptera litura	100%	100.00%	PQ816165.1

Table 3. BLAST results for specimen RNL31.

Scientific Name	Query Cover (%)	Percent Identity (%)	Accession
Herpetogramma stultalis	100%	99.9%	KP850692.1
Herpetogramma stultalis	100%	99.8%	MK950837.1

Scientific Name	Query Cover (%)	Percent Identity (%)	Accession
Herpetogramma stultalis	100%	99.8%	MK019401.1
Herpetogramma phaeopteralis	100%	97.1%	LC697837.1
Herpetogramma salbialis	100%	97.0%	HM893962.1
Herpetogramma bipunctalis	99.85%	96.9%	MK950820.1

Table 4. BLAST results for specimen RNL32.

Scientific Name	Query Cover (%)	Percent Identity (%)	Accession
Microgastrinae sp.	100%	100.00%	PV080500.1
Microgastrinae sp.	100%	100.00%	HM430512.1
Glyptapanteles sp.	100%	99.9%	ON489304.1
Glyptapanteles sp.	100%	98.9%	MH138746.1
Microgastrinae sp.	100%	95.9%	HM430495.1

Multiple Sequence Alignment (MSA)

MSA of all COI gene sequences was conducted using the MUSCLE algorithm in Geneious Prime v2025.2.2. The alignment showed that most nucleotide positions among the four samples (RNL1, RNL2, RNL31, RNL32) were conserved, with a high level of sequence similarity. No large gaps were observed in the alignment, indicating successful amplification and sequencing without significant deletions or insertions. Minor nucleotide substitutions were observed at specific positions, particularly in sample RNL31, representing normal intraspecific variation or natural mutations in mitochondrial DNA.[18]

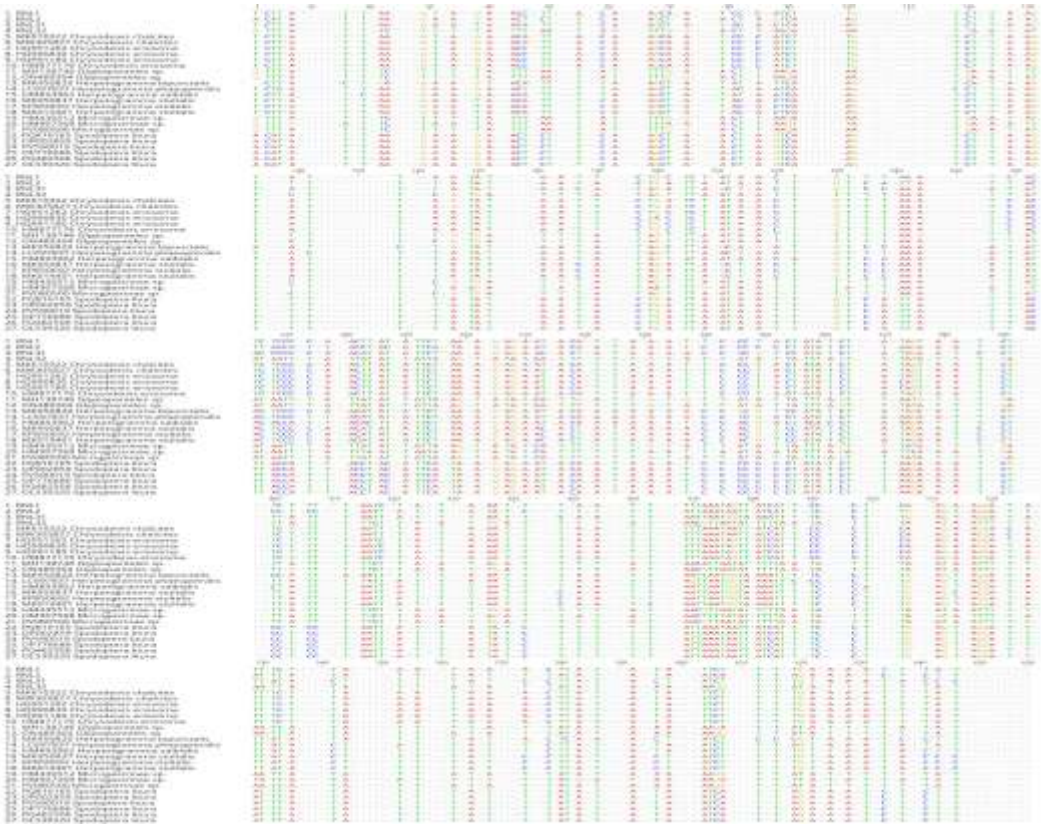

Figure 3. Multiple Sequence Alignment (MSA) of COI gene sequences of insect larvae using MUSCLE algorithm (Edgar, 2004).

Sequence Similarity Analysis

All samples showed high percent identity values against their reference sequences in GenBank (Table 5). Samples RNL1, RNL2, and RNL31 showed very high similarity to their respective Lepidoptera reference sequences, with values exceeding 99.9%, confirming species-level identification. Sample RNL32 showed 100% identity to *Microgastrinae* sp. (Hymenoptera: Braconidae). A percent identity greater than 98% in the COI gene is generally regarded as a reliable threshold for species-level identification in animals.[7]

Table 5. Percent identity of COI gene sequences among larval samples.

Sample / Species	Best BLAST Match	Order / Family	Query Cover (%)	Percent Identity (%)
RNL1	*Chrysodeixis eriosoma*	Lepidoptera: Noctuidae	100%	100.00%
RNL2	*Spodoptera litura*	Lepidoptera: Noctuidae	100%	100.00%
RNL31	*Herpetogramma stultalis*	Lepidoptera: Crambidae	100%	99.9%
RNL32	*Microgastrinae sp.*	Hymenoptera: Braconidae	100%	100.00%

Genetic Distance Analysis

Genetic distance analysis (Table 6) showed that Lepidoptera samples (RNL1, RNL2, RNL31) had relatively lower genetic distances among themselves compared to the parasitoid sample RNL32 (Hymenoptera: Braconidae). This pattern indicates closer evolutionary relationships within the Lepidoptera group, consistent with their shared herbivorous ecology. Sample RNL32 (*Microgastrinae* sp.) showed higher genetic distances from all three Lepidoptera samples, reflecting the deeper evolutionary divergence between Hymenoptera and Lepidoptera. These results are supported by the phylogenetic analysis showing distinct clades for each taxonomic group.

Table 6. Genetic distance among larval samples based on COI gene sequences.

Sample	RNL1	RNL2	RNL31	RNL32
RNL1 (C. eriosoma)	-	0.087	0.096	0.314
RNL2 (S. litura)	0.087	-	0.091	0.311
RNL31 (H. stultalis)	0.096	0.091	-	0.308
RNL32 (Microgastrinae sp.)	0.314	0.311	0.308	-

Phylogenetic Reconstruction

The Maximum Likelihood phylogenetic tree (Figure 4) showed clear separation of all four larval specimens into distinct clades corresponding to their identified species. Sample RNL1 grouped with *Chrysodeixis eriosoma*, while RNL2 clustered with *Spodoptera litura*, both within the family Noctuidae (Lepidoptera). Sample RNL31 formed a separate branch with *Herpetogramma stultalis* (Crambidae, Lepidoptera), reflecting inter-family divergence within the order. Sample RNL32 formed an independent cluster with *Microgastrinae* sp. (Braconidae, Hymenoptera), well separated from all Lepidoptera samples. The outgroup *Drosophila melanogaster* (PP764101, Diptera) branched at the outermost position, confirming its suitability as an outgroup (Hall, 2011). The phylogenetic topology strongly supported the BLAST and similarity analyses, validating the molecular identities of all four specimens.

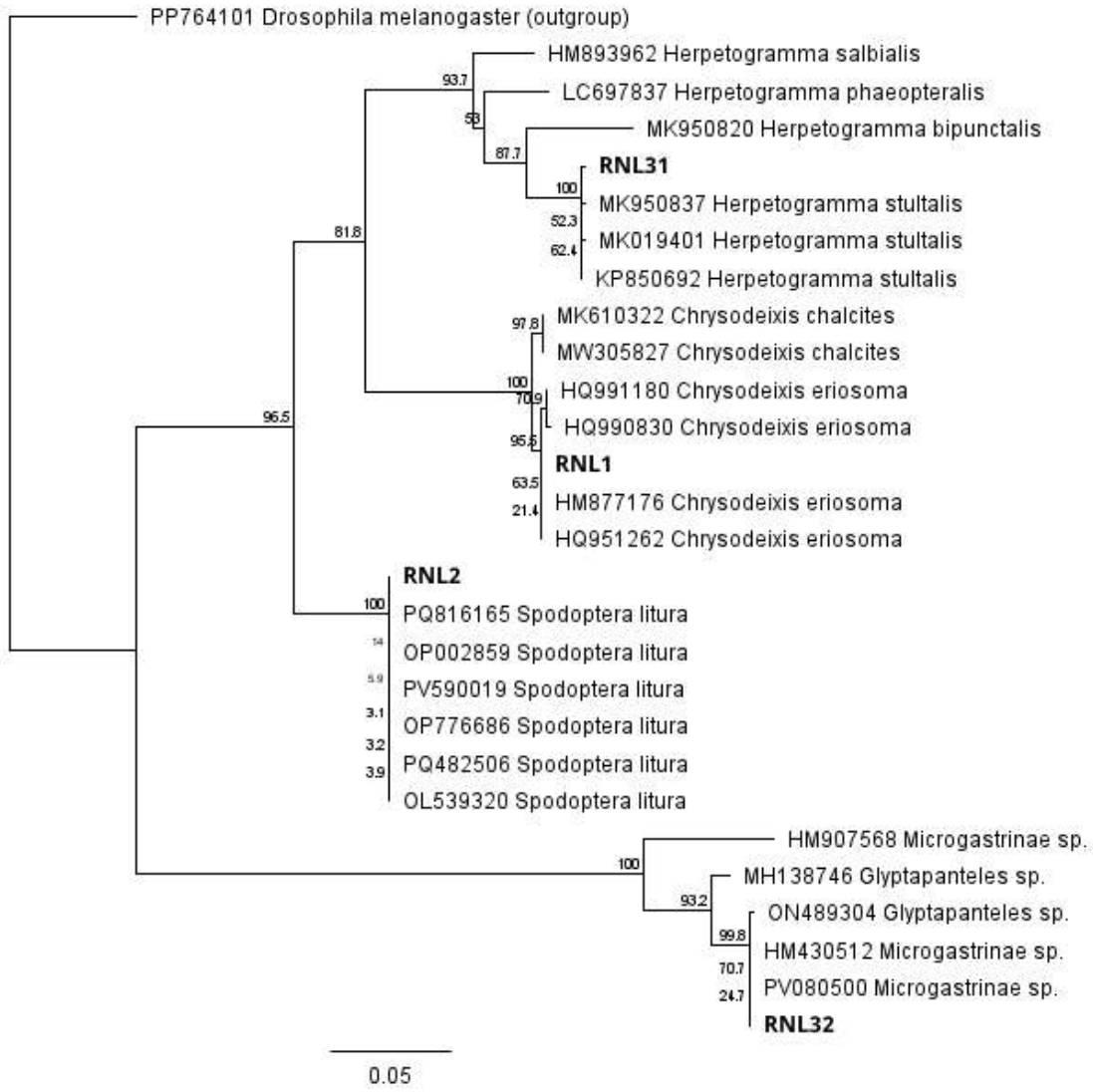

Figure 4. Phylogenetic tree of insect larvae associated with patchouli leaves based on COI gene sequences, reconstructed using Maximum Likelihood method (PhyML v3.3). Drosophila melanogaster (PP764101) was used as outgroup.

DISCUSSION

This study successfully identified four insect specimens associated with patchouli leaves in North Bolaang Mongondow Regency using COI-based DNA barcoding. Three specimens were identified as herbivorous Lepidoptera *Chrysodeixis eriosoma*, *Spodoptera litura*, and *Herpetogramma stultalis* while one specimen was a parasitoid Hymenoptera (*Microgastrinae* sp., Braconidae).

Chrysodeixis eriosoma (Noctuidae) is a polyphagous herbivore known to cause leaf perforation damage through intensive feeding activity. The 100% percent identity obtained for specimen RNL1 strongly confirms this identification. Similarly, *Spodoptera litura* (Noctuidae, RNL2) is one of the most economically important polyphagous agricultural pests in tropical Asia, capable of causing severe defoliation when populations are high.[16] In patchouli, damage to leaf tissue by these Lepidoptera larvae may directly impact essential oil biosynthesis, as leaves are the primary organ for patchouli alcohol production.

Herpetogramma stultalis (Crambidae, RNL31) showed 99.9% identity with the reference sequence. Minor nucleotide differences at certain positions represent normal intraspecific variation in mitochondrial DNA, which does not affect species-level identification (Ward et al., 2005). This species is known as a leaf-

rolling or leaf-perforating pest in various crops.

The identification of *Microgastrinae* sp. (Braconidae, Hymenoptera) as specimen RNL32 reveals an important ecological dimension in this agroecosystem. Microgastrinae are endoparasitoids of Lepidoptera larvae and represent natural biological control agents that help regulate herbivore populations.[14] The presence of this parasitoid alongside its potential Lepidoptera hosts suggests active trophic interactions within the patchouli plantation, which could be leveraged in IPM strategies.

The effectiveness of COI-based DNA barcoding demonstrated in this study aligns with previous findings from various insect groups. The high percent identity values (>99%), 100% query cover, and strong phylogenetic support for each specimen confirm that the COI gene is a reliable molecular marker for identifying insect larvae that are morphologically difficult to distinguish. This is particularly important for larval stages, which often lack species-specific diagnostic morphological characters.[12]

The phylogenetic analysis further validated molecular identifications, with each specimen forming a distinct clade corresponding to its species identity. The clear separation of Lepidoptera and Hymenoptera clades, and the positioning of Drosophila melanogaster as an appropriate outgroup, demonstrate the robustness of the Maximum Likelihood reconstruction using the PhyML algorithm.[5]

CONCLUSION

COI-based DNA barcoding was successfully applied to identify insect larvae associated with patchouli (*Pogostemon cablin*) leaves in North Bolaang Mongondow Regency. PCR amplification produced clear bands at ±650 bp in all samples. BLAST analysis identified the specimens as *Chrysodeixis eriosoma*, *Spodoptera litura*, *Herpetogramma stultalis*, and *Microgastrinae* sp. with 100% query cover and high percent identity values. MSA, similarity analysis, genetic distance, and phylogenetic reconstruction all confirmed that each sample formed a distinct clade corresponding to its species identity. The three Lepidoptera species represent potential pests threatening patchouli leaf health and essential oil production, while *Microgastrinae* sp. represents a parasitoid natural enemy. This study provides foundational biodiversity data for insect-patchouli interactions in this region and supports the development of ecologically based IPM strategies for patchouli cultivation.

Declaration by Authors

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