

Morphological and Molecular Identification of *Helopeltis* Species on Cocoa from Kaliwining Experimental Station, Jember, Indonesia

Gracia Melsiana Aldini^{1*}, Anisa Aulia Rahma¹, and Aris Budiman¹

¹Indonesian Coffee and Cocoa Research Institute, Jl. PB. Sudirman 90, Jember, Indonesia

*Corresponding author: graciamelsianaa@gmail.com

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Abstract

Severe crop loss of cocoa in Indonesia has long been reported to be caused by *Helopeltis*. It has been noted that cocoa and *Helopeltis* have existed in Kaliwining Experimental Station of Indonesian Coffee and Cocoa Research Institute (ICCRI), Jember, Indonesia, for about 100 years, and the species have developed during that time. Our study aimed for examining the *Helopeltis* species that attacked cocoa in this experimental station both morphologically, morphometrically, and molecularly and to compare the species with the one attacking cocoa in Java and Nusa Tenggara, preserved at the GenBank. *Helopeltis* was sampled from infested cocoa pods collected representatively from cocoa at the Kaliwining ES and then reared in the Crop Protection Laboratory of ICCRI. Adults of *Helopeltis*-laboratory-reared insects were identified, 30 females 30 males were selected. We examined the morphology and morphometry of the samples, and their identification was based on an insect identification key. Morphometry measurement of male and female body parts includes body length, antenna segments, head, eyes, collar, wing, tibia, and femur. In addition, the molecular identification of *Helopeltis* sp. was made through DNA extraction, amplification, and sequence analysis of DNA target regions using the COI part of mitochondrial DNA. External morphological identification, supported by morphometry of the *Helopeltis* specimen from Kaliwining, the white bands on all femora, body length, and the ratio of the first antennal segment to the posterior width of the pronotum suggested that the insect belongs to *Helopeltis bradyi*. Similarly, molecular identification using PCR amplification through specific primers from COI gene sequences confirmed that *Helopeltis* specimens from Kaliwining are classified as *Helopeltis bradyi*.

Keyword: Cocoa, *Helopeltis antonii*, *Helopeltis bradyi*

INTRODUCTION

Indonesia is a major cocoa-producing country, with 180,000 tons produced in 2022/2023 (ICCO, 2023). Nonetheless, cocoa production has been decreasing in the last three years (BPS, 2022); one of the causes is the presence of pests on cocoa plantations. *Helopeltis* has been a major pest of cocoa in Indonesia for more than 100

years (van Hall, 1914; Sulistyowati & Iswanto, 1994; Wiryadiputra, 2007; Melina *et al.*, 2016), a period that was similar to the age of the oldest cocoa experimental station in the country, the Kaliwining Experimental Station of Indonesian Coffee and Cocoa Research Institute (ICCRI), Jember, Indonesia. In other words, the cocoa and the pest have been in the experimental station for more than 100 years. During that period, the pest

had grown and developed. According to Entwistle (1985), the saturation number of species is achieved in only 20–30 years. After that, adaptation of additional insect species occurs relatively rarely, unless the planting area is notably increased.

Pancaningtyas *et al.* (2022) examined *Helopeltis* molecularly in Kaliwining and found that the species belongs to *H. bradyi*. Two kinds of *Helopeltis* that attack cocoa in Indonesia are *Helopeltis theivora* and *H. antonii* (van Hall, 1994). *H. theivora* is commonly found in places with higher temperature, while *H. antonii* is generally found in colder areas. *H. theivora* is the one causing significant losses in cocoa plantations in Malaysia Peninsular (Muhammad & Way, 1995).

Helopeltis antonii has long been reported to attack cocoa and other host plants in Java (Van Hall, 1944; Stonedahl, 1991; Wiryadiputra, 1997; Sulistyowati, 2014). The similarity of external morphological characters to other related pests can lead to misidentification (Stonedahl, 1991). This is even more likely to happen given that the early works on the taxonomy of the *Helopeltis* genus mainly relied on external morphology, especially body measurements, shape, and coloration (Signoret, 1858; Walker, 1873; Waterhouse, 1886 & 1888; Bergroth, 1889). Furthermore, recent taxonomic works on the prevalent local species of this genus have yet to be discovered.

Waterhouse (1886) described *H. bradyi* which damaged cinchona plantations in Java. Morphologically, *H. bradyi* is similar to *H. antonii*; however, the former has a slightly larger body size (Atkinson, 1897). A review conducted by Stonedahl (1991) on the oriental species of *Helopeltis* suggested that only *H. bradyi* is present in Indonesia and is mistakenly reported as *H. antonii*. Stonedahl (1991) said that nine *Helopeltis* species could now be found in Indonesia i.e. *H. antonii*,

H. chinconae, *H. bradyi*, *H. cuneata*, *H. fasciaticollis*, *H. insularis*, *H. sulawesi*, *H. sumatrana*, and *H. theivora*. On the contrary, he concluded that *H. antonii* is restricted in India, Sri Lanka, and the Andaman Islands.

The morphological similarity between *H. antonii* and *H. bradyi* from the early report of Waterhouse (1886) as well as the revision of Stonedahl (1991) regarding these two species, support the notion that *H. bradyi* is the *Helopeltis* species most likely to be found in Java. However, it is also possible that, more than 20 years after Stonedahl's (1991) review, *H. antonii* has spread in Indonesia. The latest research by Melina *et al.* (2016) reported it was *H. bradyi*, not *H. antonii*, that existed on Java island. This was evidenced by the similarity in external morphology and genitalia of *H. bradyi*. Identifying the main pests that attack cocoa plants is very important for prevention and control so that the productivity of cocoa plants can be maintained. Therefore, to determine the infesting *Helopeltis* species on cocoa at the Kaliwining Experimental Station Jember, we conducted a morphological examination of their external characters and molecular identification using COI gene sequences.

MATERIALS AND METHODS

The cocoa farms in Kaliwining Experimental Station, Indonesian Coffee and Cocoa Research Institute, Jember, East Java, are characterized by shallow water table at about 80–100 cm depth, especially during rainy seasons, situated at the toeslope of Argopuro volcano to the south with altitude about 60 m asl. The topography is plain with slope gradient estimated around 0–4% as situated at higher level than Bedadung river, the closest river nearby. It is therefore suggested that the sediment in this area is, mostly,

coming from volcanic ash derived materials. A moderate soil development stage of volcanic materials derived Inceptisols in the area together with average of 6.82 wet months and 4.31 dry months per annum, or climate type D (moderate) based on Schmidt-Fergusson classification has provided a moderate suitability level for cocoa production.

Helopeltis

Helopeltis adults utilized in this study were representatively collected from infested cocoa pods in February 2022 from all cocoa farms at the Kaliwining Experimental Station. The adult insects were then reared to be propagated in the laboratory in an air-conditioned room (ca. 23°C, 70% RH) for further research purposes, namely morphological and morphometric observations and molecular identification.

Morphological Characteristics

External morphology was observed at the Entomology Sub Laboratory, Crop Protection Laboratory, Indonesian Coffee and Cocoa Research Institute, Jember, Indonesia. The external morphological features of 60 samples containing 30 females and 30 males from the Kaliwining Experimental Station, Jember, were observed. Identification of *Helopeltis* specimen considered the external morphology of the insect based on the insect identification key (Stonedahl, 1991) and was compared with the image from the latest reference (Melina *et al.*, 2016). External morphology was observed using an Olympus SZ51 microscope equipped with OptiLab Viewer. Morphometry measurements of body parts include body length, antennal segment, head, eyes, collar, wing, tibia, and femur. Data collected were expressed in means and standard errors.

Molecular Identification

Molecular works were carried out at the Biotechnology Laboratory of Biotek Prima Indoplus, Buduran, East Java. Molecular identification of *Helopeltis* sp. was done through DNA extraction, PCR amplification, and sequence analysis. DNA target regions were amplified using a specific primer from the COI gene part of mitochondrial DNA. The COI gene sequence has very few deletions and insertions, and many parts are conserved for DNA barcoding, an identifier for each species (Hebert *et al.*, 2003). Universal primer used was LCO for forward 5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3' and the reverse HCO- 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3'. The DNA from the amplification was visualized using electrophoresis through 1% agarose gel in 1x TBE buffer with 1 kB DNA ladder as a comparative DNA marker.

The PCR products were analyzed for nucleotide sequences using the Sanger dideoxy sequencing method. The trial version of DNA Sequence Assembler v4.7 software was used for contig. The nucleotide sequence results were aligned with BLAST® (Basic Local Alignment Search Tool) available at the National Center for Biotechnology Information (<https://blast.ncbi.nlm.nih.gov>) to discover its percentage of homology. Sequences from this study and references from the National Center for Biotechnology Information (NCBI) were aligned using multiple alignment cluster W in Bioedit® 7.2.6.1 software. MEGA® 6.06 software was used for phylogeny analysis using the Maximum Likelihood method and a model with the lowest BIC (Bayesian Information Criterion) on the model test results. The phylogenetic tree was created to compare *Helopeltis* specimens from the Kaliwining Experimental Station with *Helopeltis bradyi* and *Helopeltis antonii* from GenBank. The bootstrap method with 1000

replications was utilized to evaluate the phylogenetic tree.

RESULTS AND DISCUSSIONS

External Morphological Characters

Based on the insect identification key, one of the critical external morphological characteristics of *H. bradyi* that differs from *H. antonii* is the presence of a pale band at the base of each fore, middle, and hind femora of *H. bradyi*; *H. antonii* only has this band on its fore and middle femora (Stonedahl, 1991). The pale band at the base

of each fore, middle, and hind femora was found in the Kaliwining *Helopeltis* (Figure 1B). The color on the head of Jember *Helopeltis* showed some characteristics common to *H. antonii* and *H. bradyi* (Stonedahl, 1991). The pale spots anterior to the eyes and near the collar, the pale base of the segment I of the antennae, and the patternless collar and pronotum that could be of yellow-orange, reddish brown, or dark brown colors were also indicative of both species, while the pattern on the head was similar to Stonedahl's (1991) description, the color of the head matched the picture of *H. bradyi* (Figure 1D-E; 1D-E).

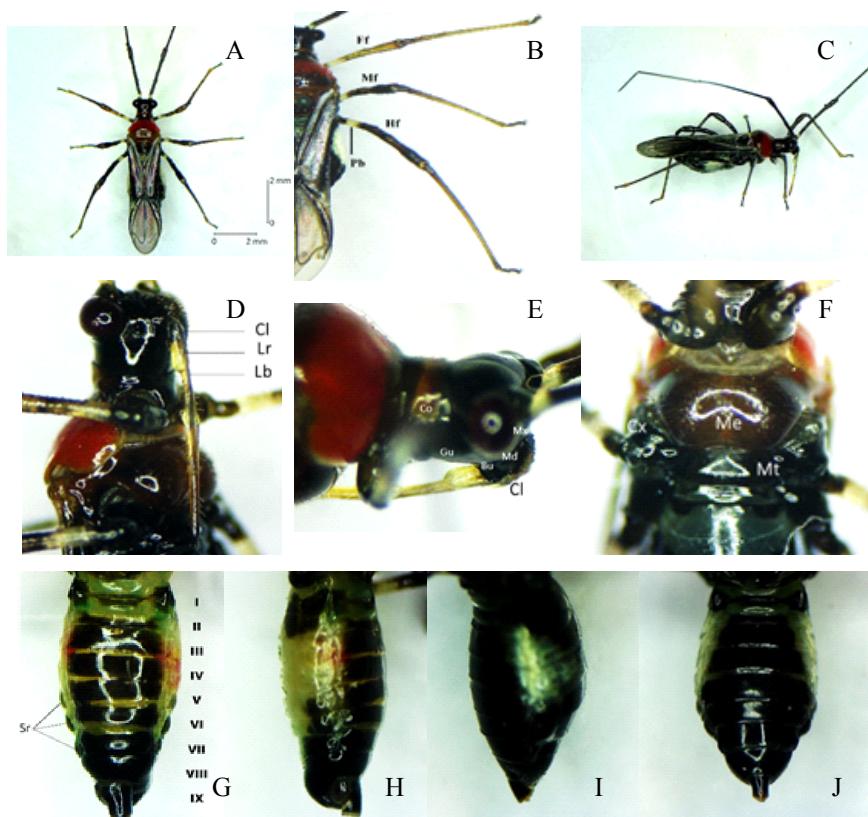


Figure 1. The external morphology of the adults from Kaliwining. A: habitus; B: leg; C: lateral habitus; D, E: frontal and lateral view of head; F: thorax; dorsal and lateral view of male (G-H) and female abdomen (I-J). Ff = fore femora, Mf = middle femora, Hf = hind femora, Pb = pale band, Cl = clypeus, Lr = labrum, Lb = labium, Co = collar, Cx = coxa, Gu = gula, Bu = buccula, Md = mandibular plate, Mx = maxillary plate, Me = mesosternum, Mt = meta sternum, Sr = spiracle. Roman numeric indicates the number of segments.

Stonedahl (1991) noted that the color pattern on the lateral side of the abdominal sterna of *H. antonii* was similar to that of *H. bradyi*. A fuscous patch was found on both sexes of both species on sterna I–III. In the Javanese *Helopeltis*, except for the last three genital segments (VII–IX), all lateral segments of mature female adults were marked with a fuscous patch, including segment VI, or on all but segment V. The patch was confined to the first three segments in the teneral female adults. It is important to note that a long and obvious patch was found on segment VI of all studied Kaliwining *Helopeltis*. Stonedahl (1991) wrote that both *H. antonii* male and female adults have a fuscous patch on sterna I–III of their abdomen, but this key indicated the possibility of the patch extending to segments IV and V. On the male Jember *Helopeltis*, beside the genital capsule (segment IX), a fuscous patch was observed on segments I–III and VI–VIII; segments IV and V were pale (Figure 1G–J).

At a glance, *H. antonii* and *H. bradyi* look very similar. In previously published studies on *H. bradyi* and *H. antonii*, only two structures, body and antennal length, could be compared directly to those of our samples. The body length of male samples (5.52–6.38 mm) fell within the range previously found for *H. bradyi* (5.5–6.9 mm), and female

body length was (6.90–7.86 mm), which was also within the range previously found for *H. bradyi* (6.6–8.6 mm) (Stonedahl, 1991; Ambika & Abraham, 1979). Like *H. bradyi* as previously reported, the Javanese samples differed from *H. antonii* by their longer first antennal segment (Melina *et al.*, 2016). This segment was also longer than the posterior width of the pronotum, with the ratio for males and females being 1.64–1.93: 1 and 1.49–1.81: 1, respectively, compared to 1.5–1.85: 1 and 1.45–1.6: 1 for *H. bradyi* and 1.20–1.45: 1 and 1.05–1.30: 1 for *H. antonii* (Stonedahl, 1991; Melina *et al.*, 2016) (Table 1).

The external characters elaborated in Table 1, white bands on all femora, body length, and the ratio of the first antennal segment to the posterior width of the pronotum, point to the Kaliwining *Helopeltis* being *H. bradyi* as described by Stonedahl (1991) and Melina *et al.* (2016).

Molecular Identification

Visualization results for *Helopeltis* sp. from Kaliwining showed a 700 bp fragment (Figure 2). This is in accordance with the research of Asokan *et al.* (2012), which showed that the mitochondrial CO1 cytochrome tested on *H. antonii* and *H. theivora*

Table 1. Measurement of body parts of *Helopeltis* (n = 60: 30 (males); 30 (females))

Body part (mm)	Male			Female		
	Mean	Range	SE	Mean	Range	SE
Body length	5.82	5.52-6.38	0.27	7.05	6.90-7.86	0.35
First antennal segment	2.64	2.53-2.86	0.12	2.68	2.46-2.92	0.19
Second antennal segment	4.62	4.44-4.83	0.14	4.26	4.10-4.49	0.20
Head width + eyes	1.18	1.06-1.24	0.05	1.26	1.14-1.39	0.05
Collar width (dorsally)	0.75	0.81-0.28	0.20	0.84	0.70-1.00	0.06
Collar + pronotum length	1.10	0.98-1.32	0.10	1.23	1.03-1.36	0.09
Pronotum (posterior width)	1.49	1.41-1.51	0.04	1.75	1.71-1.80	0.06
Right forewing	5.10	4.77-5.38	0.19	6.22	5.93-6.63	0.23
Right hindwing	4.08	3.98-4.22	0.14	4.88	4.71-5.26	0.23
Fore tibia	2.57	2.44-2.62	0.09	2.79	2.24-2.88	0.14
Fore femur	1.94	1.57-2.36	0.14	2.14	1.92-2.52	0.09
Hind tibia	3.32	3.03-3.40	0.12	3.63	3.32-3.73	0.15
Hind femur	2.63	2.55-2.94	0.08	2.67	2.58-2.82	0.17
Ratio (1 st antennal/posterior width of pronotum)	1.81	1.64-1.93	0.11	1.51	1.49-1.81	0.11

Notes: SE = standard error of the mean

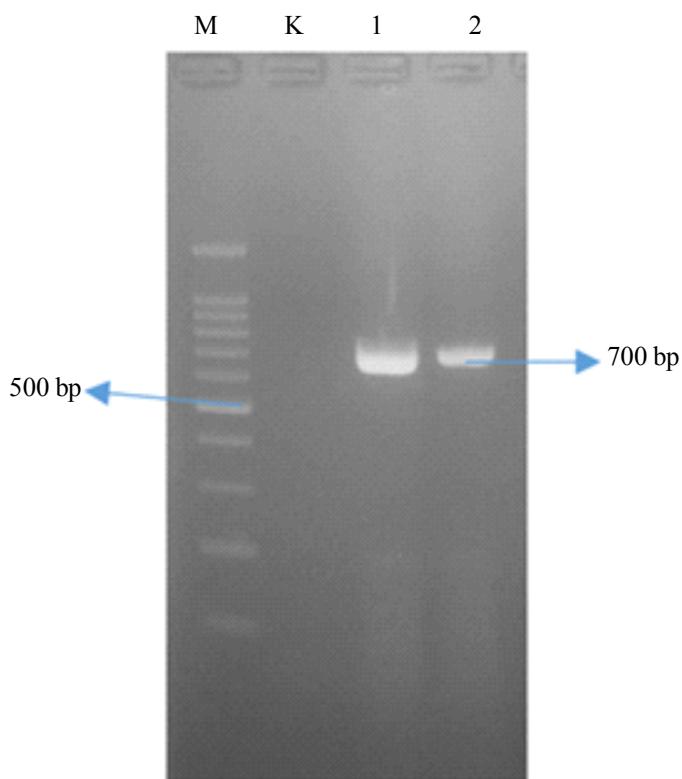


Figure 2. DNA visualization from the amplified sample. M: DNA marker, K: negative control, 1: *Helopeltis* sp. from Kaliwining 1, 2: *Helopeltis* sp. from Kaliwining 2

was at 709 bp fragment. PCR products from *Helopeltis* sp. Kaliwining were then sequenced using the BLAST program to determine the DNA base sequence. The phylogenetic results showed that the *Helopeltis* sp. from Kaliwining from BLAST results are in the same cluster as *Helopeltis bradyi*-India and have a high similarity with *Helopeltis bradyi*-Tuban up to 99%. The latest data of the two *Helopeltis* came from GenBank (Figure 3).

According to Van Hall (2001), the value of similarity can be determined from bit score parameters and identities. The higher value of the identities, the more it resembles the reference sequence in GenBank. Furthermore, Henry *et al.* (2000) state that 99–100% of identities (similarities) show the same species, while a value of 89–99% means the same genus.

Cytochrome oxidase subunit 1 (CO1) is a universal primer for PCR amplification with sizes ranging from 600-800 segments of mitochondrial DNA. This primer is used for species classification (Rubinoff *et al.*, 2006). Using phylogenetic analysis, *Helopeltis bradyi* specimens from Kaliwining were compared to *Helopeltis bradyi* and *Helopeltis antonii* specimens in the GenBank.

The phylogram from maximum likelihood analyses for 13 *Helopeltis* specimens, including *Helopeltis* specimens from Kaliwining, clustered into 2 distinct clades (Figure 3). Clade 1 comprised *H. bradyi* (specimens from Kaliwining), *H. bradyi* specimens (Kulonprogo, Batang, Wonogiri, Mojokerto, Ciamis, Banyuwangi, Serang, Tuban, Bandung, Sumbawa Barat, and Ende) from Indonesia, *H. bradyi* specimen from

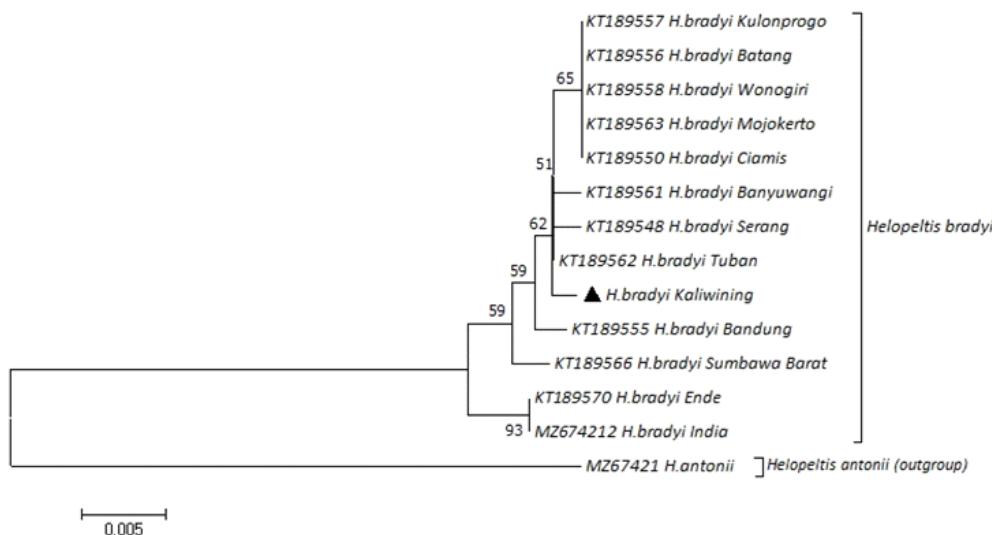


Figure 3. Phylogenetic tree of *Helopeltis* with Maximum Likelihood method from COI part of DNA with TN93+G model (BIC = 3415.617 from 25 parameters) and 1000 bootstrap. The sequence in this study of *Helopeltis* from Kaliwining, *Helopeltis bradyi* from Kaliwining was compared with *Helopeltis* from West Java, Central Java, East Java, West Nusa Tenggara, and East Nusa Tenggara, whose data were kept in GenBank.

India; and clade 2, including *H. antonii*. We strongly suggest the need for the biology of *Helopeltis* studies to delimit *Helopeltis* species further.

Identification of *Helopeltis* sp. from the Kaliwining Experimental Station molecularly has also been carried out by Pancaningtyas (2022) using COI mitochondrial gene barcoding DNA primers. Results of our present study support that study and confirm that *Helopeltis* sp. originating from the Kaliwining Experimental Station is *Helopeltis bradyi*.

CONCLUSIONS

Identification of *Helopeltis* species from Kaliwining Experimental Station, including external characters, white bands on all femora, body length, and the ratio of the first antennal segment to the posterior width of the pronotum and molecular identification, confirms the

species as *Helopeltis bradyi*. Molecular identification using PCR amplification through specific primer from COI gene sequences shows that the *Helopeltis* specimen from Kaliwining is classified as *Helopeltis bradyi*.

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