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Molecular Evidence Points to Strong Resemblance in the Parasitoid Species of Rice and Cogongrass Gall Midges, *Platygaster* spp. (Hymenoptera: Platygasteridae)

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ABSTRACT

The rice gall midge, *Orseolia oryzae*, and the cogongrass gall midge, *O. javanica*, cause gall formation on rice and cogongrass (alang-alang) (*Imperata cylindrica*). Two different species parasitize these two gall midges but closely related platygasterids, *Platygaster oryzae* on the rice gall midge and *P. orseoliae* on the cogongrass gall midge. Both the gall midges and their parasitoids are often found in the adjacent area, raising a question about the relationship between the two gall midges and their parasitoids. This research aims to study the molecular identity of the rice and cogongrass gall midges, along with their platygasterid parasitoids, based on partial sequences of the mtCOI gene. Samples were collected from rice and cogongrass in the adjacent area in Cianjur, West Java Province, and a rice field with no cogongrass in Bogor, West Java Province. Successful DNA amplification was achieved using universal primers for mtCOI. Nucleotide sequencing analysis revealed that the rice gall from Bogor and Cianjur shared 100% similarity and 93.2-99.3% similarity with the rice gall from other countries. Notably, the parasitoids *P. oryzae* collected from rice in Bogor and Cianjur shared 97.2% similarity with *P. orseoliae* collected from cogongrass in Cianjur. These findings suggest that the *platygaster* parasitoids associated with the rice gall and the cogongrass gall midges are identical, serving as potential natural enemies for both pests. This study represents the first molecular identification report of rice and cogongrass gall midges and their platygasterid parasitoids from Java Island, Indonesia.

1. Introduction

Gall midges (Diptera: *Cecidomyiidae*) are the economically significant pests affecting agricultural crops. One of the most widely recognized species is the rice gall midge (*Orseolia oryzae* Wood-Mason), which has a limited distribution in Asia, including Indonesia (Kolesik and Gagné 2020). Commonly referred to as the silver shoot, this midge causes rice shoots to develop into silvery white leek-like structures. An infestation during the vegetative phase disrupts panicle formation, preventing the panicle from developing (Ilham *et al*. 2001).

Another species, *O. javanica,* known as the cogongrass gall midge, is a monophagous insect that exclusively infests cogongrass (*Imperata cylindrica*) (Mangoendiharjo 1980). The infestation of *O. javanica* in cogongrass reduces the plant's photosynthetic capacity and depletes carbohydrate reserves in the rhizome (Arini 2017). Consequently, *O. javanica* can be a biological control agent for cogongrass (Overholt *et al*. 2016). In natural settings, the development and population density of gall midges are influenced by parasitoids, altitude, rainfall intensity, and agricultural practices (Syah and Hidayat e*t al*. 2019; Hidayat *et al.* 2020). The primary parasitoid of gall midges belongs to the genus *Platygaster* (Hymenoptera: Platygasteridae) (Reksosoesilo 1985; Soenarjo 1986). Previous studies have identified cogongrass midge

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parasitoids morphologically (Buhl and Hidayat 2015; Simamora *et al*. 2022).

The mtCOI gene is widely used as a genetic marker in molecular studies due to its conserved regions, allowing for comparison of genetic characteristics within and between species (Folmer *et al*. 1994; Smith *et al.* 2024). The conserved region of the mtCOI gene is identifiable across nearly all phyla within the Animalia kingdom, making it a useful DNA barcode (Hebert *et al.* 2003). One of the key advantages of the mtCOI gene is its specificity in identifying organisms at the species level, thanks to its well-conserved gene sequence (Hebert *et al.* 2003; Galtier *et al.* 2009). The mtCOI gene has been employed in taxonomic studies of beetles, butterflies, spiders, moths, blowflies, cotton bollworms, honeybees, whiteflies, thrips, and stem sawflies (de Mandal *et al*. 2014; Kumar *et al*. 2017; Hidayat *et al*. 2023). Currently, nucleotide sequence information for the mtCOI gene of *O. oryzae*, *O. javanica*, and their parasitoids from Indonesia is not available in the GenBank database. Thus, this research is necessary to obtain molecular characterization and to examine the genetic homology among *O. oryzae*, *O. javanica,* and their parasitoids from Indonesia.

2. Materials and Methods

2.1. Sample Collection

The collections were conducted at two locations: Location 1, situated around Situ Gede, Bogor (6°33′ 8.1″S, 106°44′46.5″E), consisted solely of rice field plantations; Location 2, located in Gekbrong, Cianjur (6°52′06″S, 107°02′41″E), comprised rice field plantations surrounded by cogongrass (*Imperata cylindrica*). Rice gall midges were collected from both the rice fields in Bogor and Cianjur (Locations 1 and 2). In contrast, cogongrass midges were collected from the cogongrass surrounding another rice field plantation in Cianjur (Location 2). The galls were provided with moist cotton at the root or stalk and placed in ventilated plastic containers. The galls were then reared until the imagos and their parasitoids emerged. Images and parasitoids were stored individually in Eppendorf tubes at -20°C in 96% alcohol for further morphological and molecular characterization.

2.2. Morphological Identification

Insects from galls were identified to genus and species by examining their morphological features, using references such as the *Illustrated Key to West-Palearctic Genera of Pteromalidae* (Bouček & Rasplus 1991), *Annotated Keys to the Genera of Nearctic Chalcidoidea* (Gibson *et al*. 1997), and other relevant sources. Specimen observations were conducted with an Olympus SZ51 stereo microscope, a Leica M205C microscope, and a Leica DFC450 digital camera connected to LAS V.4.4.0 software.

2.3. Molecular Identification

2.3.1. Total DNA Extraction

Total DNA was isolated using the GeneJet Genomic DNA Purification Kit following the manufacturer's protocol (Thermo-Fisher Scientific, Memphis, US). A minor modification was made by adding 180 µl of digestion solution and 20 µl of proteinase K. The insect was then crushed using a sterile pistil and incubated at 65°C for 1 hour. The total DNA was stored at -20°C in the final stage for further amplification.

2.3.2. Amplification of mtCO1

Total DNA was amplified using a universal primer of COI, LCO1490-F (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'), and HCO2198-R (5'-TAA TCA ACT GGA TGA CCA AAA AAT CA-3') (Folmer *et al.* 1994). PCR cocktail consisted of 2 × Dream Taq Green master mix (Thermo-Fisher Scientific, Memphis, US) (12.5 µl), 10 µM of each primer (1 µl), 1 µl of DNA template, and then added nuclease-free water up to 25 µl of total volume. The amplification program consisted of pre-denaturation at 94°C for 5 minutes, then continued with 35 cycles of denaturation at 94°C for 1 minute, annealing at 52°C (gall midges) or 50°C (parasitoids) for 35 seconds, and elongation at 72°C for 90 seconds. The amplification was then ended with post-elongation at 72°C for 7 minutes. The positive amplicons were then sent for nucleotide sequencing.

2.3.3. Nucleotide Sequence Analysis

Each nucleotide sequence was trimmed and assembled to produce a contig in Geneious Software (Biomatters Ltd., NZ). Each contig is then put in the Basic Local Alignment Search Tools (BLAST) program in the GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to search and choose highly similar sequences. Multiple pairwise sequence alignments were done by aligning the contigs and chosen sequences using the ClustalW program in Geneious Software. Each sample's identity was then visualized using SDT matrix software (SDTv1.2) (Muhire *et al*. 2014). A Maximum-Likelihood phylogenetic tree

was constructed using MEGA 11 software with 1000 bootstrap replicates (Tamura *et al*. 2021).

3. Results

3.1. Rice and Cogongrass Gall Midges and their Parasitoids

The cogongrass gall midge, *O. javanica*, is a species known for infesting young cogongrass, commonly called the cogongrass gall midge. In general, cecidomyiid has distinct morphological features, including reduced venation on wings covered in long hairs. Images of gall midges possess elongated legs, with the first tarsomere shorter than subsequent segments. Larval infestations lead to gall formation, providing a habitat for pupal development and ultimately affecting the cessation and mortality of cogongrass growth. Reddish, pointed-pipe-shaped galls on young cogongrass characterize infestation symptoms.

On the other hand, the rice gall midge, *O. oryzae*, is smaller than *O. javanica* (Figure 1), with body coloration ranging from brownish-yellow to reddish-orange. Male abdomens elongate cylindrically, while females have a more rounded abdomen. Fine dark hairs cover the body, antennae, legs, and wings. The antennae consist of 12 segments, featuring a thin-walled flagellum attached to the pedicel and scape. *O. javanica* imagos exhibit a darker body color and relatively larger size, with female imagos measuring approximately 6.53 mm and male imagos around 3.34 mm compared to other *Orseolia* species.

Parasitoids were obtained from field-reared galls emerging from within the reared galls: rice gall (Bogor), rice gall (Cianjur), and cogongrass gall (Cianjur) (Figure 2A and B). The identified parasitoids belong to the order Hymenoptera and family Platygasteridae. The parasitoids are koinobiont to the family Cecidomyiidae (Diptera), meaning they do not directly kill the host; instead, the female parasitoid lays eggs on the host's eggs or early instar larvae, and the imago emerges from the host's pupa. Male and female adults can be distinguished based on antenna shape and the presence of an ovipositor. Male adults lack an ovipositor, and the fourth antenna segment is unmodified.

The parasitoid species attacking rice gall midges (Bogor and Cianjur) is *P. oryzae*. The *P. oryzae* imago attacking rice gall midges differs in shape and size from the *Platygaster* attacking cogongrass gall (Figure 2). *P. orseoliae* morphological characteristics include a body length of nearly 1 mm, blackish-brown body color, and brown antennae and legs (Figure 2B). The *P. oryzae* imago has a very small body size, approximately 0.8 mm long, and a dark body color (Figure 2B).

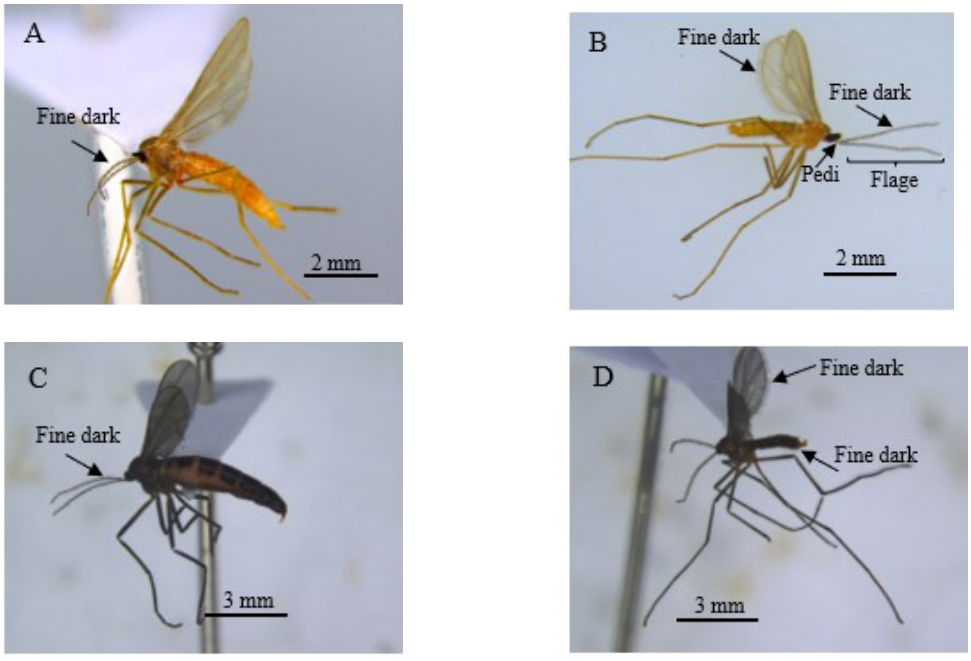

Figure 1. Adults of gall midge from rice *O. oryzae*: (A) female, (B) male; and cogongrass *O. javanica*: (C) female, (D) male

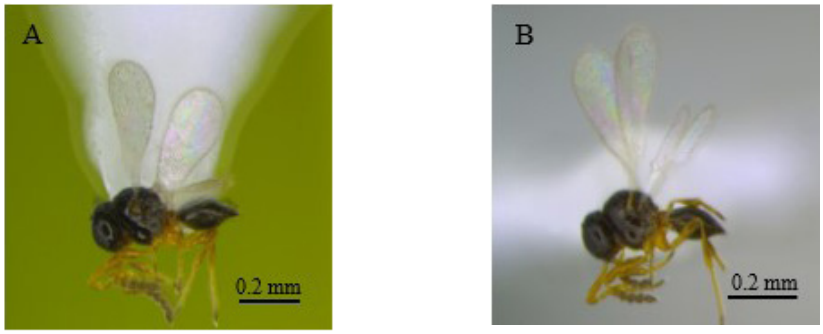

Figure 2. Parasitoids of gall midges of rice imagoes: (A) *P. oryzae*, and cogongrass (B) *P. orseoliae*

3.2. Molecular Identification of Gall Midges from Rice and Cogongrass

The universal primer of mtCOI successfully amplified the target gene from gall midges and *Platygaster* parasitoid. The DNA band produced according to the desired target was ± 710 bp fragment (data was not shown). After trimming and removing primer sequences, the alignment of mtCOI sequences (538 nt) showed that there were single nucleotide variations within the gall midge species on several points (Figure 3). We highlighted the conserved C/T pattern of *O. oryzae* (Bogor and Cianjur) and *O. javanica* (Cianjur) on positions #73, #183, #247, #367, #403, #419. The exception of this pattern was found in *O. oryzae* from India, whose C/T pattern was more closely related to *O. javanica.*

Nucleotide sequence analysis showed that rice gall midges *O. oryzae* collected from Bogor and Cianjur shared 100% identity (Figure 4). Furthermore, mtCOI nucleotide sequences of *O. oryzae* species from Indonesian rice (Cianjur and Bogor) shared 93.2-99.3% similarities among *O. oryzae* from other countries (Figure 4). Meanwhile, *O. javanica* shared 90.5% and 90.1%-91.4% with *O. oryzae* from Indonesia and another country, respectively. Both *O. oryzae* and *O. javanica* nucleotide sequences have been deposited in the GenBank with accession numbers LC761943.1, LC761944.1, and LC765198.1, respectively. The phylogram analysis placed *O. oryzae* from rice in one cluster with another *O. oryzae* from Thailand, while *O. javanica* was in a separate sub-group but still in the Cecidomyiidae family (Figure 5).

3.3.Molecular Identification of Parasitoids from Rice and Cogongrass

The alignment sequence of *platygaster* parasitoids from *O. oryzae* and *O. javanica* (582 nt) showed the conserved single nucleotides that only belong to both Indonesian platygaster samples (Figure 6). There were "C" sequences that were only found in Indonesian *platygasters* in the position between #130 and #310. Interestingly, the sequence "ATTC" in the #86-#89 was only found in the *P. orseolie* from *O. javanica* (Cianjur). Moreover, the insertion sequence of "CCATAGAGG" in the position between #357 and #365 differentiated between Eulopidae and Platygasters. Both *P. oryzae* from Cianjur and Bogor have been lodged in the GenBank under the accession numbers LC765199.1 and LC765200.1, respectively, while *P. orseolie* from Cianjur under the accession number LC769361.1.

Platygaster samples from Indonesia have a range homology value of 78.18-84.2%, with *Platygaster* sp. from other countries (Figure 7). Nucleotide sequence alignment of parasitoids *P. oryzae* from rice gall midge shared the highest similarity with *P. oreseolie* from cogongrass midge (97.54%) (Figure 7). This data indicates that the close relationship between the two species was even possible, and the possibility of *P. oryzae* and *P. orseoliae* being the same species. The neighbor-joining phylogram revealed that both *P. oryzae* and *P. orseoliae* were grouped in one group (Figure 8).

4. Discussion

This study provides the first molecular identification of the rice gall midge (*Orseolia oryzae*), cogongrass gall midge (*O. javanica*), and their Platygasterid parasitoids in Indonesia using partial mtCOI DNA sequences. The mtCOI gene was chosen for its widespread use and availability in GenBank compared to the 16S rRNA gene (Hebert *et al*. 2003; Hajibabaei *et al*. 2016). Folmer primers (LCO1490-F and HCO2198-R) were utilized for their broad applicability across various invertebrates (Folmer *et al*. 1994; Hoque *et al.* 2022;

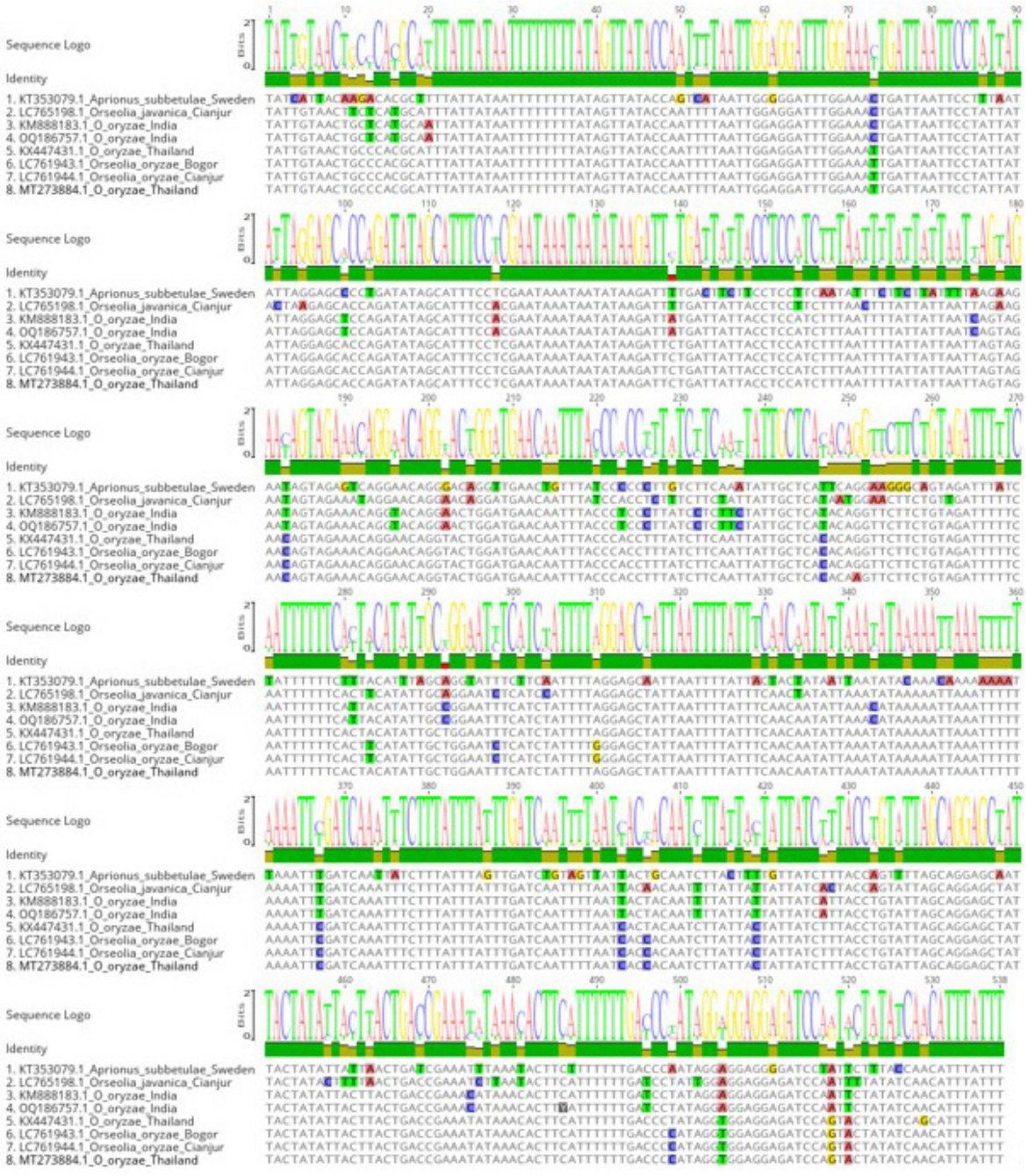

Figure 3. Nucleotide sequence alignment of *Orseolia oryzae* from rice and *Orseolia javanica* from cogongrass with other orseolia from overseas visualized with Geneious Prime software. Aprionus subbetulae was used as an outgroup

Brower *et al*. 2024). Previous research has demonstrated that Folmer primers provide better species assignment accuracy (80.7%) compared to Clarke primers (28.7%) (Smith *et al*. 2024).

The study found that the rice gall midge (*O. oryzae)* and cogongrass gall midge (*O. javanica*) share a 90.0% similarity in their mtCOI sequences. In comparison, their associated parasitoids show an even higher similarity of 99.5% (Figures 4 and 7). The mtCOI gene is considered conserved at the species level with a threshold of ≥3% (Song *et al*. 2008). These findings were further supported by phylogenetic tree analysis (Figures 5 and 8), which confirmed that the parasitoids *Platygaster oryzae* and *P. orseoliae* are closely related.

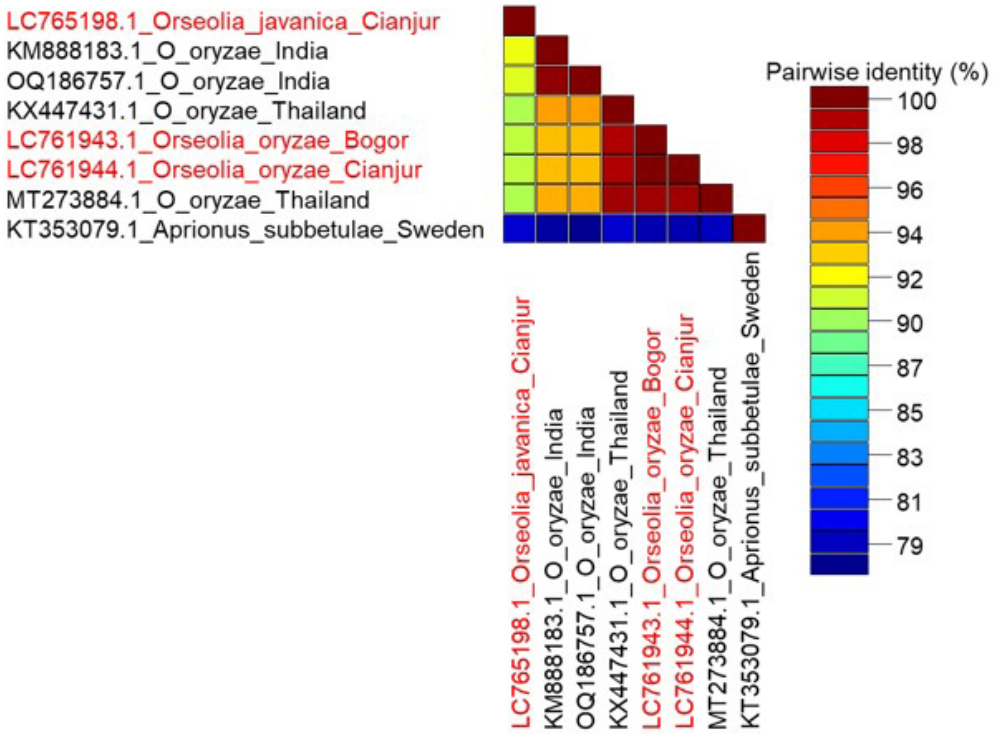

Figure 4. Sequence identity matrix of *Orseolia oryzae* from rice and *Orseolia javanica* from cogongrass. The percentage of identity was presented in color code. Orseolia samples from Cianjur are written in red

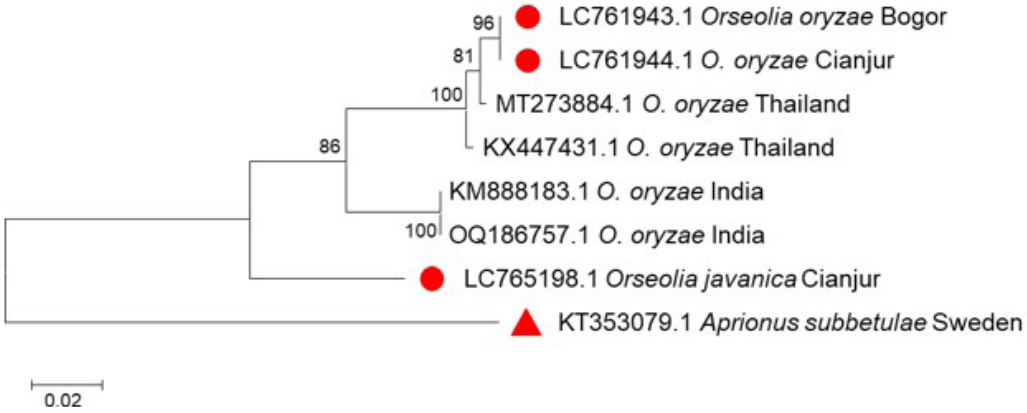

Figure 5. Neighbor-joining phylogenetic tree of *Orseolia oryzae* from rice and *Orseolia javanica* from cogongrass. The phylogeny was implemented in MEGA 11 and bootstrap with 1000 replicates. Orseolia samples from Cianjur and Bogor were written in red

Building on the work of Buhl and Hidayat (2015), who identified *P. orseoliae* as a new species from *O. javanica*, our study shows that the genetic similarity between the rice and cogongrass gall midges is 90.0%, while their parasitoids share a 99.5% similarity based on partial mtCOI sequences. The mtCOI gene sequences are considered conserved at the species level with a threshold of ≥3% (Song *et al.* 2008), supporting the close genetic relationship between the parasitoid species.

The molecular and phylogenetic analyses indicate a high level of genetic similarity between *P. oryzae* and *P. orseoliae*, with a nucleotide sequence similarity of 97.54% (Figures 6 and 8). This suggests that these parasitoids may be the same species with different host preferences or represent a recent divergence. The close genetic relationship could imply a shared evolutionary history or even a potential to be considered a single species with varying ecological roles (Brower 2006; Song *et al.* 2008). Conserved single nucleotide

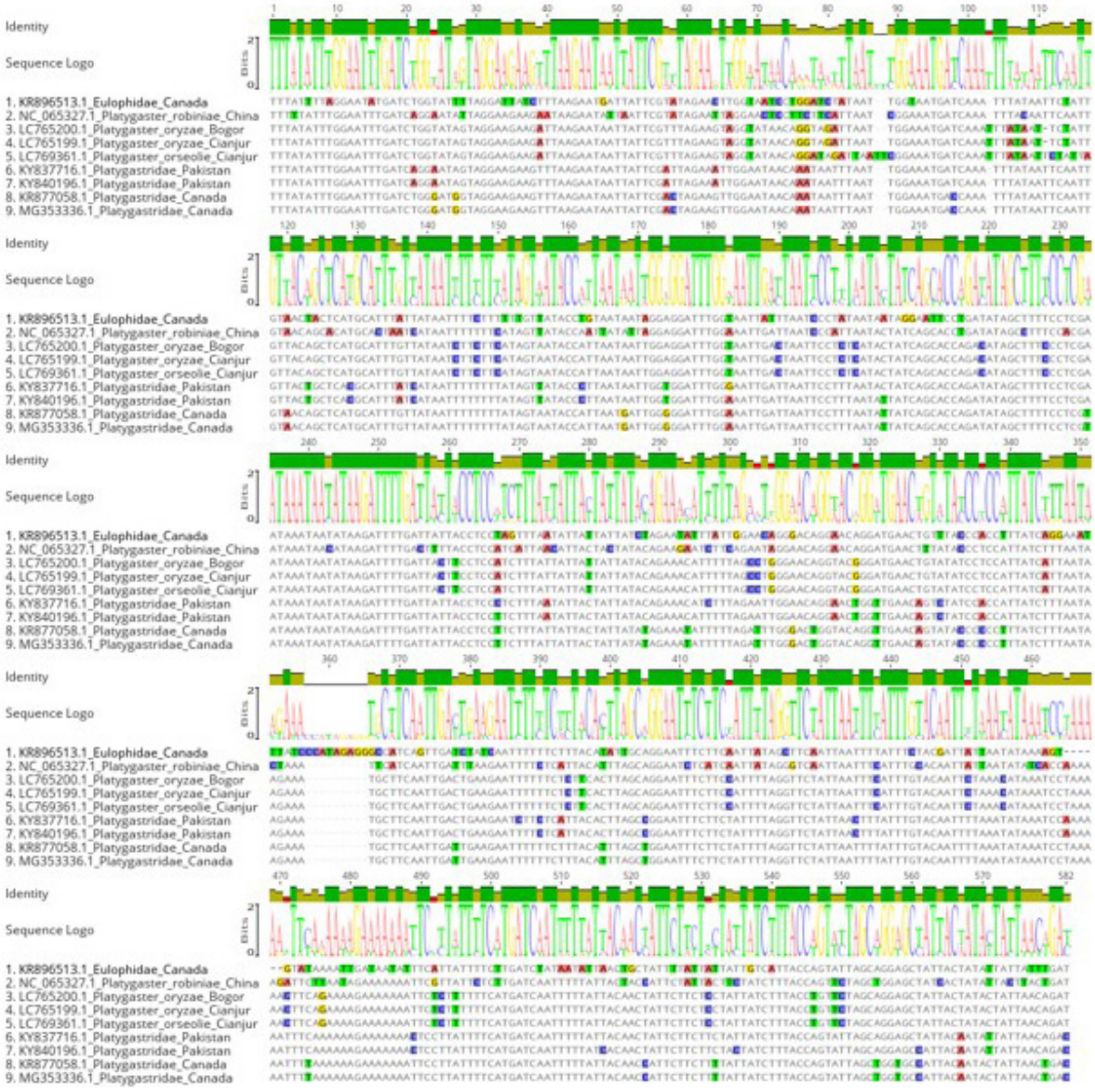

Figure 6. Nucleotide sequence alignment of *Orseolia oryzae* from rice and *Orseolia javanica* from cogongrass with other orseolia from overseas visualized with Geneious Prime software. Eulophidae family was used as an outgroup

variations specific to Indonesian samples reinforce the notion of a shared genetic lineage despite differences in host specificity.

Morphological characteristics of *O. oryzae* and *O. javanica* observed in this study are consistent with previous descriptions, with *O. javanica* being larger and darker than *O. oryzae* (Sama & Yata 2012). These differences highlight their adaptation to distinct host plants-rice and cogongrass. The cogongrass gall midge, *O. javanica*, is significant for its impact on cogongrass, resulting in reddish, pointed galls inhibiting plant growth and survival (Day & Moxley 2002). The parasitoids *P. oryzae* and *P. orseoliae* play crucial roles in biological control. *P. oryzae* was identified as the predominant parasitoid associated with rice gall midges in both Bogor and Cianjur, whereas *P. orseoliae* was linked to cogongrass gall midges in Cianjur. *P. oryzae* has been previously noted for its high parasitism rates, making it a key agent in the natural control of rice gall midge populations (Soenarjo 1986; Reksosoesilo 1989).

The high genetic similarity between *P. oryzae* and *P. orseoliae* suggests that these parasitoids may have overlapping host ranges, which could enhance their effectiveness in biological control. This potential for cross-host parasitism highlights the complexity of host-

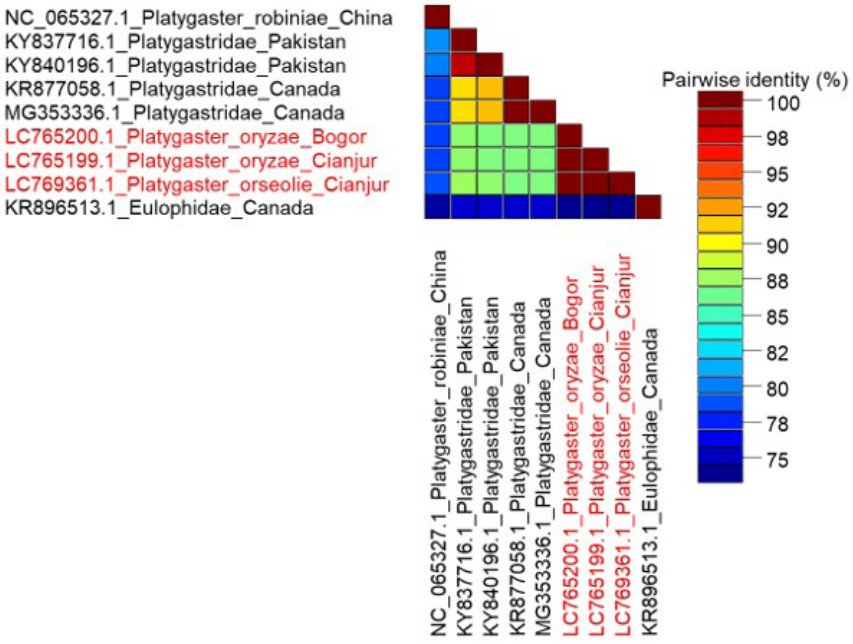

Figure 7. Sequence identity matrix of *Platygaster oryzae*, parasitoid of *Orseolia oryzae* and *Platygaster*. *Orseolie*, parasitoid of *Orseolia javanica*. The percentage of identity was presented in color code. Platygaster samples were written in red

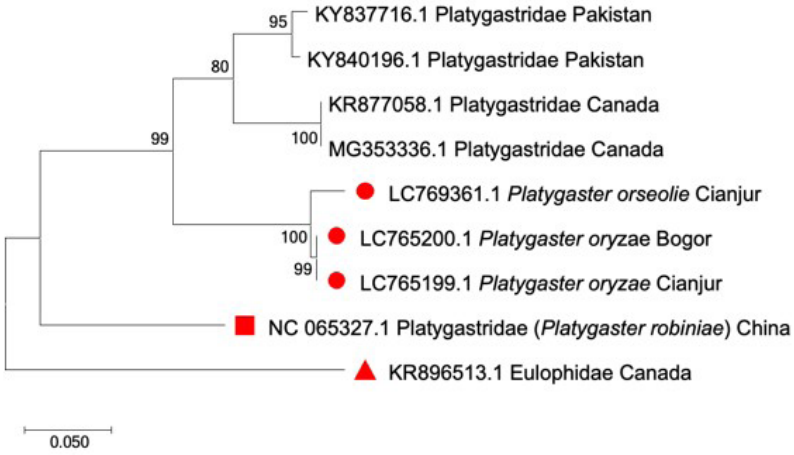

Figure 8. Neighbor-joining phylogenetic tree of *Platygaster oryzae*, parasitoid of *Orseolia oryzae* and *Platygaster*. *Orseolie*, parasitoid of *Orseolia javanica*. The phylogeny was implemented in MEGA 11 and bootstrap with 1000 replicates. *Orseolia* samples from Cianjur and Bogor were written in red

parasitoid interactions and underscores the importance of understanding these relationships for effective pest management strategies (Goulet & Huber 1993; Hebert *et al*. 2004). Future research should investigate the ecological and behavioral aspects of these parasitoids, focusing on host preferences and potential cross-host parasitism. Further studies on the genetic diversity of gall midges and their parasitoids across various regions could provide deeper insights into their population dynamics and distribution.

In summary, this study successfully identified the molecular relationships between the rice gall midge (*Orseolia oryzae*), the cogongrass gall midge (*O. javanica)*, and their Platygasterid parasitoids using partial mtCOI DNA sequences. The high genetic similarity among the parasitoids suggests that they may be the same species, offering valuable information for biological control strategies in rice and cogongrass fields. These findings enhance our understanding of these species' genetic relationships and evolutionary

dynamics, contributing to the development of effective pest management strategies and emphasizing the need for molecular and morphological studies to understand pest species and their natural enemies better.

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