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Article

Cryptic species diversity of rice hopper parasitoids in Southeast Asia

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Abstract: On-going intensification of rice production systems in Southeast Asia is causing devastating yield losses each year due to rice hoppers. Continuing development of immunity to resistant rice varieties and pesticide application further complicate this problem. Hence, there is a high demand for biological control agents. Egg parasitoid wasps are among the most important natural enemies of rice hoppers such as *Nilaparvata lugens* and *Nephotettix* spp. However, our knowledge on their diversity is still very limited due to their small size and the lack of available morphological information. Classifying these parasitoids is the first step to properly understand their role in the rice agroecosystem. We used traditional morphological identification as well as DNA sequencing of COI and 28S genes to investigate the diversity of four important hopper egg parasitoid genera in the Philippines. Parasitoids of the genera *Anagrus* spp., *Oligosita* spp., *Gonatocerus* spp. and *Paracentrobia* spp. were collected in eight study landscapes located in Luzon. We found discrepancies between the morphological and the molecular analysis. Morphological and molecular results were only valid for *Paracentrobia* spp. *Anagrus* spp. and *Gonatocerus* spp. showed more genetic diversity, than expected after the morphological analysis, indicating cryptic species. The sequences for *Oligosita* spp. revealed less variation than expected. This is the first study on molecular diversity of rice parasitoids in the Philippines. More research combining morphological, behavioural and genetic methods as well as the establishment of a comprehensive DNA database is urgently needed to assess the performance and suitability of these organisms as biocontrol agents.

Keywords: DNA barcoding; genetic diversity; hymenopteran parasitoids; *Nephotettix* spp.; *Nilaparvata lugens*; rice

1. Introduction

Rice is the main food resource for more than half of world's population [1,2]. The rice production system of Asia is one of the most important food production systems on Earth [3]. The brown planthopper (BPH; *Nilaparvata lugens*, Stål 1854) and the green leafhopper (GLH; *Nephottetix* spp.) are among the economically major rice pests. These insects cause immense damages in the Asian rice paddies through xylem sap feeding resulting in wilting of the rice crops which subsequently die [4] as well as through the transfer of devastating viruses among fields [5,6]. So far, the introduction of rice varieties resistant to BPH and GLH has not been successful as these pests rapidly adapt to the new varieties [7,8]. Pesticide application further enhances the problem by disturbing the rice agroecosystem, which can increase outbreak risk of hopper [7,9,10].

An alternative solution to the perpetual increasing application of synthetic pesticides in Southeast Asia [1] is the biological control of rice pests using their natural enemies. BPH and GLH

are typically attacked by a wide range of natural enemies, such as spiders, predatory bugs, dragonflies and egg parasitoid wasps from the *Mymaridae* and *Trichogrammatidae* family [11]. Among these enemies, parasitoid species are of particular interest. These organisms are very mobile and can disperse over large distances [12]. In addition, adults feed on pollen and nectar or the honeydew of their sap-feeding hosts, whereas their larvae develop in the hopper eggs and disrupt the hoppers' life cycles at the earliest possible stage [11]. In two previous studies on egg parasitoids of rice hoppers, egg parasitism levels of more than 60% have been observed [13,14]. This is supported by Drechsler & Settele [15], who showed that parasitoids can play a major role in controlling hoppers pests in rice agroecosystems.

Despite their importance, knowledge about species composition and diversity of parasitoid wasps especially in the tropics is still limited (but see Nishida, Wongsiri and Wongsiri [16] as well as Gurr *et al.* [11]).The morphological species identification requires extensive taxonomic expertise and is hampered by the small size (<1.5 mm) of the wasps as well as the limited amount of literature. To date, molecular information on rice parasitoids from the Philippines is completely lacking. Molecular methods have become a promising tool to resolve species identities [17–19]. Different barcodes such as the mitochondrial cytochrome c oxidase I (COI) and fragments of the small or large subunits of the ribosomal RNA (18S or 28S rRNA) genes have been applied to construct hymenopteran and dipteran parasitoid phylogenies [20,21]. However, molecular analyses might not only improve species identification but might also reveal cryptic species [22,23]. Smith et al. [24] suggested to combine barcoding with morphology and natural history. A similar conclusion was drawn by Padial and colleagues [25] in their review on an integrative taxonomy for improvement of species discovery and description.

In a previous study, we investigated parasitoid wasps of *Nilaparvata lugens* (BPH) and *Nephotettix* spp. (GLH) in eight rice production landscapes located in Luzon, Philippines (Sann et al. 2017, unpublished data). We found that BPH was parasitized by the Chalcid genera *Anagrus* spp. and *Oligosita* spp., while GLH was parasitized by the Chalcid genera *Gonatocerus* spp. and *Paracentrobia* spp. In the present study, we analyzed the diversity of these genera by traditional morphological and molecular techniques. The present study provides the first molecular identification of parasitoid wasps in rice paddies in the Philippines, combined with a taxonomic identification based on dichotomous keys. Moreover, the results of this study highlight the benefits of using molecular approaches for a rapid identification of parasitoid diversity and form a basis for further molecular studies on parasitoids wasps in the Philippines.

2. Materials and Methods

2.1. Study System and Sampling

This study was embedded in the project LEGATO, which focused on a sustainable rice production [26]. Parasitoids were collected in eight study landscapes located in the Laguna province, Luzon, Philippines (Figure S1). Sampling took place during the rice growing and fallow period of the dry season from February to June 2013. In brief, rice plants of the variety Taichung Native (1) (TN1) were grown in a greenhouse for 6 weeks, trimmed to three tillers and covered with small tubular insect cages (85 cm high, 15 cm diameter). Greenhouse cultures of BPH and GLH were reared on TN1 as previously described by Heinrichs et al. [27]. The BPH culture consisted exclusively of *Nilaparvata lugens*, while the GLH culture consisted of *Nephotettix virescens* (Distant) and *Nephotettix nigropictus* (Ståhl). Both populations came from wild individuals caught in the rice fields of the Laguna Province. Different GLH species can only be distinguished by the male genitalia and the establishment of a pure population is very difficult.

Four gravid females of either BPH or GLH were released into each cage for 48 h to lay eggs. Subsequently, the plants were transferred to three different plots per study site, to cover the naturally occurring landscape diversity in the Laguna province. Three plants infested with BPH

eggs and three plants infested with GLH eggs were distributed randomly within each plot. After 72 h, all plants were returned to the greenhouse and placed back inside separate insect cages. The parasitoids hatched 13-17 days after the field exposure of the eggs. Adult parasitoids were collected daily within this period, morphologically identified to genera level and stored at -80°C. In total, 19,455 parasitoids were collected and initially separated into the four genera *Paracentrobia* spp., *Gonatocerus* spp., *Oligosita* spp. and *Anagrus* spp. using morphological keys [28–30].

2.2. Preliminary morphological identification

Fifty parasitoids from each of the four identified genera (*Paracentrobia* spp., *Gonatocerus* spp., *Oligosita* spp. and *Anagrus* spp.) were randomly selected. The 200 parasitoids were slide mounted and identified to species level using a microscope at the International Rice Research Institute (IRRI) using the protocols previously described [28–30].

2.3. DNA extraction and amplification

Total genomic DNA was extracted from 105 whole single parasitoid individuals (Table S2) employing the Phire Animal Tissue Direct PCR Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Parasitoids were suspended in 20 µl TE buffer (100 mM Tris, 10 mM EDTA). To increase DNA extraction efficiency, 1 µl Proteinase K (20 mg mL^{-1}) was added. Parasitoid samples were carefully homogenized with sterile micro pestles and incubated over night at room temperature.

Two independent gene fragments were amplified from the extracted DNA: one located on the COI subunit I (COI I, amplicon length 670bp) and the other one on the expansion regions D2-3 of the 28S ribosomal subunit (28-D2, amplicon length 610 bp). The COI region was amplified using the primer pair HCO2198/LCO1490 [31]. The PCR reaction mixture (25 µl) contained 2.5 µl of 10-fold Ex Taq Buffer (Takara Biotechnology, co., LTD, Dalian, China), 25 mM $MgCl_2$, 2.5 mM of each of the four dNTPs (deoxynucleotide triphosphates), 10 µM of each primer, 1 U TaKaRa Ex Taq polymerase Buffer (Takara Biotechnology), and approximately 25 ng parasitoid DNA. The following thermal cycling scheme was used: initial denaturation at 94°C for 3 min, 5 cycles of denaturation at 94°C for 45 s, annealing at 45°C for 45 s, followed by extension at 72°C for 1 min, and25 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, followed by extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min.

The D2-D3 region of 28S rDNA was amplified as described for the PCR above using the primers D2-3549 [32] and D2-4068 [33]. The following thermal cycling scheme was used: initial denaturation at 94°C for 3min, 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, followed by extension at 72°C for 1 min. The final extension was carried out at 72°C for 6 min. Negative controls were performed by using the reaction mixture without template. Obtained PCR products were controlled for appropriate size and then purified using the peqGOLD Gel Extraction kit as recommended by the manufacturer (Peqlab, Erlangen, Germany; now VWR).

Sequencing was performed at the Göttingen Genomics Laboratory using an ABI 3730xl system and a BigDye terminator chemistry version 3.1 (Thermo Fisher Scientific).

2.4. Data analysis

Forward and reverse DNA sequences were processed with Gap5 v 1.2.14-r [34]. DNA sequence alignment and analysis was performed using MEGA 6 [35]. The best model to construct parasitoid identities was determined assuming partial deletion, site coverage cut-off of 95% and the branch swap filter set to 'very strong'. Additional Chalcidoidea (the super family containing all four genera) amino acid sequences as reference material were selected by using the National Center for Biotechnology Information (NCBI) BLAST tool [36] and the BOLD Identification System (http://www.boldsystems.org) (Table S3). Maximum likelihood trees were generated under the

assumption of the best fitting model and with 1000 bootstraps. Finally, median-joining networks were constructed using the software NETWORK v. 4.6.1.2 [37].

The genetic diversity between and within species was estimated using additional sequences from the superfamily Chalcidoidea (Table S3). The appropriate models to calculate the distances were determined with MEGA. The 28S distances were calculated based on the K2 model, COI distances were calculated based on the GTR model. The values were compared using a Kruskal-Wallis test and the Dunn's post hoc test [38] in R version 3.2.3 **[39]**.

To prove whether the local diversity of parasitoid species was covered by the sample size used in the analyses, we performed a rarefaction analysis (Figure S4, Figure S5) in R version 3.2.3. using the packages vegan **[40]** and drc **[41]**

2.5. Sequence data deposition

All sequence data have been submitted to the NCBI GenBank databases under accession number XXXX-YYYY.

3. Results

3.1. Morphological Analysis

In total, 200 parasitoids were determined morphologically. Only one species was found for the genera Gonatocerus spp. and Paracentrobia spp., while three species were found for the genus Anagrus spp. and Oligosita spp.: Anagrus flaveolus (11 specimen), Anagrus optabilis (6 specimen), Anagrus frequens (33 specimen), Oligosita aesopi (41 specimen), Oligosita naias (7 specimen), Oligosita shibuyae (2 specimen), Gonatocerus orientalis (50 specimen), Paracentrobia andoi iishi (50 specimen) (Table 1, Table S1).

Table 1. Species idenetified by the morphological analysis within 50 specimen per genera (GLH: green leafhopper, BPH: brown planthopper).

Species	Host	Examined individuals
Paracentrobia andoi	GLH	50
Oligosita aesopi	BPH	41
Oligosita naias	BPH	7
Oligosita shibuyae	BPH	2
Anagrus frequens	BPH	33
Anagrus optabilis	BPH	6
Anagrus flaveolus	BPH	11
Gonatocerus orientalis	GLH	50

3.2. Genetic Analysis

Molecular analyses were successfully performed on a total of 105 parasitoid samples (Table S2). We failed to sequence 162 specimen, which could not be processed further. A total of 86 (COI) and 105 (28S) sequences were used to create the neighbouring joining trees. The final dataset for comparison of the two gene fragments included the sequences from 74 parasitoid individuals.

Rarefaction analysis for the local hopper egg parasitoids diversity revealed that sampling saturation was reached for the gene 28S (Figure S2). In contrast, the curve for COI was not saturated (Figure S3).

3.3. Comparison between the morphological and genetical approach

The genetic analyses based on COI and 28S fragments revealed that the sequences clearly segregate according to the morphological pre-assigned genera (Figure 1-4, Figure S4-S5). Oligosita spp. exhibited the lowest genetic diversity with two sequences differing by one base pair for COI, while all other sequences were identical. In contrast, three Oligosita species were identified using the morphological approach. Similar to Oligosita spp., Paracentrobia spp. sequences were uniform, with only two highly similar haplotypes for the 28S and three highly similar haplotypes for the COI gene sequence (Figure 1-4, Figure S4-S5). This is in accordance to the morphological data, where only one Paracentrobia spp. species was found. On the other hand, the sequences from Gonatocerus spp. exhibited more variability than the morphological data for both genes. Three clusters could be unambiguously identified, with one cluster occurring prevalently (83.3% for 28S, 84.2% for COI) compared to the other two clusters (Figure 1-4, Figure S4-S5). Anagrus spp. was by far the most genetically diverse genus, with 7 different haplotypes identified for the 28S gene sequence and 12 haplotypes for the COI sequence (Figure 1-2, Figure S4-S5, Table S2).

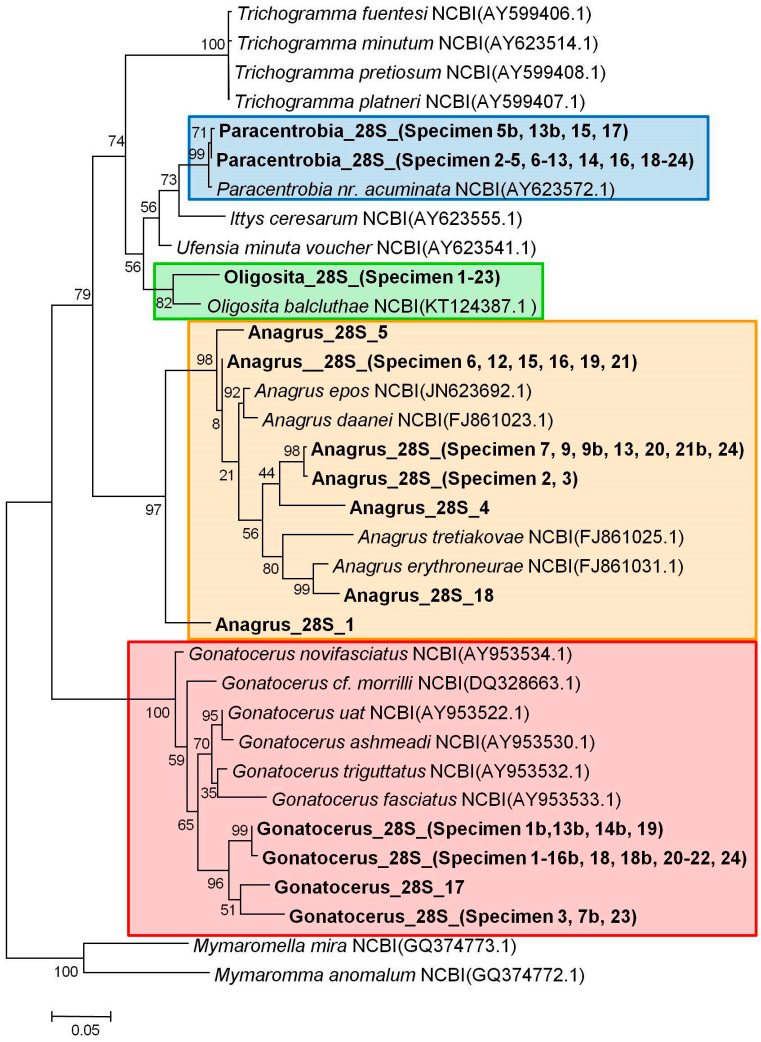

Figure 1. Maximum likelihood tree for the 28S sequences (bootstraps =1000, TN93 model), including 105 rice parasitoids and 17 outgroup specimens. Maximum likelihood bootstrap values are given for each node. Sequences with the same haplotype have been pooled together.

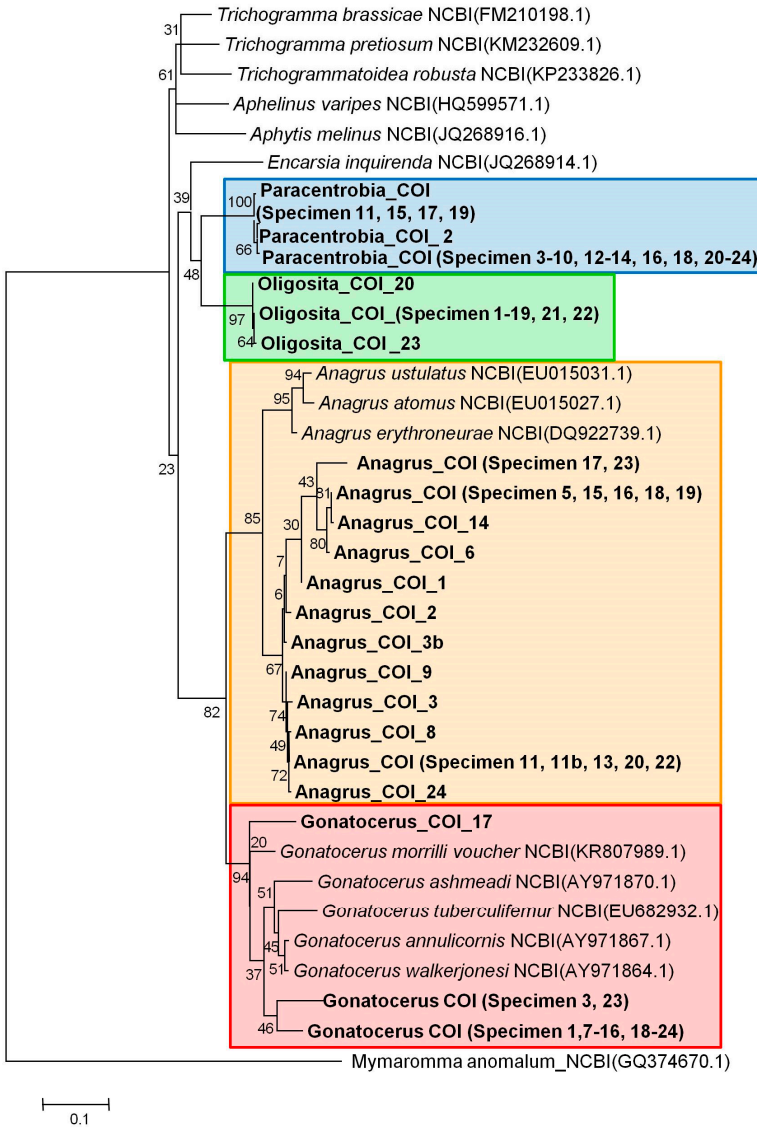

Figure 2. Maximum likelihood tree for the COI sequences (bootstraps =1000, GTR model), including 85 rice parasitoids and 15 outgroup specimens. Maximum likelihood bootstrap values are given for each node. sequences with the same haplotype have been pooled together.

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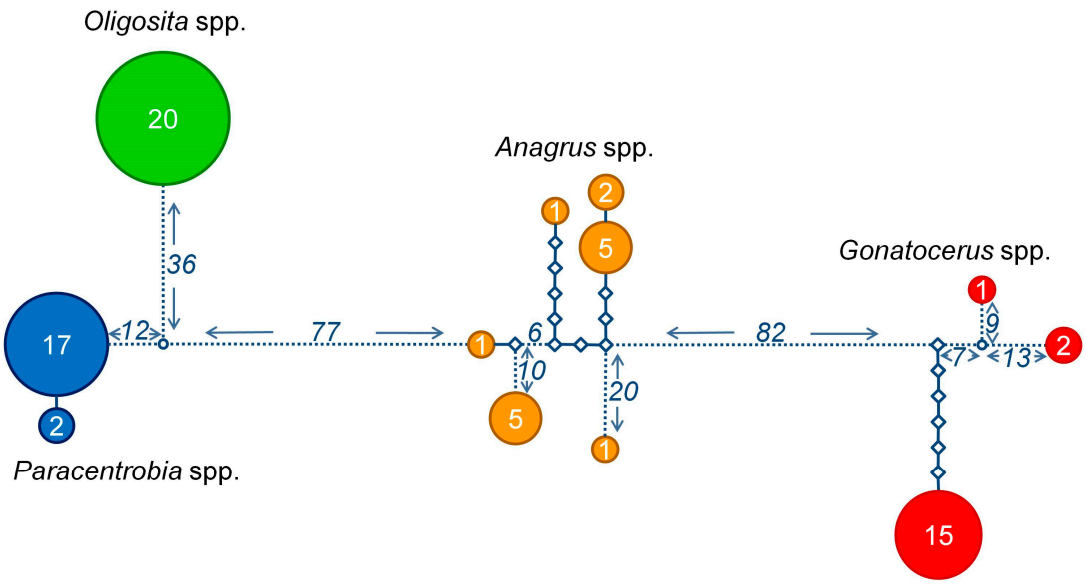

Figure 3. Overall 28S haplotype distances. Network representing the amount of substitutions between the different 28S sequences obtained from different parasitoid genera: Paracentrobia spp. (blue), Oligosita spp. (green), Anagrus spp. (orange) and Gonatocerus spp. (red). The sample size for each haplotype is written inside the respective circles. Each diamond represents a substitution. The numbers next to the dotted lines are the number of substitutions not represented in detail in the figure.

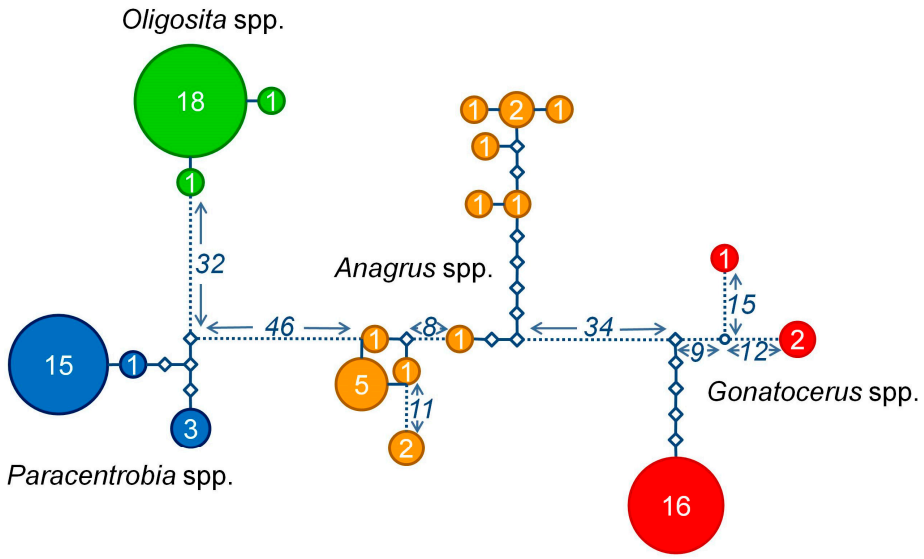

Figure 4. Overall COI haplotype distance. Network representing the amount of substitutions between the different COI sequences obtained from different parasitoid genera: Paracentrobia spp. (blue), Oligosita spp. (green), Anagrus spp. (orange) and Gonatocerus spp. (red). The sample size for each haplotype is written inside the respective circles. Each diamond represents a substitution. The numbers next to the dotted lines are the number of substitutions not represented in detail in the figure.

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The degree of genetic diversity found in Anagrus spp. and Gonatocerus spp. sequences was particularly high. The pairwise distance was 0.075 ± 0.012 SE (28S) and 0.050 ± 0.009 SE (COI) for Anagrus spp. and 0.069 ± 0.012 SE (28S) and 0.100 ± 0.016 SE (COI) for Gonatocerus spp. In contrast, the pairwise distance was 0.000 ± 0.000 (28S) and 0.005 ± 0.003 SE (COI) for Oligosita spp. and 0.001 ± 0.001 (28S) and 0.009 ± 0.004 SE (COI) for Paracentrobia spp. (Figure 5). Paracentrobia spp. and Oligosita spp. were significantly different from the congeneric Chalcidoidea data but not from the conspecific Chalcidoidea (Figure 5, Table 2). The genetic differences of Gonatocerus spp. and Anagrus spp. were within the same order of magnitude as the congeneric Chalcidoidea data.

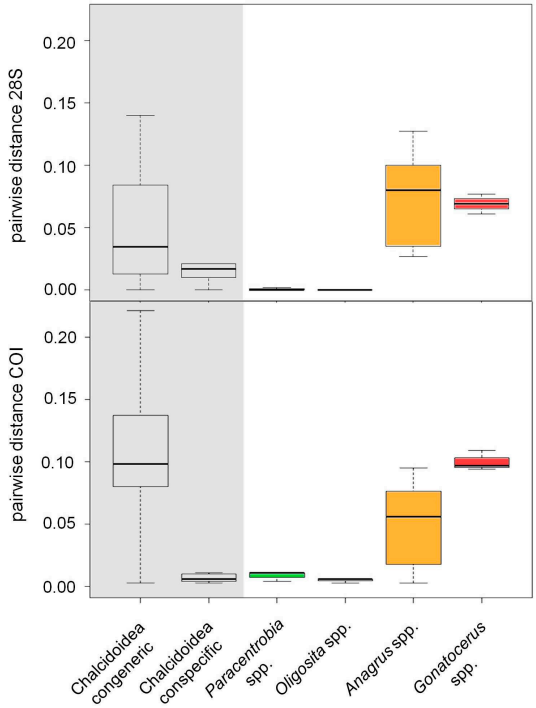

Figure 5. Pairwise molecular distances between individuals from different species of the same genus in the Chalcidoidea (congeneric), different members of the same species in the genus Chalcidoidea (conspecific) and individuals collected in this study. Pairwise distances were calculated by using the K2P+G (Kimura 2-parameter model with Gamma distribution) model for the 28S sequences and the T92+G (Tamura 3-parameter model with Gamma distribution) model for the COI sequences.

Table 2. Results of the Dunn's test (p) with degrees of freedom (df) for pairwise comparison of the pairwise genetic distances calculated within the parasitoid genera examined in this study and the pairwise genetic differences calculated for the congeneric and conspecific Chalcidoidea sequences (n.s. stands for not significant data).

		Paracentrobia spp.		*Oligosita* spp.		*Anagrus* spp.		*Gonatocerus* spp.	
		p	df	p	df	p	df	p	df
28S	Chalcidoidea congeneric	**0.003**	2	**0.003**	2	**0.021**	2	n.s.	2
	Chalcidoidea conspecific	n.s.	2	n.s.	2	**<0.001**	2	**0.05**	2
COI	Chalcidoidea congeneric	**0.017**	2	**0.002**	2	**< 0.001**	2	n.s.	2
	Chalcidoidea conspecific	n.s.	2	n.s.	2	**< 0.001**	2	**0.006**	2

4. Discussion

The genetic analysis of the diversity within the four Chalcidoidea genera studied showed discrepancies with the morphological analyses. This finding is in line with previous studies on hymenopteran parasitoids **[23,42,43]**. For example Mottern and Heraty **[44]** found that one species of the parasitoid previously described as *Cales noacki* were actually ten different Cales species. Previous studies showed that DNA barcoding can be misleading resulting in potentially more species than really exist **[45,46]**, which might play a role in the present study. The COI gene, as the classical barcoding gene, has shown its utility in many studies **[42,47,48]** and it constitutes the basis for the BOLD (www.barcodinglife.org) database. The 28S gene is valuable to distinguish among closely related species of Chalcidoidea **[49–51]**. In addition, it is more conserved and more accurate than the COI gene for this group of insects **[43,44,52]**. This is supported by our results as we found that sampling saturation was only reached for the gene 28S, but not for the COI gene.

Our study showed further that an accurate identification relies on both, molecular and morphological techniques. A similar conclusion was drawn previously [21,23]. In the tropical rice agroecosystem, more research combining morphological, behavioural and genetic methods are necessary to improve the identification of cryptic species **[53]**. Once a reliable and comprehensive DNA database is established, the identification of hopper parasitoids using DNA sequencing will be a vital step towards assessing the performance and suitability of these organisms as biocontrol agents **[54,55]**. For instance, it was hypothesized that *Anagrus* spp. can switch between alternative hopper species **[56,57]**. The strong diversity found in the present study by using molecular tools suggests, however, that *Anagrus* spp. is a complex of cryptic species. This is of high importance as the discovery of so far unknown cryptic species could expand the list of potential biological control agents **[44]**.

Although we were not able to secure the existence of new species of *Anagrus* and *Gonatocerus* by mating tests and/or further morphometric analyses, our data strongly suggest that the two genera include so far unknown species. The level of genetic difference for both 28S and COI sequences among samples from the *Anagrus* spp. and *Gonatocerus* spp. genera exceeded the threshold of 0.17-2% within sequence variation which is generally accepted to delineate individuals of the same species **[17,48,58]**. The genetic differences determined in the present study for the *Anagrus* spp. and *Gonatocerus* spp. genera were more representative of congeneric Chalcidoidea, with values separating genera from this superfamily generally assumed to be 5.8-11.25% **[17,48,58]**.

We concluded that the specimen morphological identified as *Gonatocerus orientalis* are likely to belong to a complex of cryptic species, as commonly reported in hymenopteran parasitoids **[19,24,59]**. We further suggest that there was at least one more species for *Anagrus* spp., that was not accounted by using the morphology alone. Interestingly, we observed the opposite for samples from *Oligosita* spp., indicating that *Oligosita* spp. is one species that varies morphologically and/or shows sexual dimorphism, maybe due to a high degree of phenotypic plasticity **[60,61]**.

In conclusion, our results clearly demonstrated that molecular identification should be used in combination with morphological methods for assessing the diversity of rice hopper parasitoids. However, further studies using an integrative approach are needed to cover the whole diversity of parasitoids as well as to find sustainable solutions to problems caused by the BPH and GLH. To validate the potential application of parasitoid wasps as biocontrol agents, it is of crucial importance to have a comprehensive knowledge on their ecology and diversity. We hope that this study will encourage further research by providing the first barcodes for egg parasitoid species from rice paddies in Southeast Asia.

Supplementary Materials: The following are available online, Table S1: Species found, according to morphometric analysis, throughout the Laguna province, Philippines., Table S2: Sequences obtained from parasitoids of the rice fields in the Philippines., Table S3: Sequences obtained from the NCBI GenBank., Figure S1: Distribution of the eight study sites throughout the Laguna province., Figure S2: Rarefaction curve for the 28S gene fragment., Figure S3: Rarefaction curve for the COI gene fragment., Figure S4: Maximum likelihood tree for the 28S sequences., Figure S5: Maximum likelihood tree for the COI Sequences.

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Author Contributions: S.V. conceived and designed the experiments; C.S. performed the experiment; C.S. and F.W. did the laboratory work, C.S., A.B. and S.E. analyzed the data; R.D. contributed materials and analysis tools; C.S. wrote the paper with S.V., F.W., A.B. and S.E. contributing to the development of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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