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Influence of cropping systems on insect predators’ diversity and their trophic relationships

Daniela Hipolito Maggio

Thesis presented to obtain the degree of Doctor inScience. Area: Entomology

Piracicaba
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Versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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To Onofre Maggio, Ana Lucia Hipolito Maggio, Bruna Hipolito Maggio,Hugo Fernandes and my lovely grandmother Aurora Moretti Maggio (in memory).

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CONTENTS

RESUMO

Influência dos sistemas de cultivo na diversidade de insetos predadores e suas interações tróficas

As áreas de agricultura tropical são sistemas complexos para manejo porque possuem alta diversidade de espécies (principalmente artrópodes) que interagem entre si. A falta de conhecimento sobre a diversidade de espécies e interações ecológicas promove a escolha de práticas agrícolas simplistas e com impacto negativo na comunidade de artrópodes e ambiente (por exemplo, aplicação intensa de pesticidas). As interações ecológicas entre as pragas e seus inimigos naturais (predadores e parasitoides) são particularmente interessantes, pois podem ajudar na adoção de estratégias de controle eficientes e sustentáveis. Utilizando marcadores moleculares e ferramentas de sequenciamento, é possível identificar as espécies de predadores e o seu conteúdo intestinal para aferir com precisão quais presas foram ingeridas por cada espécie de predador e descobrir a presença de outras interações ecológicas, como a predação intraguilda. Assim, o objetivo do presente estudo foi avaliar o impacto de diferentes sistemas de cultivo de milho na riqueza e abundância de espécies, interações presa / predador de importantes predadores generalistas da cultura do milho. Para atingir esses objetivos, foram amostradas cinco áreas de milho orgânico e convencional no estado de São Paulo, com armadilhas pitfalls secas e coleta ativa. Foram analisadas a riqueza e abundância usando todas as amostras. As principais presas e predadores tiveram seus DNA barcodes produzidos através da amplificação do gene COI. Para a detecção de predação foram desenhados primers específicos para essas pragas mais comuns no milho, e feitas reações de PCR com o conteúdo intestinal dos predadores para detectar a presença de DNA das principais pragas do milho. Bioensaios foram feitos para identificar a meia-vida do DNA das presas no intestino dos predadores. Para avaliar o impacto de diferentes sistemas de cultivo na teia trófica, foram utilizados os predadores coletados em armadilhas pitfall seca e coleta manual. O DNA do conteúdo intestinal foi extraído e em seguida, as bibliotecas de Amplicon foram preparadas para metabarcoding, e as sequências editadas e comparadas com as sequências disponíveis no NCBI por bioinformática. Os primers desenhados para *Spodoptera frugiperda* e *Diabrotica speciosa* foram eficientes na amplificação da presa. Foi possível detectar em campo a presença de predação por *Coccinellidae* e *Doru luteipes* sobre *S. frugiperda*. Com o metabarcoding foi possível detectar eventos de predação em insetos de importância econômica e de não importância. Eventos de predação intraguilda foram detectados principalmente de Tachinidae em *Harmonia axyridis* e entre coccinélides. As ferramentas moleculares são importantes para elucidar as interações tróficas, permitindo compreender como as espécies interagem em campo. Além disso, resultados como os aqui encontrados podem auxiliar em trabalhos de controle biológico futuros e também no manejo integrado de pragas.

Palavras-chave: DNA metabarcoding, Milho, PCR, Presa, Predador, Controle biológico

ABSTRACT

Influence of cropping systems on insect predators' diversity and their trophic relationships

Tropical agriculture areas are complex systems for management because they have a high diversity of species (mainly arthropods) interacting among them. The lack of knowledge about the species diversity and ecological interactions promotes the choice of simplistic agriculture practices and with negative impact in species community and environment (e.g. intense pesticide application). Ecological interactions between pests and their natural enemies (predators and parasitoids) are particularly interesting because may help adopting efficient and sustainable control strategies (e.g. conservative biological control). Using molecular markers and sequencing tools it is possible to analyze the predator gut content to identify with precision, which preys were ingested and find out if there are other ecological interactions such as intraguild predation. Thus, the aim of the present study was to evaluate the impact of different maize cropping systems on species richness and abundance, prey/predator interactions of important generalist maize predators. To achieve these objectives, five areas of organic and conventional corn were sampled in the state of São Paulo, with dry pitfall traps and active collection. Richness and abundance were analyzed using all samples. The main prey and predators had their DNA barcodes produced through the amplification of the COI gene. For the detection of predation, specific primers were designed for these most common pests in corn, and PCR reactions were performed with the intestinal contents of predators to detect the presence of pest DNA. Bioassays were done to identify the half-life of prey DNA in the gut of predators. To evaluate the impact of different farming systems on the food web, predators collected in dry pitfall traps and manual collection were used. DNA from the intestinal contents was extracted and then the Amplicon libraries were prepared for metabarcoding, and the sequences edited and compared with the sequences available at the NCBI by bioinformatics. The primers designed for *Spodoptera frugiperda* and *Diabrotica speciosa* were efficient in prey amplification. It was possible to detect in the field the presence of predation by Coccinellidae and *Doru luteipes* on *S. frugiperda*. With metabarcoding it was possible to detect predation events in insects of no economic importance and only one species of agricultural pest. Intraguild predation events were detected mainly from Tachinidae in *Harmonia axyridis* and among coccinellids. Molecular tools are important to elucidate trophic interactions, allowing us to understand how species interact in the field. In addition, results such as those found here can help in future biological control work and also in integrated pest management.

Keywords: DNA metabarcoding, Maize, PCR, Prey, Predator, Biological control

1. INTRODUCTION

Tropical regions have a great diversity of organisms and their interactions maintain the stability and characteristics of the community, increasing the functionality of ecosystems (Arshad *et al*, 2018; Bellamy *et al*, 2018; Rooney & MCcann, 2012). The agricultural practice in these places becomes challenging because of the complex management due to the diversity of species that can interact in a beneficial or harmful way in an agricultural context. In addition, the simplicity of the agricultural environment facilitates the introduction of invasive species that can also influence the dynamics of organisms in the environment (Acosta *et al*, 2017; Rooney & MCcann, 2012).

Several species have their food webs established in these places and play important roles in the ecosystem, which may or may not cause economic damage (Bellamy *et al*, 2018; Arpaia *et al*, 2017). Due to the reduction of plant diversity, the habitat becomes more simplified, which can impact the arthropod community in several ways. The cropping system also impacts the biodiversity of the area and can be crucial in the management of target arthropods such as pests, natural enemies, detritivores and pollinators (Van Lenteren *et al*, 2018; Acosta *et al*, 2017).

Many studies compare the effects of cropping systems on local community diversity, indicating that organic cropping systems are able to reduce pest abundance or increase predator abundance, consequently increasing prey/predator interaction (Gallé *et al*, 2019; Jacobsen *et al*, 2019). Conventional cropping systems may have negative effects on some species, such as pollinators, or benefit some species as observed by Clough *et al*. (2007). These changes in community structure can trigger changes in ecological interactions positively and/or negatively.

In all habitats, different organisms interact, establishing ecological interactions between them. An important trophic interaction in the agricultural context is the predator-prey interaction, where predatory organisms are important to keep herbivore population densities below that which promotes economic damage to cultivated plants (Van Lenteren *et al*, 2018). Prey-predator interactions are the basic principle for biological control programs, whether conservation, augmentative or classical (Ives *et al*, 2005). The predators' food webs can be specific or, like most, generalists, feeding on several species of herbivores (Venzon *et al*, 2001). From the knowledge of food webs, it is possible to establish pest control tactics that favor the increase or

attraction of local predators (natural enemies) from the management of the environment and mitigate the negative effects on the community (Van Lenteren *et al*, 2018; Gallo *et al*, 2002; Cohen *et al*, 1994).

Among the ecological interactions of a community, intraguild predation is a non-harmonious interaction that can be influenced by the abundance of prey in the area (Polis *et al*, 1989). In this case, predators compete with each other for food resources, which may result in population or community imbalances (Polis *et al*, 1989). These trophic interactions are complex and difficult to notice, as they also depend on temporal and spatial variations (González-Chang *et al*, 2016; Polis *et al*, 1989). Study carried out by Yang et al. (2017) detected intraguild predation among ladybird species, with *Harmonia axyridis* (Coleoptera: Coccinelidae) being the species with the most predators in its gut content.

Due to the difficulty in observing trophic interactions in the field, it is possible to analyze the gut content of predators with molecular tools and reconstruct their food web, identifying predation events and discovering the organisms that make up the diet of this predator (Krehenwinkel *et al*, 2017; González-Chang *et al*, 2016; Traugott *et al*, 2012; Greenstone *et al*, 2007). In the past, analyzes were made from the identification of prey body parts found in the predator's gut (Greenstone *et al*, 2007). With the advancement of molecular tools, techniques such as Polymerase Chain Reaction (PCR) or Enzyme-Linked Immunosorbent Assay (ELISA) have been widely used for analysis of intestinal content of predators present in crop areas (González-Chang *et al*, 2016; Greenstone *et al*, 2014 and 2007).

Using PCR and ELISA tools, it is also possible to study the decay rate of prey DNA detection in the predator's intestinal contents (Greenstone *et al*, 2014). With this, it is possible to understand the efficiency of ingestion of prey and also how long after the ingestion of that prey we will find its DNA in the intestinal contents of the predator (Greenstone *et al*, 2014 and 2010). In addition, detectability is influenced by predator factors such as the digestive system, life stage, predation frequency, as well as environmental factors (Weber & Lundgren 2009; Hosseini *et al*, 2008; Hagler & Naranjo, 1997). Study carried out by Nanini *et al*. (2019) showed that the detection of the decay rate of *Diaphorina citri* (Hemiptera: Psyllidae) DNA in the intestinal contents of *Hippodamia convergens* (Coleoptera: Coccinelidae) and *Chrysoperla externa* (Neuroptera: Crysopidae) are close, but when compared with field samples,

the most frequent predator of *D. citri* was *H. axyridis*, while no lacewings were positive for *D. citri.*

One of the most modern techniques applied in the construction of trophic networks is the DNA metabarcoding technique, which, through high throughput sequencing platforms, allows the sequencing of samples of small DNA fragments (Porter & Hajibabaei, 2018; González-Chang *et al*, 2016; Vo & Jedlicka, 2014; Boyer *et al*, 2013). Thus, using the DNA metabarcoding technique, it is possible to identify the species consumed by a predator by sequencing the DNA from the predator's gut contents (Kamenova *et al*, 2018; Porter & Hajibabaei, 2018), revolutionizing the understanding of trophic networks (Toju & Baba, 2018; Toju, 2015).

However, this methodology's accuracy depends on the DNA barcoding data banks (e.g., GenBank or BOLD Systems). In the case of arthropods, the information about the sequencing of COI gene fragments (*cytochrome c oxidase subunit I*) from different species associated with the environment is essential to improve the identification success at the species level. Countries from North America (Canada and United States) and Europe are making great efforts to characterize the species diversity using a DNA barcoding approach because it allows a continuous monitoring of biodiversity. However, in countries located in tropical regions, such as Brazil, these studies are scarce, with the presence of just specific efforts for some arthropod groups. The exception is Costa Rica, which has a DNA barcoding consortium to characterize its biodiversity and offer important information to tropical regions.

Studies carried out by Sow *et al*, 2020 using metabarcoding with primers for insects, showed that different generalist arthropods predators and insenctivorous vertebrates fed on different crops pests, non pests and predators in millet fields. Batuecas *et al*, 2022 also used metabarcoding but a combination of primers for animals and plants, and showed that the generalist predators from peach crops had in their diet a diversity of insect prey, including insects of economic importance, other predators and a range of plants. In both studies was elucidated how species interacted between them, and the intraguild predation was present in the majority of food web analyzed.

The metabarcoding is a very helpful tool as a start to understand the trophic interactions in the field. Studies in this area can be a resourceful tool for biological control, in a perspective of integrated pest management (IPM). Understanding the predators' dynamic, their feeding habits and the local species are important issues

in biological control (Batuecas *et al*, 2022). It is necessary to know the local natural enemies and the trophic interactions when the conservation biological control is applied in the crop, so you can create the conditions necessary for them, as shelter, corridors, and diversity of food resource, to guarantee their permanence in the area (Gontijo, 2019; Palmu *et al* 2014; Purtauf *et al* 2005).

Knowing what impact of management in agricultural areas can cause in the local community and their interactions, is crucial for successful biological control. Thus, considering the importance of knowing and understanding the local species, their trophic interactions, and the possible impact of crops systems, this study had the aim to analyze the diversity of predator species and their ecological interactions, using traditional sampling and molecular marker tools, in maize crops.

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2. A MOLECULAR MARKER TO IDENTIFY *Spodoptera frugiperda* (JE Smith) DNA IN PREDATORS' GUT CONTENT[1]

Abstract
Spodoptera frugiperda is a serious pest of maize and other crops worldwide. The integration of control tactics is recommended for *S. frugiperda* suppression because reports of insecticide and Bt plant-resistance are frequent. Biological control agents would be an alternative to improve *S. frugiperda* control in agricultural areas. We constructed a species-specific molecular marker to detect *S. frugiperda* DNA in predators' gut content and estimated the predation rates of ladybugs and earwigs on *S. frugiperda* in maize crops. Predators were sampled in Pirassununga, São Paulo state, Brazil, in 2020 and 2021. Using the species-specific molecular marker in laboratory conditions, we estimated the half-life time to detect *S. frugiperda* DNA in the gut contents of *Hippodamia convergens* as 6.16 h and *Doru luteipes* as 25.72 h. The weekly predation rate of *S. frugiperda* by predators in maize crop varied from 0 to 42.1% by ladybugs and from 0 to 9.2% by *D. luteipes*. Predation events on *S. frugiperda* by predators were more frequent during the maize reproductive stage. Our results confirmed that predators might contribute to *S. frugiperda* suppression in maize fields. However, further studies of prey–predator interactions and agricultural landscapes are essential for a better understanding of predator dynamics in crops.

Keywords: earwigs; ladybugs; half-life detectability; biological control; predator–prey interaction

2.1. Introduction

Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae), popularly known as the fall armyworm, is native to the Americas. In 2016, *S. frugiperda* was detected in Africa and reached the status of a cosmopolitan species after invading and dispersing in regions of Europe, Africa, Asia, and Oceania [1–3]. Its polyphagous and voracious feeding behavior, high reproductive rate, and long-distance dispersal capacity make *S. frugiperda* a serious agricultural pest [4–6]. In Brazil, *S. frugiperda* has been an economically important pest of maize and cotton crops. However, in recent years, this species has increased in abundance in soybean crops, making its management more challenging [7].

Different tactics are used for *S. frugiperda* control, including insecticides, transgenic plants, and natural enemies. Insecticides are the most common management strategy for *S. frugiperda*. However, their effectiveness is limited

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Maggio, D.H.; Rossetti, V.Z.; Santos, L.M.A.; Carmezini, F.L.; Corrêa, A.S. A Molecular Marker to Identify *Spodoptera frugiperda* (JE Smith) DNA in Predators' Gut Content. Insects 2022, 13, 635. https://doi.org/10.3390/insects13070635

because *S. frugiperda* larvae feed inside the maize whorl where insecticides may not penetrate [8]. Furthermore, in recent years insecticide-resistant populations of *S. frugiperda* have often been reported [9–12]. Transgenic *Bacillus thuringiensis* (Bt) plants is another worldwide control strategy for *S. frugiperda* management. However, as with insecticides, *S. frugiperda* populations resistant to Bt crops have been reported in Puerto Rico, Brazil, Argentina, and the USA [6,13–15].

Biological control agents may also be effective for *S. frugiperda* population suppression. Parasitoids and predators, such as *Telenomus remus* (Hymenoptera: Scelionidae), *Trichogramma* species (Hymenoptera: Trichogrammatidae), Tachinidae (Diptera), *Podisus nigrispinus* (Hemiptera: Pentatomidae), *Doru luteipes* (Dermaptera: Forficulidae), and *Harmonia axyridis* (Coleoptera: Coccinelidae), are described as promising biological control agents of *S. frugiperda* [16–20]. In maize areas, the most common predators are earwigs, ladybugs, ground beetles, and lacewings, which feed on different important agriculture pests [17,21–23].

Although predators are frequent in agricultural areas, the real contribution of these natural enemies to *S. frugiperda* suppression is still difficult to estimate in the field. One approach used to identify and estimate the predation rate of predators on a group of prey is an analysis of predator gut contents [24–26]. In the past, these analyses were carried out by identifying prey body parts found in the predator's gut, although it was difficult to identify the prey to species level [24]. Therefore, molecular tools have been widely used in the analysis of predator gut contents because they allow rapid and precise identification based on the design of species-specific primers, multiplex PCR markers, and, more recently, metabarcoding [24,25,27,28].

Based on the worldwide economic importance and the reports of control failures of *S. frugiperda*, predators may be important components in the development of IPM plans to suppress this pest. We designed a specific molecular marker to detect the predation rate of generalist predators on *S. frugiperda* in maize crops. Specifically, earwigs and ladybugs are two types of predators that are frequently cited as natural enemies of *S. frugiperda* [16,18], but they are inadequately studied in the field. Our specific aims were: (1) design species-specific primers to detect *S. frugiperda* DNA in the gut contents of generalist predators; (2) estimate the detectability half-life in *S. frugiperda* DNA and in the gut contents of an earwig, *D. luteipes*, and a ladybug, *Hippodamia convergens* (Coleoptera: Coccinelidae)—both common generalist predators found in maize crops—and to confirm the utility of this

molecular marker; and (3) estimate the predation rate of *S. frugiperda* and for predators collected weekly in maize crops.

2.2. Materials and Methods

2.2.1. Field detection of *S. frugiperda,* and predator collection

Insect monitoring and collections were carried out on maize farm in Pirassununga. The collections were made in two consecutive fields: in the winter, crop from April to July 2020, a total of 14 weeks of sampling; and in the summer, crop from December 2020 to February 2021, 8 weeks of sampling. In the winter crop, the maize plants developed more slowly due to the low rainfall (46 mm) and temperature (19 °C); consequently, the sampling period was longer than in the summer crop (mean rainfall 217 mm and mean temperature 25 °C). Sampling started in the maize vegetative stage V2 and continued until the harvest. The vegetative stage lasted until week 7 in winter and week 4 in summer. Both crops were cultivated under conventional practices but without insecticide applications for insect control.

A delta trap baited with the sex pheromone Bio Spodoptera (Bio Controle, Indaiatuba, Brazil) was installed in the middle of the area to detect the presence of *S. frugiperda* during the experiment. The sticky liner was replaced weekly and the sex pheromone replaced every 3 weeks or less if the pheromone was depleted. In the laboratory, the moths on the sticky liner were photographed and identified using morphological traits such as body and wing colors.

Earwig and ladybug individuals were manually collected randomly along the plant weekly from the maize vegetative stage V2 until the harvest. The maize crop area was divided into three sub-areas of 20 m^2 each, and predators were collected actively to preserve their gut contents by immediately freezing the specimens. Each predator collected was immediately placed in a 1.5 mL tube and stored in a plastic bag on ice until arriving at the laboratory. At the laboratory, the predators were stored in 99.9% ethanol at –20 °C and taxonomically identified.

To estimate the predation rate by predators on *S. frugiperda,* the predators' DNA was extracted, and the specific marker to detect the presence of *S. frugiperda* DNA in the predators' gut contents was applied exactly according to the above method.

2.2.2 Molecular analysis of gut contents

2.2.2.1. DNA extraction

All insects used in this study were cleaned by submersion in 2% sodium hypochlorite for 2 s, next in 70% ethanol for 2 s, and then in autoclaved distilled water for 5 s and allowed to dry on clean tissue paper. These procedures were carried out to cleanse any external DNA from the insect body.

DNA was extracted using the entire body except for the wings, antenna, and legs, with the CTAB protocol adapted from Corrêa et al. [29]. Each individual was submerged in 500 µL of CTAB buffer, 10 µL of proteinase K 20 mg mL–1 (Invitrogen, Waltham, MA, USA), and 2 µL of β-mercaptoethanol. The samples were incubated at 65 °C for 2 h. Then, 3 µL of PureLinkTM RNase A 20 mg mL–1 (Invitrogen) was added to each sample, which was incubated at 37 °C for 2 h and then at 65 °C for 30 min. The samples were centrifuged at 14,000 rpm for 5 min and the liquid phase was transferred to a new tube, mixed with 500 µL of chloroform and isoamyl alcohol (24:1), and centrifuged at 14,000 rpm for 20 min. The supernatant was transferred to a new tube, and the isoamyl alcohol step was repeated. Then, the supernatant was transferred and mixed with 100% isopropanol and incubated at –20 °C overnight. The samples were centrifuged at 14,000 rpm at 4 °C for 30 min and the supernatant was discarded. The DNA pellet was cleaned with 400 µL of cold 70% ethanol. The cleaning step was repeated with cold 100% ethanol and then the samples were allowed to air-dry. The dried DNA pellet was resuspended in 30 µL of autoclaved distilled water.

2.2.3. Primer design and optimization

The *S. frugiperda* species-specific primers were designed using sequences of the *cytochrome c oxidase subunit I* (COI) gene available in GenBank (access numbers: KF624877.1, GU439148.1, JQ559528.1, JF854747.1, HQ964527.1, and MH753323.1) from different regions of Brazil and the world, Spo_frugi-F: CCCATCTTTAACTTTATTAATTTCT, and Spo_frugi-R: TGAGAAAATAGCTAAATCTACTGAACTA. The primers were analyzed using Net Primer (Premier Biosoft, San Francisco, CA, USA) to determine the melting temperature and secondary structures. The specificity of *S. frugiperda* primers was tested in two ways. First, sequences in GenBank from *Spodoptera dolichos* (access

numbers: JQ559274.1, HQ568393.1, and KJ634288.1), *Spodoptera eridania* (access numbers: KJ634289.1, JQ551023.1, and KF261171.1), *Spodoptera cosmioides* (access numbers: JF854980.1 and KF261200.1), *Spodoptera albula* (access numbers: KF261151.1, KF261154.1, GU658150.1, and JF855904.1), and *E. kuehniella* (access number: GU828613.1) were aligned with the designed primers in software Sequencher 4.0.1 (Gene Codes Corp., Ann Arbor, MI, USA) (Figure 2.1). Second, the specificity of the primers was tested with PCR assays with DNA from *H. convergens*, *D. luteipes*, *H. axyridis*, *Cycloneda sanguinea*, *Anticarsia gemmatalis*, *Helicoverpa zea*, *Dalbulus maidis*, and *E. kuehniella* and four other *Spodoptera* species, *S. albula*, *S. cosmioides*, *S. dolichos*, and *S. eridania*.

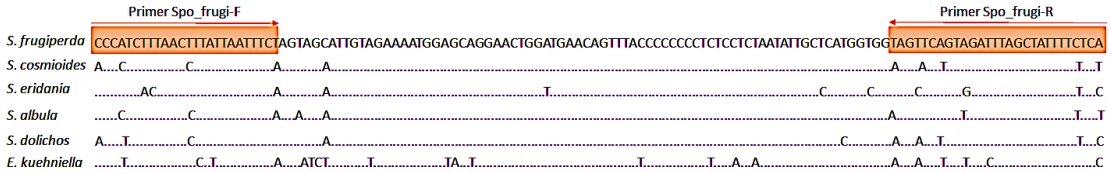

Figure 2.1. Part of COI gene sequences from *Spodoptera frugiperda*, *Spodoptera cosmioides*, *Spodoptera eridania*, *Spodoptera albula*, *Spodoptera dolichos*, and *Ephestia kuehniella*, aligned to design the pair of primers specific for *S. frugiperda*. Orange rectangles indicate the pair of forward (Spo_frugi-F) and reverse (Primer Spo_frugi-R) primers. Dots indicate pairs of bases similar to *S. frugiperda* COI sequences; letters indicate the respective different nucleotide bases in each species.

The PCR assays for this study were optimized in a total volume of 14 µL, with 7.1 µL of DNA, 1.5 µL of 10X magnesium-free buffer (Sinapse Inc., Hollywood, FL, USA), 1.5 µL MgCl2 (25 mM, Sinapse Inc.), 1.2 µL dNTP (2.5 mM, Sinapse Inc.), 1.2 µL of each primer (5 pmol), and 0.3 Taq DNA polymerase 5U/µL (Sinapse Inc.). The conditions used in the PCR assay were: 94 °C for 3 min; then 35 cycles of 94 °C for 30 s, 57 °C for 45 s, 72 °C for 2 min; and 72 °C for 10 min for the final extension in the Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA, USA). The amplification (fragment size: 130 bp) was evaluated by electrophoresis on 2% agarose gel. PCR assays to detect the presence of *S. frugiperda* DNA in predator gut contents were carried out simultaneously with three control samples: water and DNA

predators that did not have contact with *S. frugiperda* as two negative controls, and a positive control DNA of *S. frugiperda*.

2.2.4. Feeding studies

Adults of *H. convergens* were sampled in a maize field in Pirassununga (22°03′59.8″ S 47°25′58.4″ W), São Paulo state, Brazil. Theadults ladybugs were reared in plastic Petri dishes for oviposition and fed with inviable eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) and a mixture of water, honey, and brewer's yeast in cotton. *H. convergens* eggs were removed to new containers and held until the larvae emerged. The larvae were kept in glass tubes and fed with inviable eggs of *E. kuehniella* until they reached the fourth instar. All adults and larvae were reared in Bio-Oxygen Demand (BOD) incubators with a photoperiod regime of 14:10 (L:D) and 60 ± 10% relative humidity (RH) at 26 ± 1 °C.

Doru luteipes adults were collected in the field and maintained in dark plastic containers, with artificial diet that contained 35% cat food, 27% wheat germ, 23% brewer's yeast, 14% milk in powder, 0.5% nipagin, and 0.5% sorbic acid [30]; and an egg box, folded paper, and plastic straws with moistened cotton on one side. The adults oviposited in the moist cotton, and the straws with the eggs and the mother earwigs were maintained in another container until the nymphs reached the second instar. All the containers were kept in a climate-controlled room with 70 ± 10% relative humidity (RH) at 26 ± 1 °C in the dark.

A total of 70 *H. convergens* fourth-instar larvae and 66 *D. luteipes* nymphs were starved for 24 h. They were placed in individual Petri dishes and were offered a single *S. frugiperda* neonate larva for each individual of *H. convergens* and *D. luteipes*. The predators were transferred to microtubes containing 99.9% ethanol after 0.5, 2, 4, 12, 24, and 48 h of feeding on *S. frugiperda* and stored at –20 °C. During the period between the feeding and transfer to microtubes, the individuals were kept with no food in a BOD incubator. Each time group of *H. convergens* contained 10 individuals. Groups 0–12 h of *D. luteipes* contained 10 individuals, the 24 h group contained 9, and the 48 h group contained 7. The DNA extraction and PCR to detect the presence or absence of *S. frugiperda* DNA in the predators' gut contents were carried out according to the methods described above. Additionally, during these bioassays, some positive PCR fragments were sequenced by Sanger

sequencing to confirm that the amplified fragment with these primers corresponded only to *S. frugiperda*. The feeding results were analyzed by using the package "drc" in R Studio [31,32] to determine the half-life of the DNA in the predators' gut contents.

2.3. Results

2.3.1. Primer specificity and half-life for *S. frugiperda* DNA

The primers Spo_frugi-F and Spo_frugi-R designed for *S. frugiperda* were highly species-specific in our PCR conditions, and they were able to only amplify the target species when tested with DNA from other species of *Spodoptera* and other moths, earwigs, and ladybugs (Figure 2.2). Only predators that had fed on *S. frugiperda* in the laboratory (positive control) had a positive amplification in the PCR assay. The half-life detection time (DT_{50}) for *S. frugiperda* DNA in *H. convergens* gut contents was DT_{50} = 6.16 h (± 1.46, t = 4.21, $p < 0.01$), and in *D. luteipes*, it was DT_{50} = 25.72 h (±2.94, t = 8.73, $p > 0.001$).

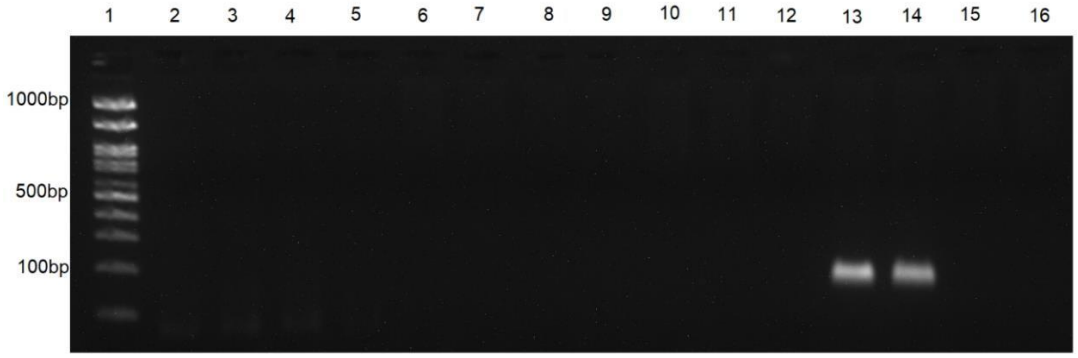

Figure 2.2. Electrophoresis gel showing the specificity of the primers Spo_frugi-F and Spo_frugi-R when tested with a PCR assay using DNA from different predators, months, and *Spodoptera frugiperda*. Column 1, ladder; columns 2–12, DNA of *Hippodamia convergens, Doru luteipes, Harmonia axyridis, Cycloneda sanguinea, Anticarsia gemmatalis, Helicoverpa zea, Dalbulus maidis, Ephestia kuehniella, Spodoptera albula, Spodoptera cosmioides,* and *Spodoptera eridania*, respectively. Column 13, DNA of *S. frugiperda*; column 14, gut contents of *H. convergens* that had fed on *S. frugiperda*; column 15, DNA of *Spodoptera dolichos*; column 16, negative control.

2.3.2. Insect field collection and predator gut-content analysis

In the winter crop, a total of 388 predators were sampled. Of these, *H. convergens* was the most abundant predator with 228 samples collected, followed by 124 for *C. sanguinea*, 47 for *H. axyridis*, 27 for *Eriopis connexa*, and 2 for *D. luteipes*. The predators appeared in week 5 with 3 ladybugs; week 6 with 16 ladybugs; week 7 with 25 ladybugs; week 8 with 78 ladybugs; week 9 with 69; week 10 with 61; week 11 with 40 ladybugs and 1 earwig; week 12 with 54 ladybugs; week 13 with 41 ladybugs; and week 14 with 39 ladybugs and 1 earwig (Figure 2.3a). The weeks that were most abundant in predators were weeks 8 and 9 when the maize was in the beginning of reproductive stage. The populations of *H. axyridis* and *C. sanguinea* fluctuated similarly, reaching their highest in week 8 and decreasing continuously in the following weeks. The population of *E. connexa* fluctuated constantly during the maize crop cycle (Figure 2.3a).

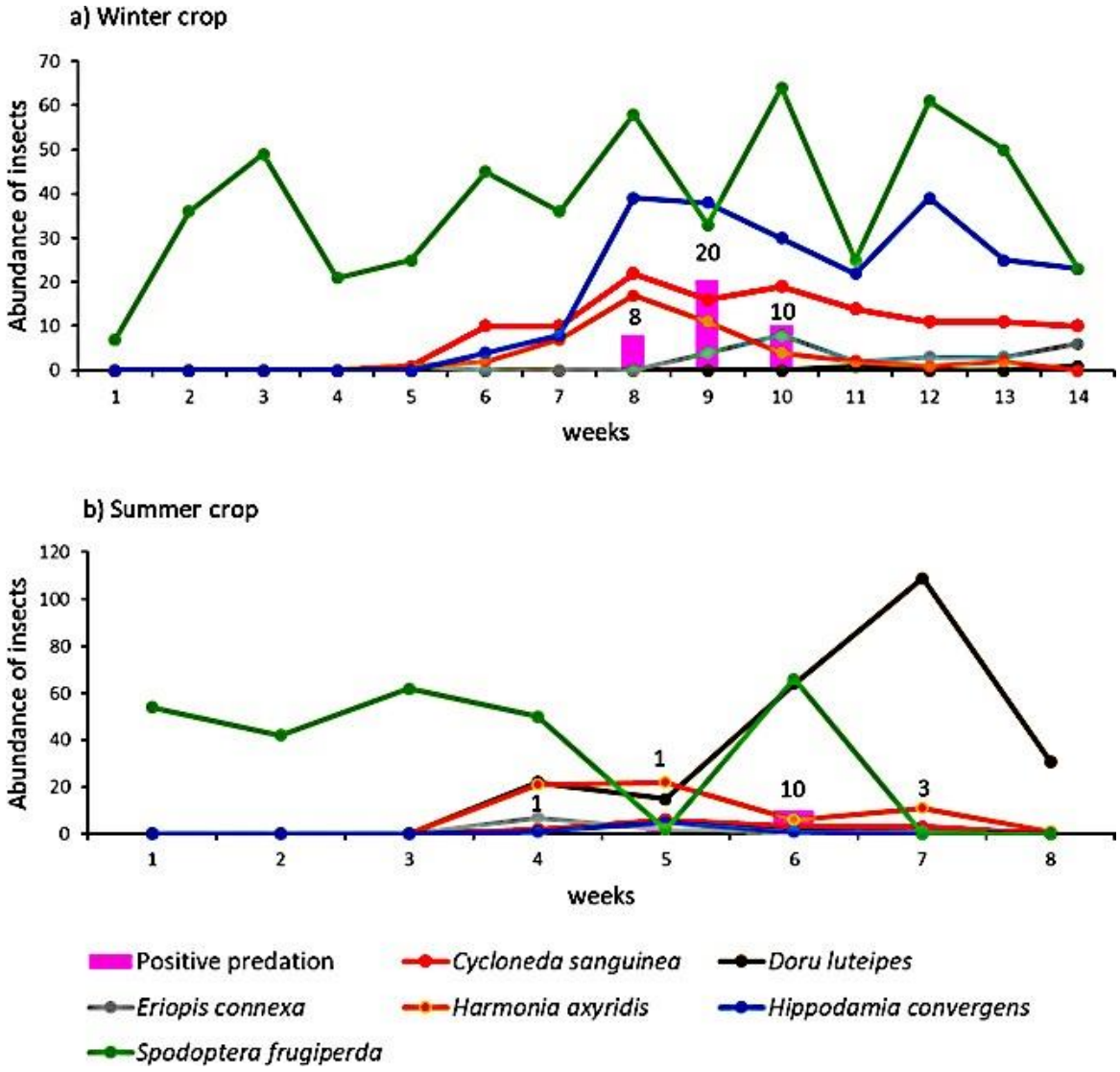

Figure 2.3. Total number of individuals of *Doru luteipes, Hippodamia convergens, Harmonia axyridis, Cycloneda sanguinea,* and *Eriopis connexa* sampled per week in Pirassununga during (**a**) winter crop (April to July 2020) and (**b**) summer crop (December 2020 to February 2021). Pink bars and the numbers on the top of the bars represent the total predation events on *S. frugiperda* identified as a positive result in PCRs from the field samples per week. The lines with dots represent the predator species sampled per week in the field.

Predation events in the winter crop occurred only in weeks 8, 9, and 10, and all ladybug species fed on *S. frugiperda*. *H. convergens* had the most positive results (24 positives) with predation rates varying between 5 and 42%, followed by *H. axyridis* (6 positives) with predation rates between 11 and 36%. The predation rate for *C. sanguinea* (four positives) was 18.2% and for *E. connexa* (two positives) was 25% (Table 2.1).

Table 2.1. Weekly abundance and predation rates for each predator that showed positive results for predation on *Spodoptera frugiperda* in winter and summer crops.

	Winter Crop						Summer Crop							
	Week 8		Week 9		Week 10		Week 4		Week 5		Week 6		Week 7	
Predator	Ab [1]	P.R [2]	Ab	P.R	Ab	P.R	Ab	P.R	Ab	P.R	Ab	P.R	Ab	P.R
Hippodamia convergens	39	5.1± 0.06	38	42.1 ± 0.156	30	26.7± 0.158	1	-	5	-	1	-	-	-
Harmonia axyridis	17	11.8 ± 0.153	11	36.4± 0.284	4	-	21	-	22	-	6	-	11	-
Cycloneda sanguinea	22	18.2± 0.161	16	-	19	-	2	-	6	-	3	-	3	-
Eriopis connexa	-	-	4	-	8	25± 0.3	7	-	1	-	1	-	1	-
Doru luteipes	-	-	-	-	-	-	22	4.5± 0.087	15	6.7± 0.126	64	1.6± 0.088	109	9.2± 0.03

[1] Ab = abundance; [2] P.R (%) = predation rates in percentage ± IC (confidence interval).

In the summer crop, a total of 334 predators were sampled, of which 241 were *D. luteipes* and 93 were ladybugs. The most abundant ladybug species was *H. axyridis*, with 61 individuals (Figure 2.3b). The predators appeared in week 4, at the end of vegetative stage, with 31 ladybugs and 22 earwigs; week 5 with 35 ladybugs and 15 earwigs; week 6 with 11 ladybugs and 64 earwigs; week 7 with 15 ladybugs and 109 earwigs; and week 8 with 1 ladybug and 31 earwigs. The most abundant weeks were 6 and 7 for earwigs, and weeks 4 and 5. Predation events were detected in 14 *D. luteipes*, with the predation rate varying between 1% to 9%. No predation was detected in ladybug individuals. One predation event occurred in the maize vegetative stage, week 4, and three predation events in reproductive stage, weeks 5, 6, and 7, where week 6 had the most predation events (Table 2.1).

2.4. Discussion

We developed a molecular marker for the specific identification of *S. frugiperda* in predator gut contents. This marker can distinguish *S. frugiperda* from other *Spodoptera* species and is useful for identifying and monitoring this cosmopolitan pest by means of a single PCR method. The *S. frugiperda*-specific primers were based on the *cytochrome c oxidase subunit I* (COI) gene and were successfully used as a DNA barcoding region in animals [33–36]. One advantage of the molecular marker developed here is the small fragment size amplified, favoring PCR amplification and identification of insects even when the DNA was degraded, which is an essential trait of a molecular marker for gut-content analysis studies [28,37].

The DNA half-life for detection of *S. frugiperda* in *H. convergens* and *D. luteipes* gut contents differed between predators, with rates of 6.16 h and 25.72 h for *H. convergens* and *D. luteipes,* respectively. DNA half-life for detection of other prey in *H. convergens* gut contents showed a similar detection time for *Rhopalosiphum maidis* (Hemiptera: Aphydiidae), 8.78 h [28]; and for *Diaphorina citri* (Hemiptera: Psyllidae), 6.11 h [38]. Here, the DNA half-life detection time in gut contents was estimated for the first time in *D. luteipes*, and it was longer than in *H. convergens*.

Differences in DNA half-life detection times in predator gut contents are expected because the DNA half-life is driven by environmental conditions and by biological traits of the predator [39,40]. Insect life stage, metabolism, mouthpart type,

and gut size and shape are some variables that affect the process of DNA degradation in the predator gut and, consequently, the DNA half-life detection time [27,38,41]. However, DNA half-life detection assays in laboratory conditions are useful and essential for determining if the molecular marker is efficient in detecting low concentrations of prey DNA in predator gut contents, as we confirmed here for this *S. frugiperda* marker.

Predation on *S. frugiperda* was detected in all predator species collected in maize fields using DNA gut-content analysis. However, the higher predation rates were detected in *H. convergens* and *H. axyridis*—even higher than in *D. luteipes,* reported as an efficient predator of *S. frugiperda* [16]. The higher predation rate detected in these ladybug species compared with *D. luteipes* is likely even higher, in view of the 4.0x longer DNA half-life in gut contents of *D. luteipes* compared to *H. convergens* in laboratory conditions. Therefore, ladybugs must be considered as biological-control agents for *S. frugiperda*, especially *H. convergens* and *H. axyridis*, the two most abundant coccinellid species in our samples.

In the field, generalist predators can feed on a variety of sources such as pollen, decomposing animal and plant tissues, and different prey species. High rates of prey consumption by a predator in the laboratory or greenhouse, as reported for *D. luteipes* on *S. frugiperda* [16], may not indicate a high predation rate in the field because it also commonly exhibits scavenging behavior. Alternative prey types or feeding behavior may also affect the predation rate of a predator on a target prey [42,43]. For example, ladybugs may prefer to feed on aphids rather than other prey, as these predators are commonly reported as biological-control agents for aphids [44].

The presence of *S. frugiperda* in the field was constant throughout the maize cycle. This species damages leaves and cobs, but maize is the most susceptible in the vegetative and initial reproductive stages, when *S. frugiperda* may feed on the meristem of young plants, stunting or killing them, and damage the kernels, causing enormous losses [45]. However, in both collection seasons, the predators were found in the area from six weeks after the maize sprouted, and their peak was in the maize reproductive stage. Predators are also attracted to the plant by the pollen, nectar, and synomones [46,47]. At the beginning of the reproductive stage, maize produces pollen that attracts insects, including different predator species [48–50]. This delay in the arrival of predators, observed in both seasons in comparison to *S. frugiperda*,

may reduce the predators' control efficiency when the maize is most susceptible to the pest. Thus, strategies to advance their arrival and retain the predators in the area should be adopted to improve the biological control of *S. frugiperda* in the first weeks of plant development [42,51,52].

2.5. Conclusions

We successfully constructed a molecular marker to detect *S. frugiperda* DNA in predator gut contents. Using this molecular marker, we confirmed that earwigs and ladybugs, especially *H. convergens* and *H. axyridis*, preyed on *S. frugiperda* in the maize field. Our study indicated that predators might be considered a reliable component of IPM for *S. frugiperda*. Therefore, a better understanding of the dynamics of prey and predator in the landscape is crucial for managing predators and implementing conservative and applied biological control programs in maize fields.

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3. INSECTS DIVERSITY AND DNA BARCODING IN ORGANIC AND CONVENTIONAL MAIZE CROPS

Abstract

The agriculture impacts directly in arthropods diversity and the management can contribute to the impact positively or negatively. Organic crops have a reduced input and use more biological products, and conventional crops still have a greater input and use chemical pesticides. Considering the importance of crop management, this study aimed to identify the species, produce the DNA barcode for the main species from maize, and check if there was difference in species diversity between organic and conventional maize crops. To achieve those goals, samples were done with dry pitfall and manual sampling in 5 organic farms and 5 conventional farms in 4 different cities in São Paulo state during the maize reproductive stage. The arthropods were separated and identified into species, families, when possible. The DNA from the main species from maize were extracted using CTAB protocol and Sanger sequencing. The biodiversity was calculated by 6 diversities indexes: Shannon, Simpson, species richness, abundance, evenness and dominance. The organic areas had a greater diversity in Shannon index, Simpson index, evenness and dominance, but no significant difference in species richness and abundance. The most common species sampled in both areas were *Doru luteipes* (Dermaptera: Forficulidae), followed by Formicidae. Coccinillidae was more abundant in organic than conventional. In conclusion, organic maize had a greater diversity perhaps because of the reduced input and surrounding vegetation that this management allows.

Keywords: Biological control, Shannon index, DNA barcode, arthropods diversity.

3.1. Introduction

The loss of biodiversity and the lack of knowledge about it are directly related to the necessity of studies in this area. The agriculture for a long time has impacted the areas, simplifying the habitat with monocultures and consequently decreasing local biodiversity (Acosta *et al*, 2017; Rooney & MCcann, 2012). Habitat loss and fragmentation are factors that impact the community and can change its composition and functionality (Acosta *et al*, 2017; Arpaia *et al*, 2017; Bellamy *et al*, 2018). Currently, agricultural management techniques are more focused on habitat conservation and pesticide reduction. Organic farming is known for its greater diversity of species, mainly of arthropods including natural enemies (Gallé *et al*, 2019).

Organic crops applied in large areas of plantation present positive results in richness and abundance of species, mainly of natural enemies (Gallé *et al*, 2019; Clough *et al*, 2007). A study carried out by Letourneau and Goldstein (2001) in conventional and organic tomato crops in California (USA), showed that arthropod fauna as well as natural enemies are more abundant in organic than conventional crop, and that herbivory was similar in two crops. This suggests that organic farming management is more beneficial and practical and may decrease the dominance of species in the community. However, more studies need to be performed, mainly, in tropical regions.

The market interested in products of organic origin has been growing worldwide, which stimulates the increase of producers with organic cropping system (Fess & Benedito, 2018). An example of an organic product is corn (*Zea mays*), which is one of the main products consumed by humans and animals and of great importance to the world economy. The crops can be transgenic or conventional corn, and the transgenic has been gaining more space in cultivars (Cruz *et al*, 2014).

The pest species found in maize cropping can be divided according to the location of damage to the plant. There are subterranean species, such as the brown stink bug (*Scaptocoris castanea* (Hemiptera: Cydnidae) and *Diabrotica speciosa* (Coleoptera: Chrysomelidae), which damage the roots or even feed on the seed. Species of surfaces that are moths that feed on the leaves and reach the culm as the lesser cornstalk borer. Aerial pests ranging from chewing species such as *Spodoptera frugiperda* (Lepidoptera: Noctuidae), sucking species such as *Dalbulus maidis* (corn leafhopper) (Hemiptera; Cicadellidae) and aphids, which feed on leaves. And ear pests such as *Helicoverpa zea* (Embrapa Corn and Sorghum). The main predators found are earwigs, beetles (Coleoptera) belonging mainly to the Carabidae and Coccinellidae families, and predatory true bugs (Hemiptera) from the Reduviidae and Anthocoridae families. But also, spiders are considered important predators in crops.

DNA barcoding is a methodology to characterize or identify species by sequencing a fragment of gene *cytochrome c oxidase subunit I* (Hebert *et al*., 2003). This approach allows us to identify species using body fragments, young life stages such as larvae, nymphs, and eggs, and species still do not have taxonomic descriptions (Valentini *et al*, 2009). Furthermore, a robust DNA barcoding database

may help species diversity studies and ecological interactions (Batuecas *et al*, 2022; Valentini *et al*, 2009).

Based on the importance of knowing the diversity of insects and spiders in cropping areas and the difference between organic and conventional cropping, the aim of this study were to: 1) identify the species and produce the DNA barcode for the main pest species from maize, and 2) check if there was difference in species diversity between organic and conventional maize crops, considering that organic maize would have a greater diversity than conventional.

3.2. Material and Methods

3.2.1. Field

The samples were done in 10 areas of maize during its reproductive stage, where three organic areas and one conventional area were in Santa Rita do Passa Quatro (Farm1 -21.6798; -47.4485; Farm2 -21.6824, 47.4650; Farm3 -21.6522, -47.4828; Farm4 -21.6569, -47.4822), one organic and two conventional areas were in Pirassununga (Farm5 -21. 8876, -47.3822; Farm6 21.9815, -47.3784; Farm7 -22.0276, -47.3690), one conventional was in Santa Cruz da Conceição (Farm8 -22.0816, -47.4227) and one organic and one conventional area were in Ipeúna (Farm9 -22.4013, -47.6806). The reproductive stage was chosen due to it higher predator diversity, which are attracted by pollen and preys. In the field surrounding areas, there were sugar cane crops, sorghum crops, native forest patches and maize crops. In those sampled areas, the previous crops were soy and all organic farms followed the protocols stablished of organic crops, using only products allowed for this type of cultivation.

Each area was divided into 2 plots of 5m² and installed three dry pitfall traps in each plot. The dry pitfalls were made of plastic bottle with small pieces of wood to create a refugee for the predators and keep them alive. The pitfalls were emptied every 24h and in each plots was active sampled randomly for 45 min and the insects were collected manually. The sampling happened for two days. The organisms sampled were individualized into tubes and kept in cold bags until arrives in the laboratory, where they were storaged at -20ºC with ethanol 100%.

3.2.2. Morphological identification

The insects sampled were separated into Orders and after identified based on morphological characters into Families. The most common insects from maize were identified to the Genus and/or Species level. Only ants were classified into

morphotypes because of its great abundance.

3.2.3. DNA barcoding

It was produced the DNA barcode for the main insects' species belonging to maize, based on their importance for agriculture. The species chosen were: *Spodoptera frugiperda, S. albula, S. cosmioides, S. eridania, Harmonia axyridis, Cycloneda sanguinea, Hippodamia convergens, Eriopis connexa, Doru luteipes, Diabrotica speciosa, Dalbulus maidis* and *Lagria villosa*. Some of these species DNA barcode were not available on online databases.

3.2.4. DNA extraction

All the insects had their body washed up with bleach 2%, ethanol 70% and autoclaved distilled water to avoid external contamination. After that, a pair of legs were removed with part of the muscles to have the DNA extracted. Except for *D. maidis* that was used the entire body due to its tiny size. The DNA was extracted using the CTAB protocol adapted from Corrêa *et al*. 2014, using CTAB buffer, proteinase K 20 mg mL–1 (Invitrogen, Waltham, MA, USA), and β-mercaptoethanol. The DNA pellet was resuspended in 50 µL of autoclaved distilled water.

3.2.5. PCR and Sanger sequencing

To obtain the DNA barcode for each species, were used the universal primers HCO/LCO for the Coccinelidae species, *D. speciosa* and *L. villosa*. For *Spodoptera* species were used the universal primers LEP F/R, and for *D. maidis* a combination of HCO/LCO and LEP F/R. And for *D. luteipes* it was necessary to use degenerate primers jgHCO 2198/jgLCO 1490 (Geller *et al*, 2013). The PCR assay was optimized with 3.0 µL of DNA, 10.7 µL of autoclaved distilled water, 2.5 µL of 10X magnesium-free buffer (Sinapse Inc.), 2.5 µL MgCl2 (25 mM, Sinapse Inc.), 2.0 µL dNTP (2.5 mM, Sinapse Inc.), 2.0 µL of each primer (5 pmol), and 0.3 Taq DNA polymerase 5U/µL (Sinapse Inc.) for the primers HCO/LCO and LEPF/R. For the primers jgHCO/jgLCO it was optimized with 3 µL of DNA, 14.65 µL autoclaved distillated water, 2,5 µL 10x magnesium-free buffer (Invitrogen), 0.75 µL MgCl2 (50 mM, Invitrogen), 2 µL dNTP (2.5 mM, Sinapse Inc.), 1 µL of each primer (5 pmol), and 0.125 µL Platinum Taq DNA polymerase (Invitrogen). The thermal Cycler program was: 94 °C for 3 min; then 35 cycles of 94 °C for 30 s, 53 °C for 45 s, 72 °C for 2 min; and 72 °C for 10 min for the final extension for the primers, except for the jgHCO/jgLCO that the annealing temperature was 55ºC. The PCR amplicons were visualized in agarose gel 1.5% by

electrophoresis. The amplicons were purified with EXO/rSAP purification enzyme (Cellco Biotech) for 20 min at 37ºC and 10 min at 80ºC. The amplicons were sequenced by Sanger sequencing and the resulting sequences were edited and aligned inthe software Mega 11.

3.2.6. Diversity index

The biodiversity indexes were directly and indirectly evaluated using 6 index: species richness (S), Shannon-Wiener diversity index (H'), Pielou evenness (J'), Simpson index (1-D), dominance (D) and abundance (Ab). The classifications of dominant and rare species were based on the proportion of their abundance in the total sampled in each treatment. Species rarefaction curve was calculated with 500 randomizations using the software *EstimateS 9.1* (Colwell, R. K. 2013). Species number, Jackknife mean and jackknife standard deviation were plotted to obtain the curve. To determine if the diversity indexes between organic and conventional treatment were statistically different, firstly was performed a shapiro wilk test, to verify if the data had normal distribution and a Levene test to check if their variance were homogenous. A t- student test with the means of six biodiversity indexes from the five plots of each treatment (organic and conventional) was performed. All analyses were done in the software R Core Team (2021) with the packages "*car*" (Fox & Weisberg, 2019), "*dplyr*" (Wickham et al, 2022), and "*RVAideMemoire*" (Hervé 2022).

3.3. Results

A total of 17 DNA barcode were produced from 12 species that are considered important pests or predators in maize (See appendix A). The sequence length varied from 566 pb to 658 pb. It was sampled 2458 individuals were samples, being 1311 from organic and 1147 from conventional maize. The most abundant individualswere *D. luteipes* in all areas, followed by Formicidae. In the conventional areas, were found 2 species of Coccinellidae, *H. axyridis* and *C. sanguinea* in a total of 8 individuals in organic and conventional maize. and in the organic was sampled the same species from conventional, and *H. convergens* and other species of Coccinellidae not identified were sampled only in organic. In total 126 individuals of Coccinellidae were in organic. The organic areas had more predators as Coccinellidae, Staphylinidae, and Arachnida, except for *D. luteipes* that was more abundant in numeric number in conventional (Table 3.1).

Table 3.1: Species identification and abundance ranked divided into organic and conventional maize crops.

ORGANIC		**CONVENTIONAL**	
Identification	**Abundance**	**Identification**	**Abundance**
Doru luteipes	720	*Doru luteipes*	866
Formicidae	232	Formicidae	170
Harmonia axyridis	104	Forficulidae	15
Lygaeidae	63	Gryllidae	15
Gryliidae	38	Arachnida	14
Coleoptera larva	27	Diptera	11
Arachnida	24	Erotylidae	10
Staphylinidae	19	*Lagria villosa*	9
Cycloneda sanguinea	18	Coleoptera larva	6
Lepidoptera larva	10	*Cycloneda sanguinea*	6
Chrysomelidae	6	Cydnidae	4
Diptera	5	Staphylinidae	4
Erotylidae	5	Blattellidae	3
Diabrotica speciosa	4	Lepidoptera larva	3
Eulophidae	4	*Diabrotica speciosa*	3
Blattaria	3	Elateridae	2
Cydnidae	3	*Harmonia axyridis*	2
Elateridae	3	Pentatomidae	2
Coccinellidae	3	Lygaeidae	1
Melolonthidae	2	Melyridae	1
Reduviidae	2	Coreidea	1
Lagria villosa	2		
Alydidae	1		
Cerambycidae	1		
Eulophidae	1		
Meloide	1		
Mormidea v-luteum	1		
Scarabaeidae	1		
Hippodamia convergens	1		
Cercopidea	1		
Pentatomidae	1		
Total	**1311**	**Total**	**1147**

For the diversity measures, there were no significant difference between species abundance ($t(8) = 1.1516$ and $p > 0.05$) and richness ($t(8) = 1.6508$ and $p > 0.05$). However, for Shannon index ($t(8) = -3.1566$ and $p < 0.05$), Simpson index ($t(8) = -2.6126$ and $p < 0.05$), evenness ($t(8) = -2.9501$ and $p < 0.05$), and dominance ($t(8) = 2.6126$ and

p< 0.05) the organic areas showed a higher diversity and species dominance when compared to conventional areas (Fig. 3.1).

The species rarefaction curve obtained did not achieve the asymptote for organic areas, indicating that was necessary more efforts to samples the diversity in the local. Although, the conventional area achieved the asymptote with the samples made (Fig 3.2).

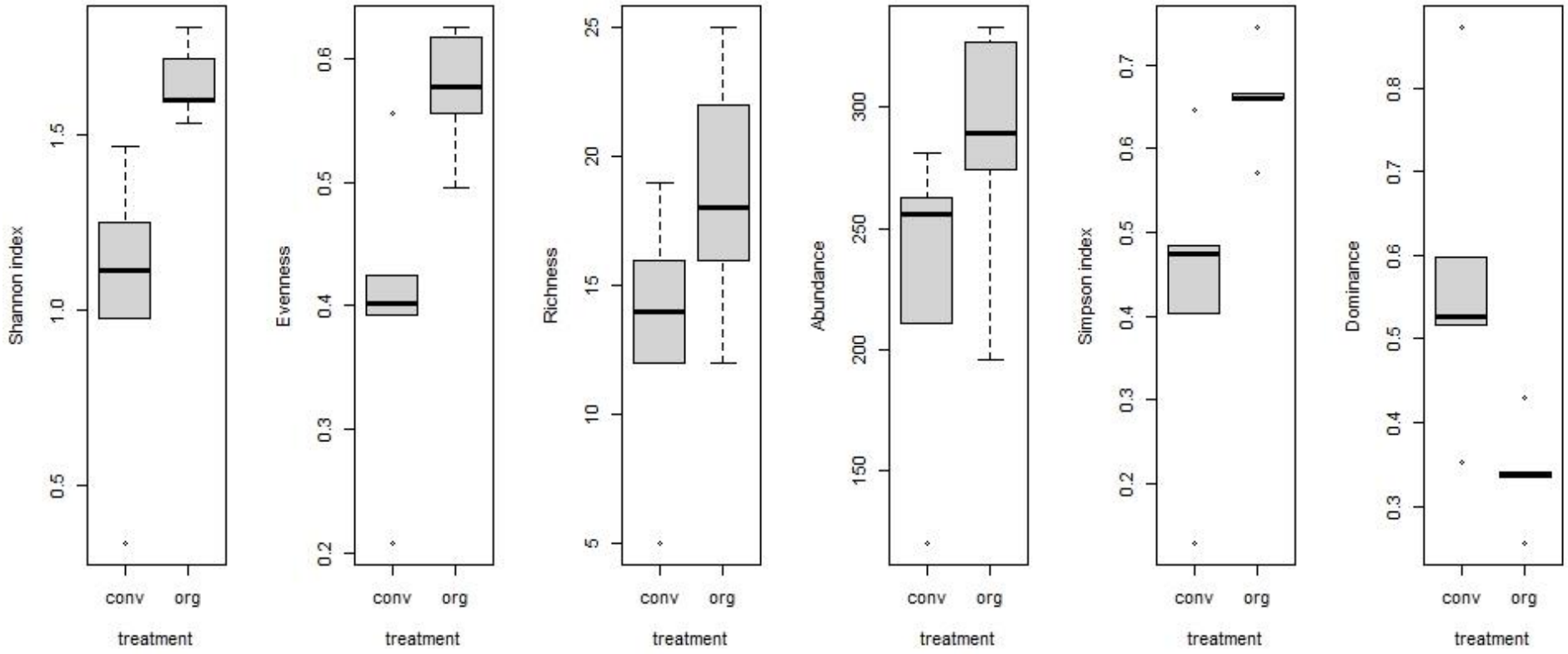

Fig 3.1 The boxplots are representing the median for Shannon index, Evenness, Richness, Abundance, Simpson index, and Dominance index for the organic and conventional areas sampled during the experiment. Shannon index ($t(8) = -3.1566$ and $p < 0.05$), evenness ($t(8) = -2.9501$ and $p < 0.05$), Species richness ($t(8) = 1.6508$ and $p > 0.05$), Abundance ($t(8) = 1.1516$ and $p > 0.05$),Simpson index ($t(8) = -2.6126$ and $p < 0.05$), Dominance index ($t(8) = 2.6126$ and $p < 0.05$).

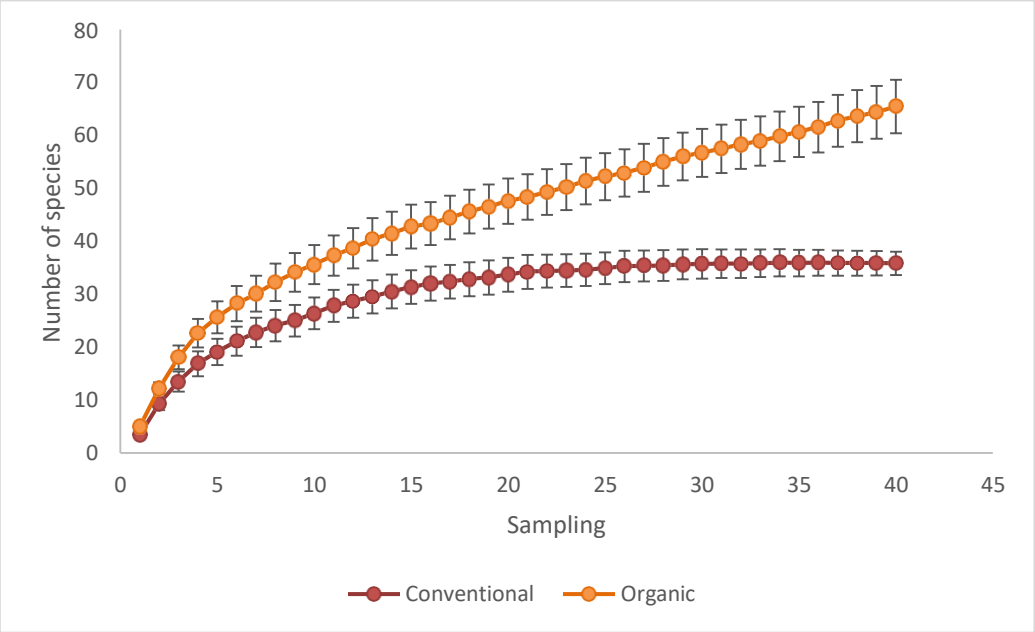

Fig 3.2 Species rarefaction curve plotted with sampling numbers, Jackknife mean, and jackknife standard deviation from organic and conventional areas of maize.

3.4. Discussion

The results showed that organic cultivation had greater diversity when compared to conventional cultivation in the Shannon, Simpson, evenness and dominance diversity indices. But there was no difference between species richness and abundance using the sampling methodology here described. The barcode DNA of the main pests and predators were successfully produced and added to the personal database for use in metabarcoding Chapter 5 in this thesis.

Despite knowing the need to study the diversity of species in a place, to understand how communities are established, there are still many undescribed insect species and also groups that are less studied. The most commonly used species identification is by morphology, but molecular identification has become an ally with the barcode (Hebert *et al,* 2003). Many species that were considered to be identical, with the barcode it was possible to identify that the genetic distance between these individuals was sufficient to classify them as different species (Bickford *et al*, 2007; Ashfaq & Hebert, 2016; Lima *et al*, 2022).

The elaboration of barcode DNA is not only useful in species identification work. Identification of trophic interactions, either by PCR or metabarcoding, need robust databases for the development of specific primers and also for comparisons with the sequences obtained (Batuecas *et al*, 2022; Marquina *et al*, 2019; Šigut *et al*, 2017, Staudacher *et al*, 2016). From the moment that it is possible to know the

species of the place, interaction studies and the management of areas would make it easier. For a correct management of a pest in agricultural crops, for example, their identification correctly is essential for the correct control.

The organic cropping system presented some higher diversity indexes than the conventional cropping system. This result was expected, as many studies have already addressed the positive influence of organic farming on the diversity of arthropods in the area, mainly natural enemies (Jacobsen *et al*, 2019; Bellamy *et al*, 2018; Popov *et al*, 2018). The non-difference between abundance and species richness between conventional and organic may have been due to the great abundance of *D. luteipes* in both areas, proving to be the most dominant species, and the organic presented only 10 species more than the conventional, which were species with low abundance.

One of the points to be highlighted is the abundance of Staphylinidae and mainly Coccinellidae found in the organic system. By having more spontaneous plants that function as shelter, corridors and alternative sources of food, the organic system favors the establishment of predators in the place (Purtauf *et al,* 2005). The presence of predators in abundance is important, especially in organic farming where the lowest use of chemical pesticides and pest control normally occurs with biological products.

Doru luteipes was the most abundant insect collected in both cultures, being a little more abundant in the conventional one. This species is found over time in corn plantations and is often associated with predation by *S. frugiperda* and *H. zea* in the laboratory (Cruz *et al*, 1995; Reis *et al*, 1988). However, in the field this predator showed low consumption rates of *S. frugiperda*, suggesting that its predatory habit is secondary (Maggio *et al*, 2022).

The practice of organic cultivation has favored the local community of arthropods and several studies address this bias. A study conducted by Jacobsen *et al*, 2019 showed that conventionally managed strawberry crops showed 10x more *Tetranychus urticae* (Acari: Tetranychidae) than organically grown strawberry that showed more natural enemies that were favored due to management and surrouding vegetation. Popov *et al*, 2018, showed that in organic cultivation of apple orchard the management was important as fertilizers and biological plant protection and that microclimatic conditions can affect the local arthropod diversity.

3.5. Conclusion

- Organic maize crop has a greater diversity compared to conventional maize crop in Shannon index, Simpson index, Dominance and evenness;
- There is no difference in species richness and abundance among organic and conventional;
- The DNA barcodes were useful to identify species and were added to DNA barcoding data banks.

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4. DNA BARCODING, DIVERSITY, ABUNDANCE AND PEST PREDATION RATE OF GROUND BEETLES (COLEOPTERA: CARABIDAE) IN ORGANIC AND CONVENTIONAL MAIZE CROPS

Abstract

The ground beetles (Coleoptera: Carabidae) are predators found in soil and known as biological control agents for having a diversified diet that includes pest insects. To know the diversity, abundance and impact of cropping systems on ground beetles' species, our aims were: (i) produce DNA barcodes for the identification of ground beetles' species collected in maize crops; (ii) evaluate the diversity and abundance of ground beetles sampled in organic and conventional maize crops; and (iii) evaluate, using molecular markers, the presence of predation of ground beetles on the main pests of maize: *Spodoptera frugiperda, Helicoverpa zea, Rhopalosiphum maidis* and *Diabrotica speciosa.* For that, five areas of organic maize and five areas of conventional maize were sampled during maize reproductive stage. There were installedsix dry pitfalls in each area for 48h. The ground beetles were separated into morphotypes/species and individualized in tubes with ethanol 100%. The DNA was extracted with CTAB protocol. For the DNA barcoding was used the first 658 pb of COI gene. To estimate the predation on the main pests by ground beetles, were used specific primers for each species. It was sampled 283 individuals of 19 morphotypes, being 259 individuals of 15 morphotypes in organic maize and 24 individuals of 10 morphotypes in conventional. The two most abundant species in the organic maize were *Selenophorus* sp1 with 118 individuals and *Selenophorus alternans* with 56 individuals. In the conventional maize, the genus with more individuals was *Pterostichus* with 6 individuals. Organic showed a higher diversity on Shannon index, Simpson index, and richness when compared to conventional areas. Also, in the organic areas there was a dominance of species, maily because of the higher number os *Selenophorus* species samped. No predation events were detected on *S. frugiperda*, *H. zea*, *R. maidis,* and *D. speciosa* by the ground beetles in both crop systems. The DNA barcoding was crucial for reaching the correct number of Carabidae species and will be a helful tool for future taxonomic studies of ground beetle's species. The difference in species diversity between cropping systems shows a positive impact of organic cropping on diversity and species richness of ground beetles in maize areas. However, ground beetles seems to have a preference on feeding on alternative preys or seed weed, instead of the main pests in maize crops.

Keywords: Conservative biological control, Species richness, predators, molecular markers.

4.1. Introduction

The agriculture landscapes have changed during a long time due to the intensive land-use. These practices can result in habitat losses and fragmentation, which impacts the local community, being able to change the species composition and function (Acosta *et al*, 2017; Arpaia *et al*, 2017; Bellamy *et al*, 2018). With the

integrated pest management (IPM), the techniques applied in crops areas to control pests are more focused in reduce the inputs and preserve the environment.

The landscape composition and farming activities, as the type and use of lands, application of pesticides and herbicides, can affect ground beetles' (Coleoptera: Carabidae) assemblages (Palmu *et al*, 2014; Cajaiba *et al*, 2018), that are considered as biological control agents. Organic managed fields enhance predators' abundance and reduces pests for some species, but for some ground beetles' species, the intensive agriculture seems to favor them in big areas instead of small areas (Döring & Kromp, 2003; Gallé *et al*, 2019; Jacobsen *et al*, 2019).

Many studies with carabids in agricultural areas showed their potential role in pest control. The ground beetles are the largest family belonging to adephagan beetles and can be used as bioindicators because they are sensitively to human changes in the habitat (Cajaiba *et al*, 2018). They are polyphagous, voracious and can feed on insect pests and weed seeds. Many studies have shown that carabids can consume meals of almost their body size and a considered number of seeds (Labruyere et al, 2016; Matta et al, 2017). Furthermore, these insects may be important predators of agricultural pests in different crops systems (crop species and cultivation systems) (Menalled et al, 2007; Cividanes, 2021).

However, in tropical regions, the diversity of carabid species is not completely known (Cajaiba *et al*, 2018). Furthermore, the taxonomic keys are focused in species identified in Europe and North America regions, promoting identification error that may compromise the species diversity and abundance quantification and the comparison with data banks from other works (Balakrishnan, 2005). Actually, the DNA barcoding approach has allowed the species identification using a standardized methodology to each Life Kingdom without the morphological characterization or binomial nomenclature (Valentini *et al*, 2009; Hebert *et al*, 2003) for example, to Kingdom Animalia the sequencing of first 658 bp (nucleotides) of *cythocrome c oxidase subunit I* (COI) is a methodology recommended to species definition (Hebert et al., 2003).

Furthermore, molecular techniques that use molecular markers with species-specific primers (previously developed) are useful and enable to detect the presence of DNA of a species without the necessity of gene sequencing, using just a presence or absence of the DNA amplification of a specific species in agarose gel (Greenstone *et al*, 2007; Traugott *et al*, 2012; González-Chang *et al*, 2016; Krehenwinkel *et al*,

2017). This approach can identify species in low financial cost and can be useful to identify ecological interactions such as herbivory, predation, and symbiosis detection (Kamenova *et al*, 2018).

The predation or consumption in the fields are difficult to identify by observation, especially carabids that can have a nocturnal feeding behavior (Cole *et al*, 2002). Thus, these molecular markers with species-specific primers enable to detect the presence of prey DNA into the predators' gut-content (Greenstone *et al*, 2007; Traugott *et al*, 2012). Another advantage of this tool is that even after hours fed on is possible to amplify the prey DNA which is fragmented and in lower concentration. This is only possible due to the small size of the amplified fragment, but not so small that is not possible to distinguish the target species from the predator or other non-target species (Greenstone *et al*, 2014).

Considering carabids as an important predator found in different managed agriculture areas and their importance as bioindicator and a potential natural enemy of pests, our general aims were to evaluate the diversity, abundance and predation rate of carabids in organic and conventional managed maize crops. The specific aims were: 1) identify the carabids species using identification keys and DNA Barcoding methodology; 2) evaluate the diversity and abundance of carabids in organic and conventional managed maize fields; and 3) estimate the predation rate of carabids on severe maize pests using DNA gut content analysis.

4.2. Material and Methods

4.2.1. Field

The samples were carried out in 10 areas of maize during its reproductive stage, where three organic areas and one conventional area were in Santa Rita do Passa Quatro, one organic and two conventional areas were in Pirassununga, one conventional in Santa Cruz da Conceição, and one organic and one conventional areas were in Ipeúna, all areas in São Paulo state, Brazil. Each area was divided into 2 plots and installed three dry pitfall traps in each plot. The dry pitfalls were made of plastic bottle with small pieces of wood to create a refugee for the predators and keep them alive. The pitfalls were emptied every 24h and the parcels were active sampled randomly for 45 min. The sampling process occurred for two days. The insects sampled were individualized into tubes and kept in cold bags until arrives in the laboratory, where were storage at -20ºC with ethanol 100%. This field was the same as described in chapter 3 with more details.

4.2.2. Morphological and molecular identification

4.2.2.1. Morphological identification

The insects were firstly separated into morphotypes and after identified in tribe, genera or species, using the identification keys available for Carabidae on Pierre Moret 2003, Choate 2003 and Reichardt 1977.

4.2.2.2. DNA extraction

The ground beetles with one or two individuals had their DNA extracted using a non-destructive protocol. The individuals were submerged in 500 µL of non-destructive buffer, 12,5 µL of proteinase k 20 mg mL^{-1} (Invitrogen) and incubated overnight at 65ºC. The individuals were removed and submerged in ethanol 100% to stop the digestion. It was added 500 µL of chloroform and isoamyl alcohol (24:1), and centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was transferred to a new tube and was added 1/10 of the supernatant volume of sodium acetate (3M, pH 5,2), 2,5 µL of glycogen 5 mg mL^{-1} (Invitrogen), and 7/10 of the volume of cold isopropanol 100% and incubated overnight at -20ºC. The samples were centrifuged at 14,000 rpm for 30 min at 4ºC, the liquid phase was discarded, was added 500 µL of cold ethanol 70%, discarded and added 500 µL of cold ethanol 100% and discarded. The DNA pellet was air-dry and resuspended in 50 µL of autoclaved distilled water.

The morphotypes with more than two individuals, the DNA was extracted using de CTAB protocol. First legs with muscles were removed from the body and macerated with liquid nitrogen. The samples were submerged in 500 µL CTAB buffer, 10 µL of proteinase k 20 mg mL^{-1} and 2 of β-mercaptoethanol and incubated for 2h at 65ºC. Then, was added 3 µL of RNAse (Invitrogen) and incubated at 37ºC for 2h and after at 65º C for 30 min. The samples were centrifuged at 14,000 rpm for 5 min at room temperature. The liquid phase was transferred to a new tube, added 500 µL of isoamyl alcohol (24:1) and centrifuged for 20 min. The supernatant was transferred to a new tube and the isoamyl alcohol step was repeated once more. The supernatant was transferred and added 400 µL of cold isopropanol 100% and incubated overnight at -20ºC. The samples were centrifuged at 14000 rpm for 30 min at 4ºC. The next steps were the same as described at the end of the non-destructive extraction. The DNA pellet were resuspended in 30 µL of autoclaved distilled water.

4.2.2.3. PCR and sanger sequencing

The universal primers LCO/HCO (Folmer et al, 1994) and LEP F/R (Hebert et al. 2004) were used to amplify the cytochrome c oxidase subunit I (COI) gene, to obtain the barcode for each morphotype. A PCR assay was optimized in a volume of 25 µL, with 3.0 µL of DNA, 10.7 µL of autoclaved distilled water, 2.5 µL of 10X magnesium-free buffer (Sinapse Inc.), 2.5 µL MgCl2 (25 mM, Sinapse Inc.), 2.0 µL dNTP (2.5 mM, Sinapse Inc.), 2.0 µL of each primer (5 pmol), and 0.3 Taq DNA polymerase 5U/µL (Sinapse Inc.). The program used in the thermocycler was 94ºC for 3 min, 35 cycles of 94ºC for 30 seconds, 53ºC for 45 seconds, 72ºC for 2 minutes and 72ºC for 10 minutes in the extending stage. The results were evaluated by electrophoresis on 1.5% agarose gel. The fragments amplified were cleaned-up using recombinant shrimp alkaline phosphatase (rSAP) and exonuclease I (Exo I) (Cellco Biotech) and sequenced by Sanger.

After, the sequences were aligned e edited in the software BioEdit 7.2 (Hall, T.A. 1999). Additionally, we checked the presence the NUNTs (Nuclear paralagous genes) in COI sequences looking for “stop codons” in COI sequences (Lopez et al., 1994).

4.2.2.4. Genetic distance

To check if the morphotypes were truly different among then, a genetic distance was performed in the software Mega 11 involving COI sequences from 18 ground beetles morphotypes. The analyses were conducted using Kimura-2-parameter model as substitution model and the variance estimation method by bootstrap method. After, the COI sequences were submitted to two data banks: the NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Barcode of Life Data System (BOLD System - https://www.boldsystems.org/) to check the sequences homology and achieved the species identification. We considered that the identification was performed to a specific level just when we obtained a homology higher than 98%.

4.2.3. Diversity and abundance indices

The biodiversity indexes was directly and indirectly evaluated using 6 indexes: species richness (S), defined as the number of morphotypes sampled, Shannon-Wiener diversity index (H’), Pielou evenness (J’), Simpson index (1-D), dominance (D) and

abundance (Ab). The abundance was based on the number of individuals samples from each morphotype. The classifications of dominant and rare species were based on the proportion of their abundance in the total sampled in each treatment. Species rarefaction curve was calculated with 500 randomizations using the software *EstimateS 9.1* (Colwell, R. K. 2013). Species number, Jackknife mean and jackknife standard deviation were plotted to obtain the curve. To determine if the diversity indexes between organic and conventional treatment were statistically different, was performed a shapiro wilk test, to verify if the data had normal distribution and a Levene test to check if their variance were homogenous. A t-student test with the means of five diversities indexes from the five plots of each treatment (organic and conventional) was performed. The richness index did not have a normal distribution, then a Mann-Whitney test was performed. All analyses were performed in the software R Core Team (2021) with the packages "*car*" (Fox & Weisberg, 2019), "dplyr" (Wickhamet al, 2022), and "RVAideMemoire" (Hervé 2022).

4.2.4. Predation rate in the field

4.2.4.1. DNA extraction

Ten morphotypes were selected to detect presence of prey DNA in their gut-content. Just morphotypes with more than 4 individuals were selected due to the reproducibility of our results. The individuals were cleaned for 2 seconds in 2% sodium hypochlorite and then in 70% ethanol. After they were submerged in autoclaved distilled water and air-dry on a tissue paper. The gut-content DNA was extracted using the entire body, except for *Galerita sp* and *Calosoma alternans* that were used only the gut by dissecting them. The DNA was extracted using CTAB protocol, as described above.

4.2.4.2. Primers

It was selected 4 maize pests to be detected in the predators' gut-content, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), *Helicoverpa zea* (Lepidoptera: Noctuidae), *Rhopalosiphum maidis* (Hemiptera: Aphididae) and *Diabrotica speciosa* (Coleoptera: Chrysomelidae). The species-specific primers to detect *S. frugiperda* DNA were Spo_frugi-F and Spo-frugi-R (Maggio et al., 2022), for *H. zea* DNA the primers Hzea-154F and Hzea-307R (Peterson et al., 2018) and for *R. maidis* the primers ClaCOIIF and ClaCOIIR1 (Chen et al., 2000). The species-specific primers

to detect *D. speciosa* DNA, D_speciosa_F: TATAGTAGGGACATCCCTG and D_speciosa_R: GTAATAGTAAAGAGGGTGGTAGT, were designed using COI sequences from different places available in GenBank. The sequences were aligned with sequences from other species belonging to *Diabrotica* genera, thus the primers only amplify *D. speciosa*. A PCR assay was carried out to test the primers specifity with DNA from *D. speciosa* (as a positive control), *S. frugiperda, Spodoptera eridania, Spodoptera albula, Spodoptera cosmioides, H. zea, Harmonia axyridis* (Coleoptera: Coccinellidae)*, Cycloneda sanguinea* (Coleoptera: Coccinellidae)*, Hippodamia convergens* (Coleoptera: Coccinellidae)*, Doru luteipes* (Dermaptera: Forficulidae) and water (as a negative control). The PCR reaction was optimized in a volume of 25 µL with 3 µL of DNA, 10.7 µL of autoclaved distilled water, 2.5 µL of 10X magnesium-free buffer (Sinapse Inc.), 2.5 µL MgCl2 (25 mM, Sinapse Inc.), 2.0 µL dNTP (2.5 mM, Sinapse Inc.), 2.0 µL of each primer (5 pmol), and 0.3 Taq DNA polymerase 5U/µL (Sinapse Inc.). The thermocycler program at 94ºC for 3 min, 35 cycles of 94ºC for 30 seconds, 55º seconds for 45 seconds, 72ºC for 2 minutes and to finishe at 72ºC for 10 minutes. The results were visualized in agarose gel 2% by electrophoresis.

4.2.4.3. Prey detection

PCR assays were carried out to detect the presence of prey DNA in the predators' gut-content. The PCR assay for the primers Spo_frugi-F/Spo_frugi-R was the same as described by Maggio et al 2022. For the primers Hzea-154F/Hzea-307R the reaction was optimized in a volume of 25 µL, with 3.0 µL of DNA, 14.62 µL of autoclaved distilled water, 2.5 µL of buffer (Invitrogen), 0.75 µL MgCl2 (50 mM, Invitrogen), 2.0 µL dNTP (2.5 mM, Sinapse Inc.), 1.0 µL of each primer (5 pmol), and 0.125 Taq DNA polymerase 5U/µL (Invitrogen). For the primers ClaCOIIF/ClaCOIIR1 was optimized in a volume of 14 µL, with 7 µL of DNA, 1.5 µL of 10X magnesium-free buffer (Sinapse Inc.), 1.5 µL MgCl2 (25 mM, Sinapse Inc.), 1.0 µL dNTP (2.5 mM, Sinapse Inc.), 1.0 µL of each primer (5 pmol), and 0.3 Taq DNA polymerase 5U/µL (Sinapse Inc.). For the primers D_speciosa_F/D_speciosa_R the PCR reaction was used as described above.

For all PCR assays, the thermocycler program was the same, expect for the annealing temperature that was 57º for Spo_frugi-F/Spo_frugi-R, 60ºC for Hzea-154F/Hzea-307R and 55ºC for ClaCOIIF/ClaCOIIR1 and

D_speciosa_F/D_speciosa_R. The denaturation stage at 94ºC for 3 min, 35 cycles of 94ºC for 30 seconds, specific-primer temperature for 45 seconds, 72ºC for 2 minutes and 72ºC for 10 minutes in the extending stage. The results were evaluated by electrophoresis on 2% agarose gel and the positive amplification were written down.

4.3. Results

4.3.1. Molecular and morphological identification

It was identified 19 different morphotypes of ground beetles belonging to 9 tribes and more than 10 Genera (Table 4.1). The morphotypes were identified to Genus and/or Species level with morphological characters and confirmed with molecular identification. The morphotype 8 was identified just the tribe with morphological characters. Overall, 30 DNA barcodes sequences were produced from 18 morphotypes, except for the morphotype 5 that was not possible to amplify the barcode region. The sequence length varied between 658 to 502 pair of bases (see appendix B).

The genetic distance analyses showed that all 18 morphotypes, with the DNA barcode sequence, are different between them, confirming that they belong to different species, as divided by morphological characters. The genetic distance variation among the two species from the genera *Selenophorus* was 0.5386. Among species from genera *Pterostichus* the variation was between 0.1297 to 0.9865, and from genera *Tetragonoderus* the variation was 1.0139. When we compare the two most abundant genera sampled, *Selenophorus* and *Pterostichus*, the genetic distance among them varied from 0.1135 to 1.2330 (Table 2).

After the data publication, all specimens/morphotypes will be deposited at Museu Luiz de Queiroz at ESALQ/USP.

Table 4.1: Ground beetles' species and morphotypes sampled in organic and conventional areas of maize identified with morphological characters and molecular identification with COI gene.

Morphotype	Tribe	Genera	Species	Barcode identification (homology similarity)*
M1	Harpalini	*Selenophorus*	Sp 1	
M2	Pterostichini	*Pterostichus*	Sp1	
M3	Harpalini	*Acupalpus*	Sp 1	
M4	Pterostichini	*Pterostichus*	Sp 2	
M5	Megacephalini	*Tetracha*	Sp 1	
M6	Lebiini	*Lebia*	*Lebia concinna*	
M7	Harpalini	*Trichotichnus*	Sp 1	
M8	Harpalini			
M9	Harpalini	*Selenophorus*	Sp 2	*Selenophorus alternans* (Private database Bold)
M10	Harpalini	*Notiobia*	Sp 1	
M11	Masoreini	*Tetragonoderus*	*Tetragonoderus laevigatus*	
M12	Pterostichini	*Pterostichus*	Sp 3	
M13	Masoreini	*Tetragonoderus*	*Tetragonoderus intersectus*	*Tetragonoderus intersectus* (MW340195 = 97%)
M14	Harpalini	*Bradycellus*	Sp 1	
M15	Odacanthini	*Colliuris*	Sp 1	*Colliuris pilatei* (MN345324.1 = 100%)
M16	Lebiini	*Cymindis*	Sp 1	
M17	Helluonini	*Helluomorphoides*	Sp 1	
M18	Carabini	*Calosoma*	*Calosoma alternans*	*Calosoma alternans* (MN344335.1 = 99%)
M19	Galeritini	*Galerita*	*Galerita brasiliensis*	

* NCBI Blast (Benson et al. 2013 - http://www.ncbi.nlm.nih.gov) and BOLD System (Ratnasinghan and Hebert. 2007 - www.barcodinglife.org)

Table 4.2. Genetic distance (Kimura 2- parameter) of ground beetles' morphotypes sampled in organic and conventional areas of maize.

	M1	M2	M3	M4	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18
M1																	
M2	0.5015																
M3	0.5025	0.1184															
M4	0.5354	0.1297	0.1153														
M6	0.5200	0.1400	0.1327	0.1520													
M7	0.0758	0.5149	0.4902	0.5089	0.4990												
M8	0.5304	0.1274	0.0667	0.0995	0.1291	0.5134											
M9	0.5386	0.1473	0.0852	0.1135	0.1273	0.5107	0.0618										
M10	0.5141	0.1201	0.0784	0.1012	0.1345	0.4990	0.0852	0.0853									
M11	0.5084	0.1236	0.0972	0.0995	0.1255	0.4840	0.1042	0.1077	0.0886								
M12	1.2330	0.9451	0.9058	0.9865	0.9817	1.2568	0.9877	1.0011	0.9810	1.0011							
M13	1.3545	0.9274	0.8929	0.9872	0.9842	1.3800	0.9484	0.9772	0.9543	1.0139	0.1194						
M14	0.5407	0.1291	0.1203	0.1464	0.1643	0.5477	0.1382	0.1455	0.1256	0.1401	0.9019	0.9542					
M15	0.5387	0.1421	0.1257	0.1319	0.1422	0.5458	0.1293	0.1439	0.1384	0.1167	1.0192	1.0433	0.1439				
M16	0.5430	0.1255	0.1112	0.0061	0.1457	0.5114	0.0921	0.1059	0.0990	0.0955	0.9750	0.9772	0.1419	0.1240			
M17	0.5099	0.1796	0.1777	0.1584	0.1836	0.5069	0.1595	0.1733	0.1796	0.1497	1.0792	1.0160	0.1981	0.2045	0.1555		
M18	0.5088	0.1531	0.1686	0.1488	0.1904	0.5240	0.1685	0.1760	0.1515	0.1570	1.0025	1.0277	0.1823	0.1941	0.1498	0.2309	
M19	0.5211	0.2076	0.2133	0.1948	0.2249	0.5384	0.1839	0.2077	0.1958	0.2040	1.0950	1.0402	0.2133	0.1960	0.1918	0.1862	0.2455

4.3.2. Diversity and abundance indices

Overall, were found a total of 283 ground beetles' individuals, being 24 from conventional and 259 from organic. The morphotypes 3, 5, 16 and 17 are exclusively from conventional and morphotypes 4, 8, 9, 10, 12, 13, 14, 15 and 18 are exclusively from organic. Those areas have in common just the morphotypes 1, 2, 6, 7, 11 and 19 (Fig. 4.1). There was no significant difference for ground beetles' abundance when compared the two treatments (Table 4.3).

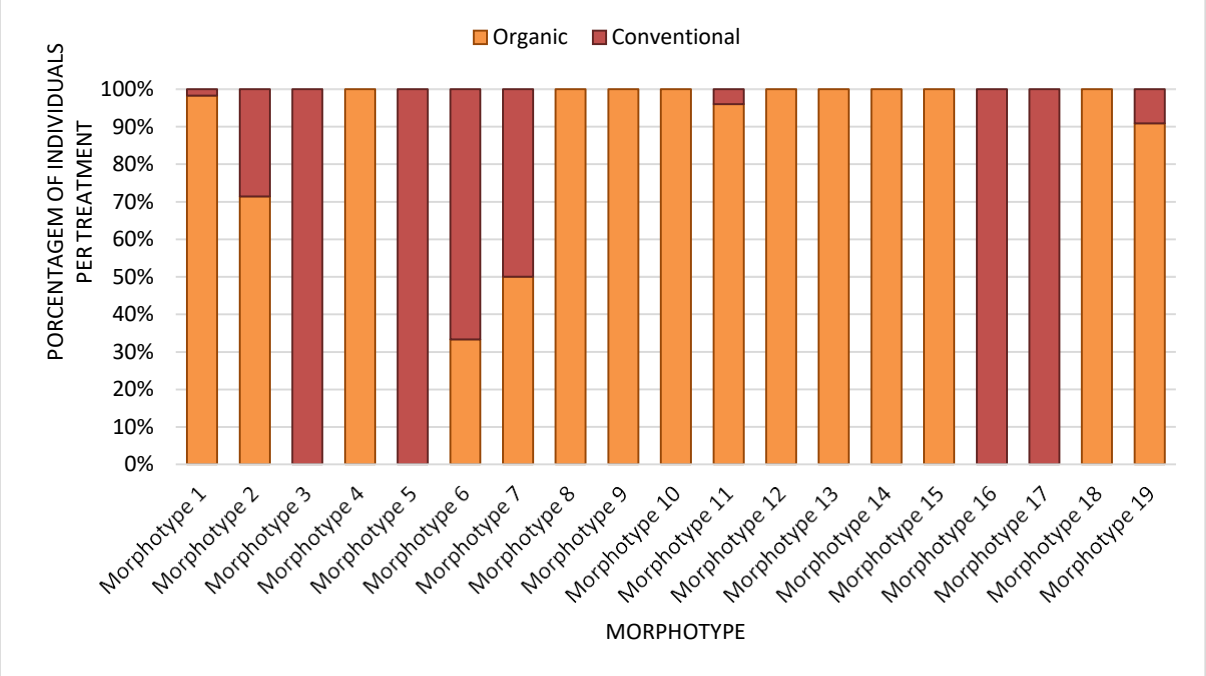

Fig 4.1. Percentage of individuals from each morphotype samples in organic and conventional areas of maize.

Table 4.3. Abundance, average abundance and species sampled in organic and conventional areas of maize.

Site	Replicates	Abundance	Average abundance	Genera	Species
Organic	5	259	52	11	15
Conventional	5	24	4.8	10	10

For the diversity measures, the organic maize had the highest species richness ($w = 0.5$ and $p < 0,05$) (Fig 4.2) with 15 species and conventional with 10 species. Despite the organic has a bigger number of species abundance than the conventional, there was no significant difference in abundance between treatments

(t(4.014) = -1.881 and p>0.05) and evenness (t(8) = 0,658 and p>0.05). The organic areas had higher values for Shannon index (t(8) = -4.095 and p<0,005) and Simpson index (t(8) = -2.696 and p<0.05) when compared to conventional areas (Fig 4.2).

The most abundant species (dominant species) sampled in the organic maize was the morphotype 1 (*Selenophorus* sp1) with 118 individuals, followed by morphotype 9 (*Selenophorus alternans*) with 56 individuals. The rarest species were morphotype 8 and morphotype 15 (*Colliuris* sp1) (Fig 4.3a). The most sampled species belongs to morphotype 2 (*Pterostichus* sp1) with 6 individuals and the least sampled were morphotypes 3, 5, 11 17 and 19 with one individual from each (Fig 4.3b). There was a significant difference between the areas associated with dominance index (D), where the organic area had more probability of individuals belong to the same species (t(8) = 2.696 and p<0.05).

The species rarefaction curve (Fig 4.4) did not achieve the asymptote in both areas indicating that was necessary more sampling in the areas. Although, in the organic the sampling was more efficient, with its curve close to the asymptote, when compared to the conventional curve.

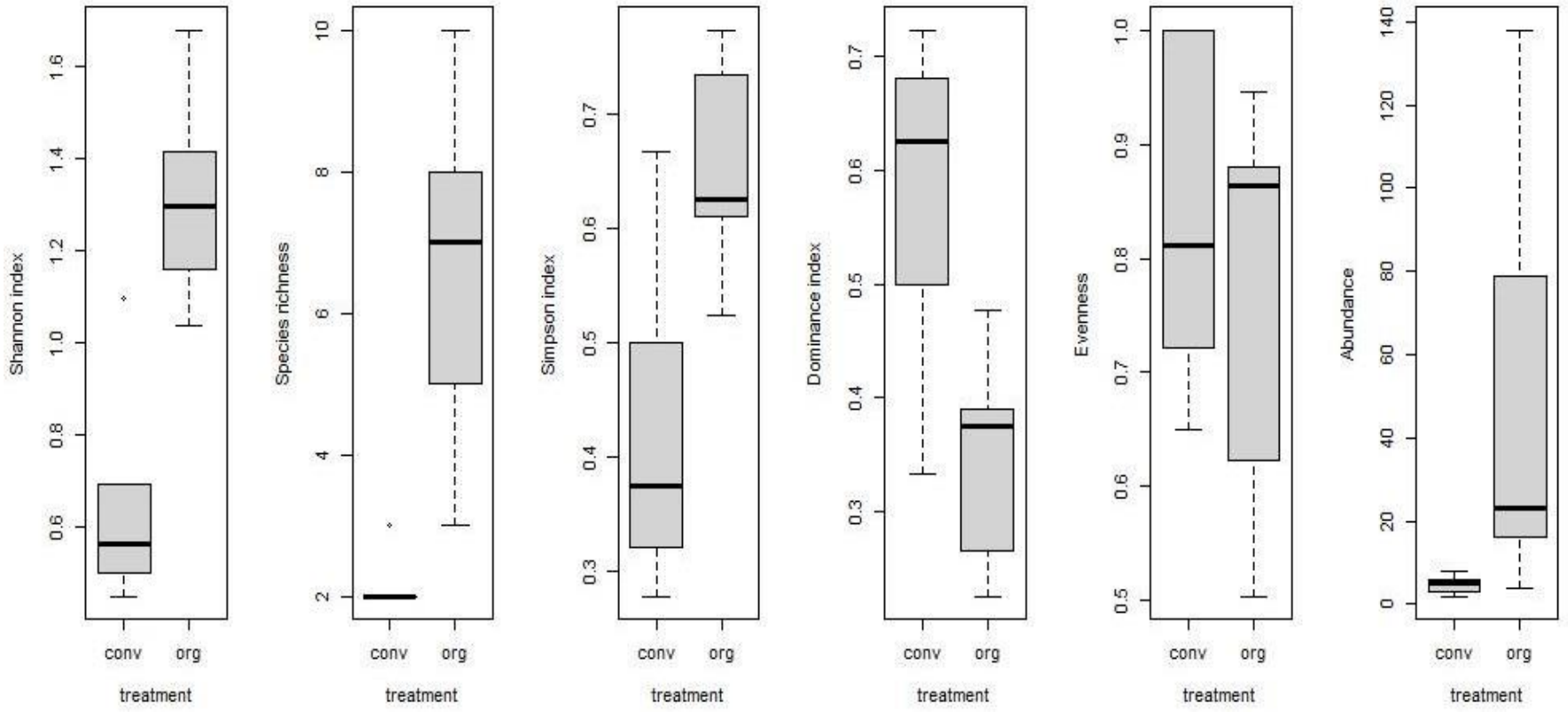

Fig 4.2. The boxplots are representing the median for Shannon index, Species richness, Simpson index, Dominance index, Evenness and Abundance for the organic and conventional areas sampled during the experiment. Shannon index ($t(8) = -4.095$ and $p<0{,}005$), Species richness ($w = 0.5$ and $p< 0{,}05$), Simpson index ($t(8) = -2.696$ and $p<0.05$), Dominance index ($t(8) = 2.696$ and $p<0.05$), evenness ($t(8) = 0{,}658$ and $p>0.05$), and Abundance ($t(4.014) = -1.881$ and $p>0.05$).

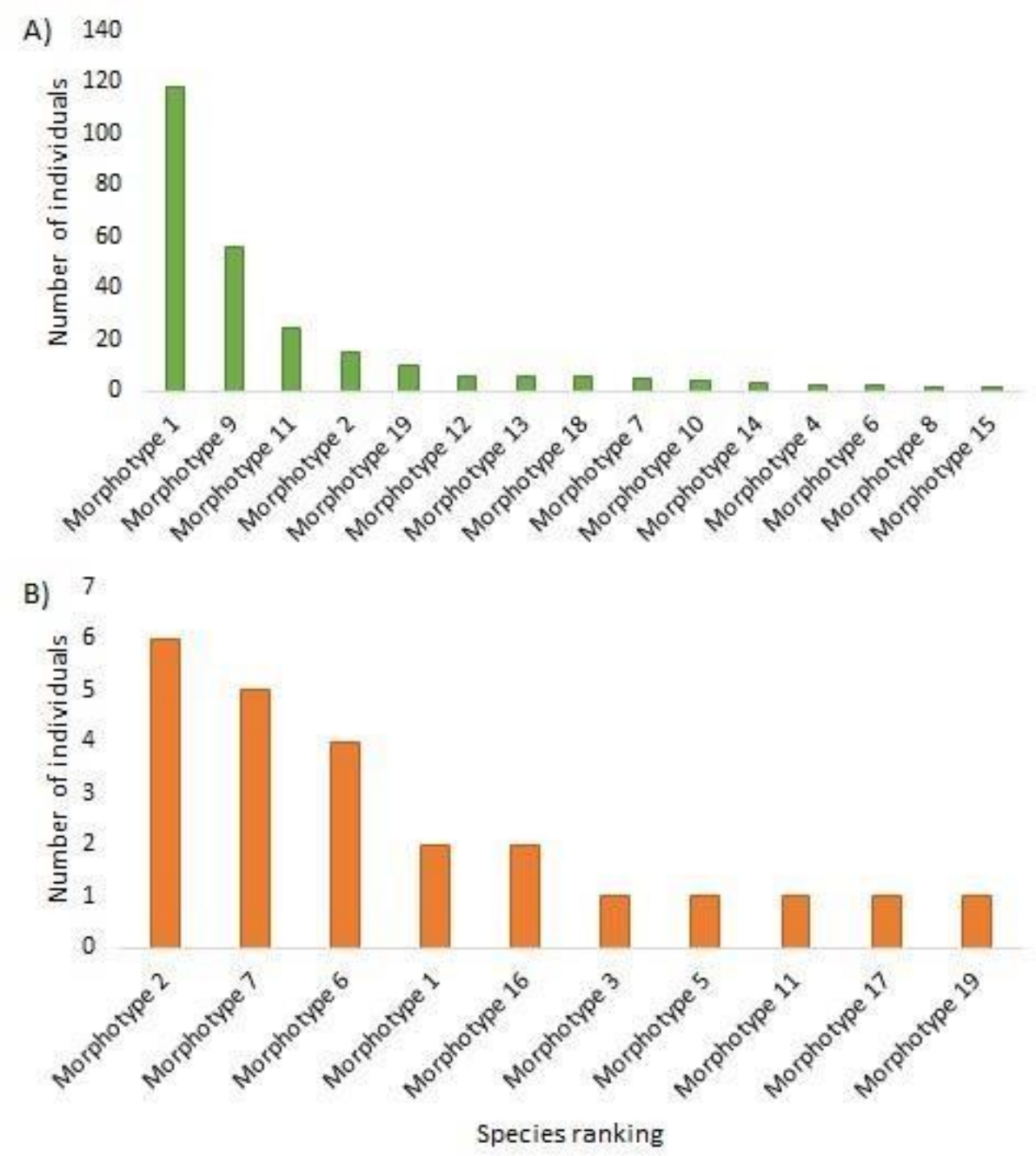

Fig 4.3. Species ranking from the most sampled species to the least sampled species per morphotype in A) Organic areas of maize, and B) Conventional areas of maize.

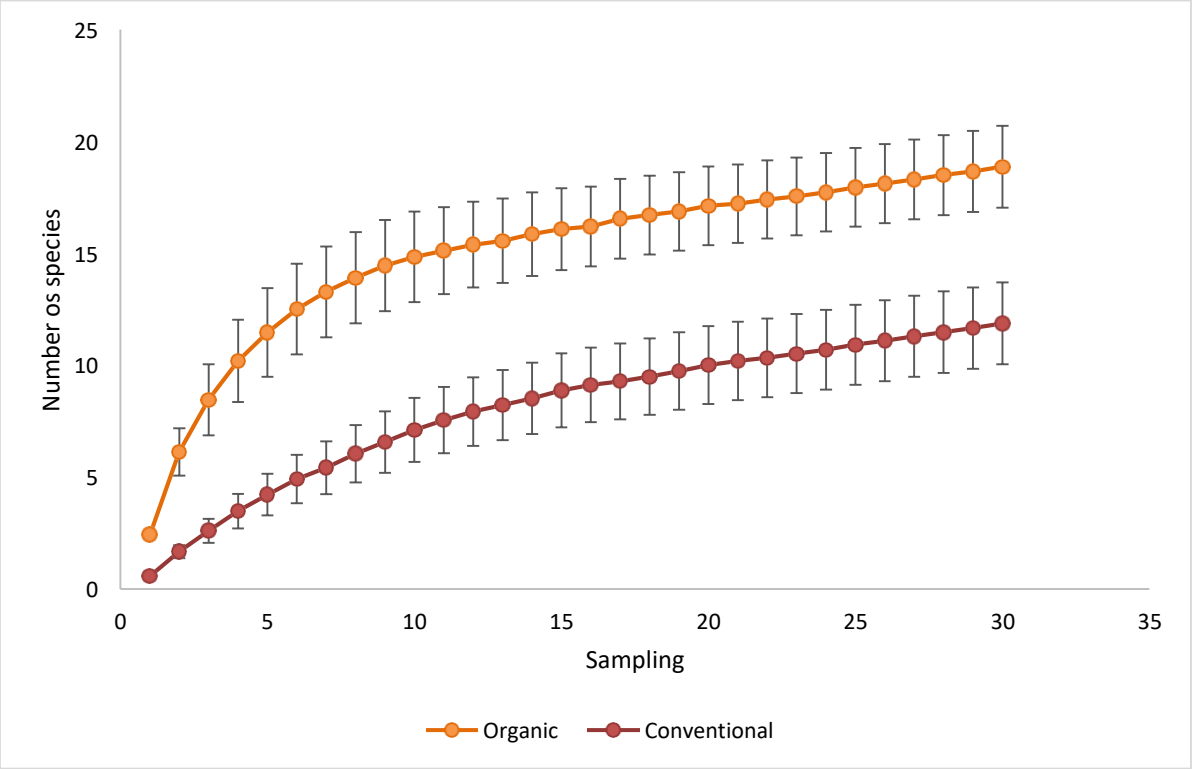

Fig 4.4. Species rarefaction curve plotted with sampling numbers, Jackknife mean, and jackknife standard deviation from organic and conventional areas of maize.

4.3.3. Predation rate in the field

Were selected 213 individuals belonging to 10 morphotypes from conventional and organic areas. For all the primers used to detect DNA of *S. frugiperda, H. zea, R. maidis* and *D. speciosa*, there were no positive results, showing no evidence of predation events by ground beetles during the time of the experiment.

4.4. Discussion

Despite the economic importance of the ground beetle family, as biological control agents, and used as bioindicators, little is known about their taxonomic identification. Few studies in Brazil are carried out regarding the description of species or taxonomic identification keys that help to identify individuals at species level. The main key used for the Neotropical region, which includes Brazil, is the dichotomous key made by Reichardt in 1977, which allows the identification of most groups by tribe and genera.

Molecular identification, on the other hand, becomes a faster alternative to identify species, since the database with DNA barcode sequences is public and

easily accessible. However, for the molecular identification achieved a correct binomial nomenclature, it is necessary to integrate the morphological identification, establishing the DNA barcode of each species. Once established, future comparisons are easier, by DNA barcoding.

However, the DNA barcoding was very helpful to confirm that all morphotypes identified are different species, same with small morphological differences observed among some specimes. It was confirmed because the genetic distance among species were higher than 0,0758, what represent different species of ground beetles as confirmed by Hendrich et al. (2015), Raupach *et al*. (2016 and 2019), Totonchian *et al*. (2021), and Lewis (2022). Despite we identified just four species a specific level, the DNA barcoding supported the estimative of diversity and abundance indexes with high precision since we were sure about the exact number of species and their abundance in each collection areas. Furthermore, these DNA barcoding may support future studies of species taxonomic identification of ground beetles and many other ecological studies based in metabarcoding methodology.

The results obtained here showed that maize cropping systems can impact the ground beetles' diversity. Organic maize crops have greater species richness and is more diversity than conventional maize crops when analyzing Shannon and Simpson indices. This can be explained by the higher availability of food resources, shelter options and also lower input than conventional cropping systems and also by the impact of pesticides on ground beetle specimens and their arthropods preys (Benton *et al*, 2003; Shah *et al,* 2003; Purtauf *et al* ,2005; Manelled *et al,* 2007). Furthermore, some species of ground beetles are favored by the presence of spontaneous plants on site (Manelled *et al,* 2007). These plants, in addition to being a source of food resources, can also act as ecological corridors, facilitating the dynamics of these insects between cultivated and forest areas (Purtauf *et al* 2005).

Areas with conventional crops usually have high input and use pesticides and herbicides. The application of these products can directly or indirectly affect the ground beetles, reducing their diversity and abundance (Shah *et al*. 2003; Manelled *et al,* 2007). Broad spectrum pesticides are able to act on non-target organisms, such as predators, which can reduce their contribution to pest control (Seagraves & Lundgren, 2012). The use of herbicides to control weeds

removes shelter and alternative food resources, which are used by predators, mainly in organic crops (Shah *et al* 2003). Finally, some pesticides may have avoidance effects on the organisms (Cordeiro *et al*, 2010).

For the abundance and evenness indices, there was no significant difference between organic and conventional cultivation. The abundance, despite being numerically higher in the organic than in the conventional one, itis possible that there was no difference due to the predominance of 2 species of *Selenophorus* in the organic. The dominance of *Selenophorus* sp 1 and *S. alternans* represents 67.2% of all ground beetles collected in the organic, this may be responsible for the significant difference in the dominance index, which measures the probability of two randomly sampled individuals belonging to the same species.

Ground beetles are very sensitive to changes in habitat and management, and may undergo changes in community composition (Eyre *et al*, 2016; Labruyere *et al*, 2016; Alignier & Aviron, 2017). Some studies have shown that the landscape in many cases can be more important than the management in the area (Palmu *et al* 2014, Purtauf *et al* 2005). The complexity of the landscape offers more shelter to predators and a source of food, factors that facilitate the dynamics and abundance of predators. In addition to factors associated with landscape, ground beetles are also strongly influenced by climatic factors, such as temperature, humidity and shade (Thiele, 1977).

Some species are more associated with cropping systems, as in the case of species belonging to the genus *Selenophorus*. Studies have shown thatindividuals of this genus are more present in agricultural areas than in forest environments (Cajaiba *et al* 2018; Martins & Cividanes, 2020). As well as the species *Calosoma alternans* and species of the genus *Tetracha*, which are known to be predators of worm in soybeans (Martins & Cividanes, 2020; Matta *et al*. 2017). In Brazil, the tribes Harpalini and Pterostichini are widely associated with agricultural areas and were the most sampled tribes in the present study, together they correspond to 78.4% of all ground beetles collectedhere.

The ground beetles have a diversified diet, being able to feed, for example, on seeds and arthropods (Wyckhuys & O'Neil, 2006; Cividanes *et al,* 2014; Matta *et al*. 2017). In the present study, it was not possible to identify ground beetles' predation events on the main pests that occur in maize, *S. frugiperda*, *H. zea*,

D. speciosa and *R. maidis*. Although no predation was detected, a study carried out with visual identification of intestinal content showed the presence of material from arthropods in the gut content of some species of ground beetles collected in areas of cultivation. Still, most of the content found was plant material (Matta et al. 2017).

Many ground beetles are associated with weed predation, mainly species of the genus *Selenophorus* (Martins & Cividanes, 2020; Matta *et al*. 2017). Possibly, the non-detection of predation during the experiment could be related to the majority of ground beetles collected belonging to this genus, and suggesting a food preference for weed seeds. In organic farming systems, chemical herbicides are not used and much of the weed removal is done manually, with more availability of food resources.

The high diversity of these predators in cultivated areas can be of great agricultural importance for pest control such as insects and weeds. The organic farming system favors the diversity of this group, due to low input, more shelter and food sources. And although no predation events were found in the main maize pests, further studies are needed to understand the relationship between the food preference of these predators in the agricultural fields.

4.6. Conclusion

- Were identified 19 morphotypes using morphological characters, and 4 morphotypes until species level with molecular identification;
- The DNA barcoding approach is helpful to definition of ground beetle species based in genetic distance.
- The organic areas had a greater diversity when compared to conventional areas in Shannon and Simpson Diversity Index, species richness and dominance;
- There was no significant difference between organic and conventional when compared abundance and evenness
- No predation events on the main maize pests were detected in the ground beetles using DNA gut content analysis.

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5. METABARCODING AS A TOOL TO IDENTIFY TROPHIC INTERACTIONS

Abstract

Understanding the trophic interactions among the species in agricultural areas, is an important tool for biological control. Many species have their food webs stablished in cropping fields, feed on prey that are considered species of economic importance in agriculture. However, not only beneficial interactions for biological control are found in the fields. Intraguild predation is a common event in the field, which a predator feed on another predator (intraguild prey) or the predator is parasitized by parasitoids. Those interactions are not easy to be identified, so molecular techniques as metabarcoding and PCR assay are very useful to detect them. Thus, the aim of this study was to identify the best sets of primers for gut content, build the food web of the main predators present in organic and conventional maize crops. To achieve these aims, predators from 5 organic and 5 conventional maize crops were sampled during the maize reproductive stage. The DNA from their gut content was extracted with CTAB protocol and a library for metabarcoding was constructed with different set of primers. Also, a PCR assay with specific-primers for *Spodoptera frugiperda* was carried out to confirm the metabarcoding results. Bioinformatics pipeline was used to analyze the data obtained from the metabarcoding sequencing and a food web was illustrated. It was possible to identify intraguild predation among Tachinidae and the predators sampled, especially *H. axyridis*. Also, the ladybirds seem to feed on different insects prey, as mosquitos, lepidopterans and Leafhoppers. The best primers set was ZBJ-ArtF1c/R2c which amplified more species from the predators' gut content. Although, metabarcoding is a technique which requires more studies to improve the amplification of insects' gut content and is still an expensive method.

Keywords: Food web; intraguild predation; biological control; prey-predator;PCR assay.

5.1. Introduction

Agriculture is one of the main economic activities in the world and the management of these areas is the focus of many studies. Biological control is part of integrated pest management (IPM) and aims to control agricultural pests and keep them below the economic injury level, using natural enemies such as predators, parasitoids and microorganisms (Lenteren *et al,* 2018). One of the biological control techniques is the conservation biological control that favors the natural enemies present in the area, so that they have favorable conditions and increase the local population (Gontijo, 2019; Lenteren *et al,* 2018).

Interactions between organisms in the field are key to successful biological control. The prey-predator relationship, predation rate and food preference are some factors that can influence management (Schmidt *et al*,

2021). In the field, some species may behave differently from that observed in the laboratory, such as, for example, feeding on a particular pest in smaller amounts in the field than expected (Maggio *et al,* 2022). Alternatively, predators may ingest other predators, so-called intraguild predation.

Intraguild predation (IGP) is often observed in nature and in the laboratory, and occurs when one predator feeds on another (intraguild prey) belonging to the same guild. Intraguild predation usually occurs when there is low availability of prey or due to the predator's more voracious behavior (Bjorklund, 2016; Polis; Myers; Holt, 1989). In this way, the variation in behavior allows better adaptation to the environment and can often be observed in food webs where intraguild predation is present (Wu; Okuyama, 2012; Polis; Myers; Holt, 1989).

Study carried out by Paula *et al*. (2016) detected intraguild predation among ladybird species using molecular tools, and *Hippodamia convergens* (Coleoptera: Coccinelidae) was the species with the most predators in its gut content. Traugott *et al*. (2012) found parasitoid DNA in the gut contents of several predators such as beetles and spiders. This indicates that these predators are ingesting parasitized prey, evidencing an intraguild predation event.

Next generation sequencing allows sequencing mixed DNA samples without the necessity of separating individuals or having a target individual, and DNA in a small amount or size (Porter & Hajibabaei, 2018; González-Chang *et al,* 2016; Vo & Jedlicka, 2014; Boyer *et al*, 2013). Mostly, fragments of the cytochrome oxidase subunit I (COI) gene are sequenced, functioning as a species identifier (barcode) (Porter & Hajibabaei,
2018; Deagle *et al*, 2014). After the samples have been sequenced, it is possible to compare with existing sequences in databases to find out which species corresponds to the sequenced fragment (Gariepy & Zhang, 2014).

Many studies are conducted with metabarcoding to understand trophic interactions and the local community. The technique can be applied to fish communities (Traugott *et al*, 2021), amphibians (Sakata *et al*, 2022), insects (Marquina *et al*, 2019; Šigut *et al*, 2017), among others. It is possible to highlighttwo major areas of research that use metabarcoding, studies with eDNA and with gut content. eDNA (environmental DNA) was based on identifying the DNA

available in the environment to understand the local community and monitoring diversity (Roger *et al,* 2022; Sakata *et al*, 2022). Gut content DNA has been shown to be a little more challenging as standardization, for example (Jeffs *etal*, 2021).

The use of metabarcoding for food webs requires not only well- established steps such as DNA extraction, PCR assay, purification and sequencing. Greater care is needed, such as cleaning the predator's body to avoid contamination of external DNA, use of more than one set of primers to amplify as many prey as possible, a robust database to compare the results obtained and also a bioinformatic pipeline capable of optimizing the sequencing results (Batuecas *et al*, 2022; Plummer *et al*, 2015; Gibson *et al*, 2014; Jones, 2012; Bohmann *et al*, 2011).

Due to the importance of understanding trophic interactions in the field, identification of gut contents and the use of molecular tools such as metabarcoding and PCR are allies in the understanding of ecological interactions that can be applied in agriculture and especially in conservation biological control. Thus, the aim of this work was to identify the best sets of primers for gut content of insects, build the food web of the main predators present in corn crops and compare whether there is a difference in diet betweenpredators from different cropping systems.

5.2. Material and Methods

5.2.1. Field

The samples were done in 10 areas of maize during its reproductive stage, in 4 cities from São Paulo state. Each area was divided into 2 plots and installed three dry pitfall traps in each plot. The pitfalls were emptied every 24h and the plots were active sampled randomly for 45 min for two days. The organisms sampled were individualized into tubes and kept in cold bags until arrives in the laboratory, where were storage at -20ºC with ethanol 100%. This field sampling was the same as described in chapter 3 in more details.

5.2.2. Preliminary experiment

To guarantee that the metabarcoding sequencing and which primers would be chosen, was performed a bioassay with 4 individuals of 4th instar *Hippodamia convergens* larvae, which were fed with *Spodoptera frugiperda* larva, *Helicoverpa zea* larva, *Aphis gossypii, Ephestia kuehniella, Corcyra cephalonica,* and *Diaphorina citri.*

Those ladybug larvae had their gut content DNA extracted with CTAB protocol following the steps described above. Those samples were used as a positive control in the sequencing because was known what there was in the gut content.

5.2.3. DNA extraction

For analysis of gut content with metabarcoding approach, we selectedthe 10 morphotypes from ground beetles with more than 4 individuals collected (total = 163 individuals), 100 *Harmonia axyridis*, 23 *Cycloneda sanguinea*, 800 *Doru luteipes* (Table 5.1.) and the 4 larvae of *H. convergens* from the preliminary experiment.

All the predators had their body washed up in bleach 2% for 3s, after in ethanol 70% for 3s and in autoclaved distilled water for 10s. This procedure was done to avoid external contamination in their body. Every time that was manipulated a predator, the forceps were washed up in bleach, ethanol, and dried with cleaned tissue. The predators were set to air drier. After that, the wings, legs, and antennas were removed with clean forceps and scalpel. For *D. luteipes*, their last segment of abdomen with the forceps was removed carefully. All the predators had their body macerated with liquid nitrogen, except for the ground beetles bigger than 2 cm, that had their gut carefully removed, to avoid that the content leaked out. The gut was removed in a magnifying glass with Petri dish, forceps, scissors, and scalpel. The gut was transferred to a 2 mLtube and macerated.

The DNA was extracted using the CTAB protocol, with 500 µL of CTAB buffer, 10 µL of proteinase k 20ng/ µL, and 2 µL of β-mercaptoethanol. The supernatant was cleaned up with chloroform and isoamyl alcohol (24:1) and centrifuged. The DNA was precipitated with isopropanol and incubated at -20 °C overnight. The pellet was washed up with ethanol 100%, 70%, and let to air dry. The pellet was resuspended in 30 µL of autoclaved distilled water. After that, the DNA samples were pooled into groups of 4 until 10 individuals from the same species, area, and treatment (Table 5.1). The DNA was quantified by Qubit™ (Invitrogen).

Table 5.1: Number of individuals that had the DNA extracted from the gut content for the metabarcoding divided by organic and conventional areas. In parenthesis the number of DNA pools for it species/morphotype per treatment.

Family	*Species /Morphotype (M)*	*Number of individuals* (sequenced pool)	
		Organic	Conventional
Carabidae	M1	60 (6)	-
	M2	10 (1)	5 (1)
	M6	-	4 (1)
	M7	-	4 (1)
	M9	40 (4)	-
	M11	20 (2)	-
	M12	5 (1)	-
	M13	5 (1)	-
	M18	5 (1)	-
	M19	5 (1)	-
Coccinellidae	*Harmonia axyridis*	100 (10)	-
	Cycloneda sanguinea	18 (3)	5 (1)
Forficulidae	*Doru luteipes*	400 (40)	400 (40)

5.2.4. Primer set tests and metabarcoding library

To amplify the prey DNA from the predators' gut content from the preliminary experiment and also 2 pools from *D. luteipes*, 2 pools of *H. axyridis* and 2 pools of ground beetle morphotype 1 were selected 3 pairs of primers, considered universal primers that amplify the COI gene, ZBJ-ArtF1c/ZBJ- ArtR2c (157bp), Uni-MinibarF1/Uni-MinibarR1 (130 bp), LEPF1/MLepF1-Rev (218 bp), MG2-LCO1490_F/MG2-univ-R1 (133 bp). And also a pair of primers 16SMAVF/R (37 bp) that amplifies the 16S gene (Table 5.2). These primers were already synthetized with the Illumina adapter, a sequence of nucleotides necessary to fit the Illumina platform for sequencing. The best pair of primers, with more accurate species identification, was selected to amplify the samples from the field.

The PCR reaction for each pair of primers was optimized with 8 µL of PCRBio (PCR Biosystems), 8 µL of ultrapure water, 0.5 µL of primer forward(10 ρmol), 0.5 µL of primer reverse (10 ρmol), and 3 µL of DNA. The thermal cycler program for the pair of primers ZBJ-ArtF1c/ZBJ-ArtR2c was 3 min at94ºC, 16 cycles of 30s at 94ºC, 30s at 61ºC decreasing 0.5ºC in each cycle (touch-down program), 30s at 72º, 24 cycles of 30 s at 94ºC, 30s at 53ºC, and 30s at 72ºC. The extension time was 10 min at 72ºC and finished at 4ºC. For the pair of primers Uni-MinibarF1/Uni-MinibarR1 was used 2 min at 95ºC, 5 cycles at 95ºC for 1 min, at 46ºC for 1 min, at 72ªC for 30s. 35 cycles of 1 min at 95ºC, 1 min at 53ºC increasing 0.5ºC in each cycle (touch-up program) and 30s at 72ºC. To finish, 5 min at 72ºC and maintained at 4ºC.For the pair of primers LEPF1/MLepF1-Rev was used 2 min at 95ºC, 25 cycles for 30s at 95ºC, 30s at 45ºC, 90s at 72ºC, and to finish 10 min at 72ºC, and maintained at 4ºC. For the primers 16SMAVF/R, the thermal cycler program was 15 min at 95ºC, 55 cycles of 30s at 94ºC, 90s at 55ºC and no elongation step. A negative control was added to all PCR assays to control contamination.

A second primer test was carried out with the set of primers MG2-LCO1490_F/fwhR1_R2/CO1-CFMR-dege_R3 (178/181 bp) (Table 5.2). This test was done with 5 pools of samples from the field, 1 *H. axyridis* from organic, 2 *D. luteipes* from conventional, 2 samples from ground beetle morphotypes 9 and 18 from organic. The PCR reaction was the same as for the other primers and the thermal cycler program was 15 min at 95ºC, 40 cycles of 30s at 94ºC, 45s at 45º, 2 min at 72ºC, and the final for 10 min at 72ºC, and maintained at 4ºC.

After the amplicons were visualized in 2% agarose gel byelectrophoresis. The samples that were amplified were sent to sequencing in a sequencing company, where samples were cleaned up with magnetic beads, to eliminate fragments that were not the target size, and quantified with Qubit. After these procedures, a second PCR was carried out, cleaned up with magnetic beads, pool normalization, and quantified with KAPA kit by qPCR. The sequencing was in Illumina platform (HiSeq) with a mean covering of 100.000 reads.

The field samples pools, 5 of *D. luteipes* from conventional, and 5 from organic, 3 pools of *C. sanguinea* from organic, and 1 from conventional, 8 pools of H. axyridis from organic, and 1 pool of each ground beetle morphotype 1, 2,9, 11,

and 19 from organic, and 1 pool of morphotype 2 from conventional, were amplified with the primers ZBJ-ArtF1c/R2c.

Table 5.2: Primers tested to amplify the predators' gut content DNA by Illumina sequencing

Primer's name	Primer's sequence (5' – 3')	Reference	Paired with	Fragment size
ZBJ-ArtF1c	AGATATTGGAACWTTATATTTTATTTTTGG	Zeale *et al*., 2011	ZBJ-ArtR2c	157 bp
ZBJ-ArtR2c	WACTAATCAATTWCCAAATCCTCC			
Uni-MinibarF1	TCCACTAATCACAARGATATTGGTAC	Meusnier *et al*., 2008	Uni-MinibarR1	130 bp
Uni-MinibarR1	GAAAATCATAATGAAGGCATGAGC			
LepF1	ATTCAACCAATCATAAAGATATTGG	Hebert *et al*., 2004	MLepF1-Rev	218 bp
MLepF1-Rev	CGTGGAAAWGCTATATCWGGTG	Brandon-Mong *et al.*, 2015		
MG2-LCO1490_F	TCHACHAAYCAYAARGAYATYGG	based on Gillet *et al*., 2015 and Tournayre *et al*., 2020	MG2-univ-R1	133 bp
MG2-univ-R1	ACYATRAARAARATYATDAYRAADGCRTG			
fwhR1_R2	ARTCARTTWCCRAAHCCHCC	based on Galan *et al*., 2018 and Tournayre *et al.,* 2020	MG2-LCO1490_F	178 bp
CO1-CFMR-dege_R3	AYNARTCARTTHCCRAAHCC			181 bp
16SMAV-F	CCAACATCGAGGTCRYAA	De Barba *et al*., 2014	16SMAVR	37 bp
16SMAV-R	ARTTACYNTAGGGATAACAG			

5.2.5. Bioinformatic pipeline

The resulting sequencing in FASTq files was analyzed with bioinformatics pipeline, in the Natural History Museum server in London/UK and the Molecular Ecology of Arthropods laboratory server in Piracicaba/BR. The packages used in the pipeline were "*cutadapt*" to remove the primers and tags from the sequences added during the PCR assays and discarded reads smaller than 50 bp for COI gene. The sequences were merged to combine forward and reverse sequences with "*pear*" package, and the sequences unassembled were discarded. The sequences were concatenated with 97% similarity to start the filtering process with the package "*vsearch*". The dereplicated, chimeras, and denoising sequences were removed. These sequences are considered as sequences with primers chimeras, possible noising from sequencing or replicated sequences. After that, the OTUs (Operational Taxonomic Units) were delimitated in clusters with 97% of similarity with the package "*vsearch*". The OTUs resulting were compared with the online database available on NCBI, and Bold Systems, and with the personal database generated by the barcode here produced. The sequence similarity with the database was considered in species level only if it achieved 97% or more. Below that, was considered only family level.

5.2.6. Specific-primers

To make sure that the results from the metabarcoding sequencing were correct, were performed PCR assays with specific primers Spo_frugi_F/R (Maggio *et al*, 2022) for *Spodoptera frugiperda* DNA, which is the most abundant pests in maize. The PCR assay was optimized in a volume of 14 µL, with 7 µL of DNA, 1.5 µL of 10X magnesium-free buffer (Sinapse Inc.), 1.5 µL MgCl2 (25 mM, Sinapse Inc.), 1.0 µL dNTP (2.5 mM, Sinapse Inc.), 1.0 µL of each primer (5 pmol), and 0.3 Taq DNA polymerase 5U/µL (Sinapse Inc.). The thermocycler program was carried out according to Maggio *et al*, 2022. The results were elucidated in 2% agarose gel by electrophoreses.

5.2.6. Trophic interactions

To identify the trophic interactions, the OTUs identified from the metabarcoding sequencing and the positive results from specific-primers PCR assays were analyzed by predator and illustrated a qualitative food network.

5.3. Results

5.3.1. Primer set tests and metabarcoding results

The preliminary experiment showed that the best primers to amplify the preys DNA was ZBJ-ArtF1c/R2c (Tables 5.3, 5.4, and 5.5). A total of 1.503.458 reads were obtained with a merge percentage of 98 – 99% and less than 3% lost in filtering process. It was possible to identify all prey that *H. convergens* had fed on in laboratory in the preliminary experiment. Also, it was possible to distinguish to species level, 8species in the predators' gut content sampled in the field and from the preliminary experiment. Other 5 identifications are from family level, 1 from order, and 2 genera.

The other primers, Uni-MinibarF1/R1 and LepF1/MLepF1-Rev (Tables 5.6, and 5.7) had a total of 1.050.480 and 538.097 reads, respectively. These primers did not have a good performance, being able to amplify only the predators' DNA and cross contamination during the amplification process, especially with the primers Uni-MinibarF1/R1, which the contamination was with DNA from Human, fish, and bacteria. The primers 16SMAV-F/R had 11236 reads and only amplified the predators' DNA and *Aphis gossypii*, but could not amplify the others prey DNA when tested in the gut content from the preliminary experiment. Also, the percentage of identity during the comparison process with the database was low in the predators' DNA.

The primers MG2-LCO1490_F/MG2-univ-R1, and MG2-LCO1490_F/fwhR1_R2/CO1-CFMR-dege_R3 (Table 5.8, and 5.9) had 154.088 and 151.668 reads, respectively, a merge percentage of 97 – 99% and 3% of loss in filtering process. These primers also amplified the predators' DNA and some species/group that the primers ZBJ-ArtFc1/R2c, indicating that they have inferior performance when compared to ZBJ-ArtFc/R2c. However, only the primer MG2-LCO1490_F/MG2-univ-R1 amplified *Forficulidae* until now, but could not amplify Tachinidae DNA that was present in almost all *H. axyridis* field samples. The primers MG2-LCO1490_F/fwhR1_R2/CO1-CFMR-dege_R3 had the worst performance in COI gene, amplifying the predators' DNA, Diptera DNA and *Wolbachia endosymbiont.*

When tested with specific-primers for *S. frugiperda* DNA, no predation event was identified in the predators' gut content field sampled. The PCR assay was carried out for all samples pools, and none of them had a positive result.

Table 5.3 OTUs identity, and number of reads per sample sequenced with primers ZBJ-ArtF1c/R2c for Coccinellidae predators. In bold are the reads considered as trophic interactions. CS: *Cycloneda sanguinea*; HA: *Harmonia axyridis*; -C: conventional; -O: organic.

OTU Identity	**CS-C**	**CS-O**	**CS-O**	**CS-O**	**HA-O**	**HA-O**	**HA-O**	**HA-O**	**HA-O**	**HA-O**	**HA-O**	**HA-O**	**HA-O**	**HA-O**
Cycloneda sanguinea	40678	47688	43526	60219	7	**94**	6	10	6	34	0	**106**	0	17
Tachinidae and/or	0	0	0	0	**267**	0	169	0	0	0	**517**	0	0	17
Cecidomyiidae	0	0	0	0	0	114	0	0	0	0	0	0	0	0
Balclutha incisa	0	0	0	0	0	**20**	0	0	0	0	0	0	9	0
Tachinidae or Anthomyiidae	0	1	5	21	**48760**	5	**55826**	7	7	**54018**	**71961**	33	0	0
Harmonia axyridis	1	6	5	0	233	34180	422	34166	9420	2610	78	22952	2211	1823
Lespesia aletiae	0	3	4	0	1	7	2	4	**34937**	0	0	0	0	0
Aedes aegypti	0	0	0	0	0	**696**	0	**4002**	2	0	0	0	0	0
Phoridae	**285**	0	0	0	0	0	0	0	0	0	0	0	0	0
Grapholita molesta	0	0	0	0	0	0	0	0	0	0	0	**123**	**39**	0
Carabidae	0	0	0	0	0	0	0	0	0	0	0	0	0	44
Anopheles sp	0	0	0	0	0	0	0	0	0	0	0	0	0	**5478**

Table 5.4 OTUs identity, and number of reads per sample sequenced with primers ZBJ-ArtF1c/R2c for ground beetles morphotyoes. M: morphotype; -C: conventional; -O: organic.

OTU Identity	**M2-C**	**M19-O**	**M2-O**	**M9-O**	**M11-O**	**M1-O**	**M1-O**	**M1-O**
Tachinidae or Anthomyiidae	0	24	31	36	17	29	0	0
Carabidae	80990	140298	21	73777	49502	61288	139107	121420

Table 5.5 OTUs identity, and number of reads per sample sequenced with primers ZBJ-ArtF1c/R2c for *Doru luteipes* predators. In bold are the reads considered as trophic interactions. DL: *Doru luteipes*; -C: conventional; -O: organic.

OTU Identity	**DL-C**	**DL-C**	**DL-C**	**DL-C**	**DL-C**	**DL-C**	**DL-C**	**DL-O**	**DL-O**	**DL-O**	**DL-O**	**DL-O**
Cycloneda sanguinea	0	0	0	0	0	0	0	0	0	0	16	0
Tachinidae or Anthomyiidae	0	0	0	0	0	0	0	0	0	0	0	19
Harmonia axyridis	0	0	0	0	0	0	24	0	0	0	0	5
Grapholita molesta	58	0	**4315**	**679**	0	0	0	0	0	0	0	0
Carabidae	82	7	0	0	0	0	24	0	0	4	53	24
Corcyra cephalonica	15	0	0	0	0	0	0	4	0	0	0	0
Hippodamia convergens	4	0	0	0	3	0	0	2	0	0	0	0
Diptera	0	74	0	0	0	0	0	0	0	27	**2013**	0
Spodoptera frugiperda	0	0	0	0	0	0	0	2	0	0	0	0
Fish	0	0	0	0	14	11	0	2	1	0	0	0
Aphis sp	0	0	0	0	0	0	0	0	9	0	0	0

Table 5.6 OTUs identity, and number of reads per sample sequenced with primers Uni-MinibarF1/R1 for the predators sampled and positive controls. D.L: *Doru luteipes*; HA: *Harmonia axyridis*; M: ground beetle morphotype; -C: conventional; -O: organic.

OTU Identity	**DL-C**	**DL-C**	**HA-O**	**HA-O**	**M1-O**	**M1-O**	**Positive control**	**Positive control**
Hippodamia convergens	65	74	61	238	31	42	279251	145386
Harmonia axyridis	83	174	341960	270500	8	90	118	110
Carabidae	0	0	0	0	0	0	0	0
Fish	0	33	0	0	0	29	0	0
Homo sapiens	0	0	0	30	0	0	0	0
Solo bacteria	0	0	0	0	0	12181	0	0
Spodoptera frugiperda	0	0	0	0	0	0	8	0

Table 5.7 OTUs identity, and number of reads per sample sequenced with primers LepF1/MLepF1-Rev for the predators sampled and positive controls. D.L: *Doru luteipes*; HA: *Harmonia axyridis*; M: ground beetle morphotype; -C: conventional; -O: organic.

OTU Identity	**DL-C**	**DL-C**	**HA-O**	**HA-O**	**M1-O**	**M1-O**	**Positive control**	**Positive control**
Hippodamia convergens	24	21	7	11	0	0	68984	238363
Harmonia axyridis	0	55	42391	155014	0	0	8	41
Carabidae	0	0	0	0	28247	4931	0	0

Table 5.8 OTUs identity, and number of reads per sample sequenced with primers MG2-LCO1490_F/MG2-univ-R1 for the predators sampled and positive controls. In bold are the reads considered as trophic interactions. D.L: *Doru luteipes*; HA: *Harmonia axyridis*; M: ground beetle morphotype; -C: conventional; -O: organic.

OTU Identity	**DL-C**	**DL-O**	**HA-O**	**M18-O**	**M9-O**
Diptera	**30544**	**33887**	6	4	1
Acari	0	0	0	0	**9**
Chironomidae	3	1	1	1	0
Harpalini	2	1	0	1	28428
Harmonia axyridis	1	7	28758	0	0
Calosoma sp	5	1	4	32321	0
Forficulidae	142	110	0	0	0
Braconidae	0	0	0	0	**13**

Table 5.9 OTUs identity, and number of reads per sample sequenced with primers MG2-LCO1490_F/fwhR1_R2/CO1-CFMR-dege_R3 for the predators sampled and positive controls. In bold are the reads considered as trophic interactions. D.L: *Doru luteipes*; HA: *Harmonia axyridis*; M: ground beetle morphotype; -C: conventional; -O: organic.

OTU Identity	**DL-C**	**DL-O**	**HA-O**	**M18-O**	**M9-O**
Diptera	**34677**	**32106**	0	2	0
Acari	0	0	0	0	**28**
Harpalini	0	0	0	0	28066
Harmonia axyridis	0	2	26096	0	0
Calosoma alternans	0	0	2	30091	0
Wolbachia endosymbiont	29	9	0	0	0

5.3.2. Trophic interactions

It was not possible to identify difference among predators from conventional and organic maize crops, especially because the most common predator between the treatments was *D. luteipes* and the primers set did not work as expected. The other common predators for both treatments were 6 individuals from *C. sanguinea* and 6 individuals ground beetle morphotype 2 for conventional maize and this reduced number interfered to analyze if the food webs were different.

It was possible to identify that the majority of the trophic interactions are associated with *H. axyridis*, where was found DNA from individuals that are considered prey: Cecidomyiidae (gall midges), *Aedes aegypti* (Diptera: Culicidae), *Balclutha incisa* (Homoptera: Cicadellidae - Leafhoppers), *Grapholita molesta* (Lepidoptera: Tortricidae - Oriental fruit moth), and *Anopheles sp* (Diptera: Culicidae). It also amplified DNA from parasitoids and other predators, indicating intraguild predation, as *C. sanguinea, Lespesia aletiae* and Tachinidae. For *C. sanguinea*, was amplified only DNA from Phoridae, a fly that parasites pollen, bees, tick, ants and other insects. For *D. luteipes* was identified DNA from Diptera, and *G. molesta,* only. And for ground beetle was found DNA from Braconidae and Acari (Fig 5.1).

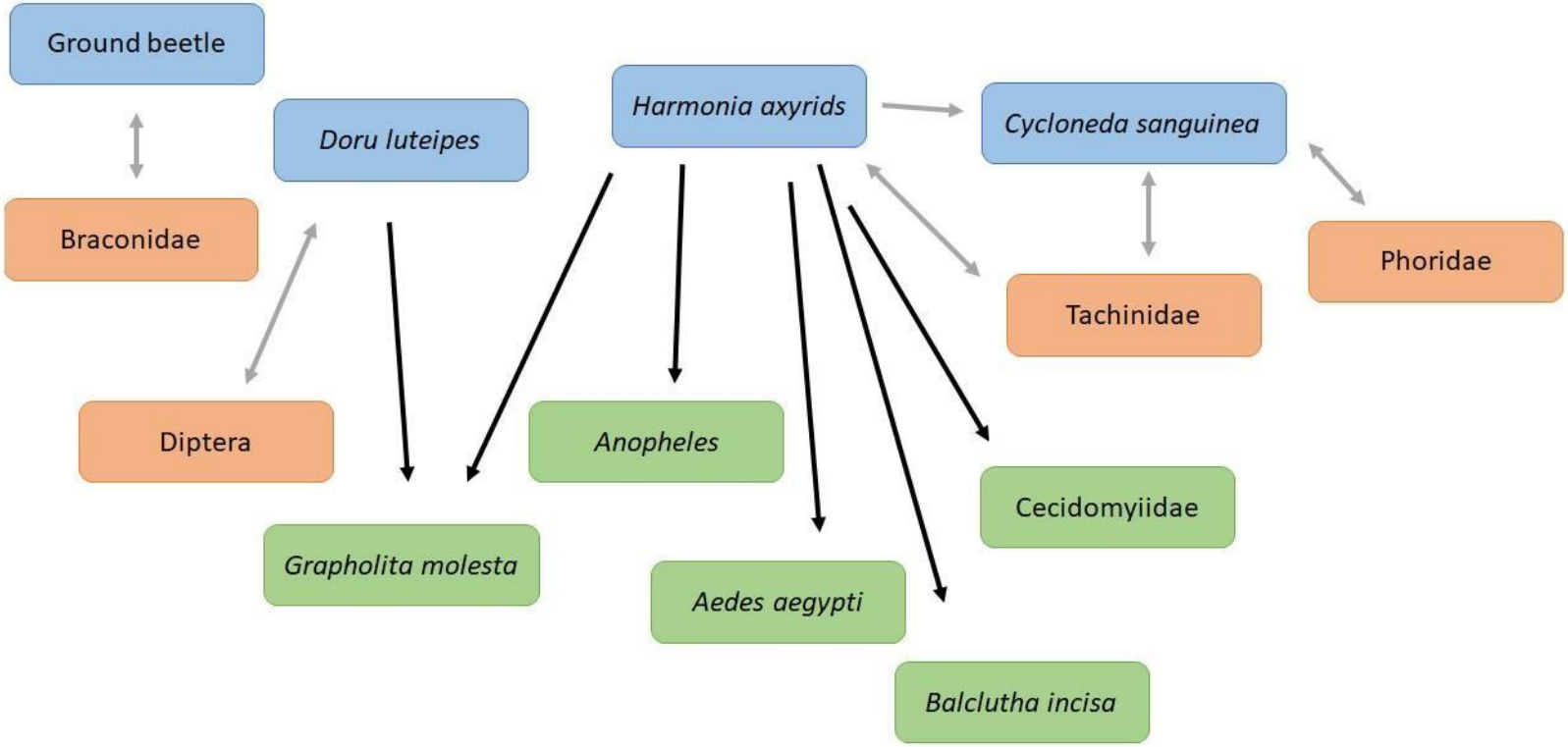

Fig 5.1 Trophic relationship of the predators sampled in the organic and conventional maize. Blue squares represent the predators sampled in this study; orange squares represent prey/parasitoids; green squares represent the prey; black arrow represents the consumption by the predator; and the gray arrow represents the intraguild predation.

5.4. Discussion

The results implied that the predators found in maize, mainly *H. axiridis*, prey on several insects, not only of economic importance, but also other non-target insects. The food web can be composed of hematophagous insects such as *A. aegypti*, herbivores such as *G. molesta*, and also predators such as intraguild predation found between *H. axyridis* and *C. sanguinea*.

Despite being able to identify some trophic interactions in the field, the molecular identification of some species, especially prey and parasitoids, was low, and in some cases it was possible to reach the taxonomic level of the family. Databases with insect barcodes have DNA sequences mainly from species of economic importance, which are the most studied. The lack of information on molecular identification makes it difficult to work on faunal and food web surveys (Piper *et al*, 2019), but still useful as a model for future studies of interactions to be carried out. These problems are amplified when studying tropical systems, where the arthropod biodiversity is not thoroughly characterized, and DNA barcoding initiatives are scarce even in agroecosystems as maize crop areas.

The choice of primers for metabarcoding is fundamental so that as many species as possible can be covered (Brandon-Mong et al. 2015). Studies with gut content and feces deal with DNA fragments degraded in small pieces, often making their detection difficult (Kamenova *et al*, 2017; Greenstone *et al*, 2014; Chen *et al*, 2000). Furthermore, amplifying the predator's DNA decreases the efficiency of metabarcoding sequencing, getting most of the predator's reads. Some works have developed blocking primers, which are able to anneal to the predator's DNA and prevent it from being amplified (Toju & Baba, 2018; Piñol *et al*, 2015). This technique, despite being efficient, is still expensive and also due to the phylogenetic proximity between insects, there may have a chance that these blocking primers also prevent the amplification of DNA from other insects.

The primers used here were able to amplify different taxonomic groups of insects, being efficient mainly in the detection of Tachinidae DNA. The ZBJ-ArtF1c/R2c primers proved to be more efficient in this study, mainly because they did not amplify *D. luteipes* DNA. The non-amplification of the predator's DNA from the gut contents is advantageous, considering that there will be more chances of amplifying other DNAs from the gut contents since the predator's DNA is found in greater quantity and concentration (Paula *et al*, 2016).

The other primers tested here were also able to amplify some groups of insects, but with less efficiency. However, the MG2-LCO1490_F/MG2-univ-R1 primers were the only ones to amplify *D. luteipes* DNA. Which, on the other hand, is interesting, as it would be possible to use this set of primers in the gut content of other predator species, which do not belong to the Forficulidae, to identify possible intraguild predation. *D. luteipes* is present in maize crops during all development stages (Cruz *et al*, 1995) and could possibly be preyed by other predators. The 16SMAV-F/R primers amplify a very small fragment (37 bp), which makes identification difficult and can even be a problem in the bioinformatics process. For the identification of a species in the gut contents, normally the size of the fragment must be from 100 to 300 bp (Kamenova *et al*, 2017; Greenstone *et al*, 2014; Chen *et al*, 2000). With this size it is possible to distinguish between the species and it is also not sizes too big or too small that they cannot be detected.

The gut content detection technique is widely used to identify trophic interactions, whether prey-predator or intraguild predation (Robeson *et al*, 2018; Galan et al, 2018; Greenstone *et al*, 2010). The use of the PCR technique is the most common, as it is based on the use of specific primers for a species or multiplex for detection of several species or groups (Nanini *et al*, 2019; Staudacher *et al*, 2016; Traugott *et al*, 2012). The technique has a low operating cost and is easy to visualize the result, as it does not require sequencing. Many field studies have shown predation rates (Traugott *et al*, 2021; Nanini *et al,* 2019; Peterson *et al*, 2018) and parasitism rates (Jeffs et al, 2021; Šigut *et al*, 2017; Traugott *et al*, 2012) in agricultural cropping areas based on the PCR technique. One of the main studies in the area using specific primers carried out by Greenstone *et al* 2010, showed that predators such as ladybirds, stink bugs, and ground beetle, sampled in the field fed on Colorado potato beetle (*Leptinotarsa decemlineata*). From this pioneering work in the area, other researchers began to use the PCR technique to elucidate trophic webs.

Metabarcoding is the most recent technique based on next generation sequencing. In this technique, as there is no single target species or group, it is possible to elucidate the trophic web more comprehensively (Schmidt *et al*, 2021). However, as it is a recent technology, some challenges still make difficult the wide application of the technique, such as the high cost of the products, more qualified

labor and the need for high performance sequencing. In addition, sequencing analysis requires the use of bioinformatics pipelines and servers.

A problem still inherent to the metabarcoding technique is known as tag-jumping, a process in which, during sequencing, the machine identifies a DNA sequence from one location and understands that it is from another, which can generate false reads (Mathieu *et al*, 2020; Schnell *et al*, 2015), and consequently false positives. when it comes to gut contents. This problem was found in the present study, mainly in the first sequencing performed. It is believed that because there was a high concentration of predator DNA and the low distance between the wells within the sequencing plate, tag-jumping occurred, and some results considered as predation, which were discarded.

Although molecular tools are widely used for the construction of food webs, some abiotic and biotic factors of predators can affect the identification of prey DNA. Biotic factors such as metabolism, developmental stage, meal size, and mouthparts, for example, are factors that influence DNA detection by both PCR and metabarcoding (Greenstone *et al*, 2014). We can use as an example the detectability half-life curve of the DNA of *S. frugiperda* in the gut contents of *H. convergens* and *D. luteipes* published by Maggio *et al* 2022. Both predators were kept under the same conditions of temperature, humidity, light and fed on only prey of the same developmental stage. The half-life curve rate in *H. convergens* was 3x higher than in *D. luteipes*. Possibly *H. convergens* may have a faster metabolism and degrade DNA faster.

For *D. luteipes*, it was expected that some evidence of predation would be found, mainly on *S. frugiperda*, since during the samplings, it was the main pest found. And as it has the highest rate of half-life detectability of *S. frugiperda* DNA to date, the chances of finding the DNA in case of ingestion would be considerable. However, the lack of predation detection of *D. luteipes* and the low predation, as found by Maggio et al 2022, may suggest that in the field, *D. luteipes* may prefer to feed on other food sources. *D. luteipes* is an insect that feeds on decaying matter, pollen and other insects.

For ladybirds, the detection of *A. aegypti*, *Anopheles* and *C. sanguinea* DNA in the gut contents of *H. axyridis* are cases of direct predation. Although it is curious to have found ladybird predation on mosquitoes, it would be possible that these predators fed on eggs or larval stages of these prey (Yang, 2006). The presence of

G. molesta DNA, both in ladybirds and in *D. luteipes*, was also an interesting fact, since *G. molesta* is a pest mainly of peach (Botton *et al*, 2005), and it may be that this pest was in corn just moving around and laying their eggs there, or the predators moved to nearby areas

that had *G. molesta* as this pest is found in the state of São Paulo. This last possibility is a little more remote, as normally predators tend to stay in one place until there is no more food resource, and only then move to other areas.

Intraguild predation was the main event found with the metabarcoding. Tachinidae DNA was present in most of the samples of *H. axyridis*. Studies have shown that *Strongygaster triangulifera* (Diptera: Tachinidae) is capable of parasitizing *H. axyridis*, other coccinellids and also Lepidoptera (Katsoyannos & Aliniazee, 1998). The DNA of this genus was detected in the samples studied here, indicating that *H. axyridis* could be parasitized or had ingested eggs or even parasitized Lepidoptera (secondary predation). Unfortunately, with these molecular data it is not possible to identify whether there is direct parasitism or secondary predation. However, this result is important, since finding interactions between predators and parasitoids in the field are extremely important for biological control practices.

In addition to Tachinidae, it was also possible to detect predation by *H. axyridis* on *C. sanguinea*. The interaction between ladybugs and intraguild predation are topics well addressed in the literature (Yang *et al*, 2017). Intraguild predation is not advantageous for biological control, but this trophic interaction is common. As the predators are exposed in the same way when they are foraging, the chances of encountering these two predators are greater. Thus, it becomes advantageous to prey on another predator, since the energy gain is greater (Polis; Myers; Holt, 1989).

From the point of view of biological control, the results obtained here were not very positive, but made it possible to understand how organisms are interacting in the field. So that interactions do not always occur according to laboratory experiments. Studies that evaluate the gut content of predatory insects feeding on other insects are still mainly performed with PCR and specific primers, and little with metabarcoding. Thus, the data here are important to guide future studies in this area and also to improve the metabarcoding technique.

5.5. Conclusion

- The best primers set was ZBJ-ArtF1c/R2c, which amplified DNA from preys and intraguild predation;
- The predators' food web presented especially preys of non-economic importance for agriculture;
- It was identified several intraguild predation, mainly from Tachinidae in H. axyridis;
- No evidence was found of difference between predators' food web between treatments due to the limited number of predators sampled.

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6. FINAL CONSIDERATIONS

Studies in the area of trophic interactions and diversity are essential to understand the dynamics of local arthropods and also to choose the correct pest management, especially for conservative biological control. This management tactic aims to increase local predators and to know which species occur and their ecological interactions helps in better pest control. Furthermore, knowing what the predators associated with predation of the key pests is helpful information for the prospection of biological control for applied biological control programs.

Crop systems can positively influence species diversity, benefiting natural enemies and pollinators, but they can also have a negative impact, decreasing local diversity of these benefitial and non-target organisms. The organic corn cropping system studied here presented greater diversity when compared to the conventional cropping system. In addition to the difference in diversity indices, predators were more abundant in organic, which was probably favored due to management, presence of shelters, spontaneous plants and alternative food resources.

The use of molecular tools helped the advancement of knowledge in the area of food webs, where PCR reactions using specific primers or groups, helped the detection of predation events. In the field, intractions are not always easy to identify. We developed and applied these molecular markers with success in our studies and they confirmed their utility to detection of specific events of predation. Thus, the specific primers developed here for *Spodoptera frugiperda* and *Diabrotica speciosa*, as well as the primers already published for *Helicoverpa zea* and *Rhopalosiphum maidis*, were able to amplify only the DNA of the target species, and were efficient to detect predation events even after a few hours of prey ingestion.

The predation rate in species of economic importance such as *S. frugiperda*, *H. zea*, with specific primers, was higher in ladybirds than in *D. luteipes* and Carabidae beetles. Which indicates that ladybirds can be good biological control agents for these pests. This information is important in the context of applied biological control since *D. luteipes* is, commonly, reported as the most important predator of *S. frugiperda* in maize crops and applied biological control programs with this organism are always recommend. However, *D. luteipes* was not confirm by our studies as an efficient predator on *S. frugiperda*, problably, due to a wide feed behaviour ad dynamic in maize areas.

Using the metabarcoding approach, the presence of predation by Coccinelidae, Carabidae and *D. luteipes* of prey reported as agricultural pests was low. However, events of intraguild predation were more present. This, in turn, occurred mainly by species of Tachinidae, in *Harmonia axyridis*, which may have been parasitized or had ingested some prey that was parasitized. The presence of intraguild predation in food webs need to be considered for biological control programs, as it reduces the number of natural enemies in the field and there is also competition between them.

The metabarcoding tool was important to understand the trophic interactions of predators in agriculture systems, but there are still limitations regarding the applicability of the tool and its execution, especially associated with primers chosen for sequencing. Many studies focus on species diversity in eDNA, some food webs with feces DNA, and studies with gut contents of insects that feed on other insects are still few. The present work tried to approach the use of this tool, despite all the limitations, and still we were able to identify not very well known intraguild interactions. More efforts are still needed to improve sequencing quality, primers that are able to amplify more groups and optimize all pre and post sequencing steps.

Finally, our studies showed the vast application potential of molecular tools for understanding ecological interactions in agricultural fields. We believe that understanding these ecological interactions is essential to improve pest management and their impact on different farming systems.

APPENDICES

APPENDIX A

DNA barcode from the main pests and natural enemies from maize crops produced in chapter 3.

>*Cycloneda sanguinea* (624 bp)
TTATATTTTCTGTTTGGTATATGRGCAGGTATAGTTGGCACTTCTCTGAGAATTCTAATTCGTCTTGAACTAGGA
ACAACTAATAGACTAATTGGAAACGATCAAATTTATAATGTAATTGTTACTGCTCATGCATTTATCATAATTTTT
TTTATAGTAATACCTATTATAATTGGAGGATTTGGAAATTGACTGGTACCCTTAATAATTGGGGCCCCAGATATA
GCTTTCCCACGTTTAAATAACATAAGTTTTTGACTTTTACCTCCTGCATTAAC:ATTACTAATAATAAGAATAAT
TGTAGAAATAGGGGCAGGTACAGGTTGAACAGTTTACCCTCCCCTATCTTCAAATTTAGCACATAATGGACCCTC
TGTTGATTTAGTAATTTTTAGTTTACATTTAGCTGGAATTTCATCTATTTTAGGAGCTGTTAATTTTATTTCAAC
TATCATAAATATGCGCCCTTCAGGAATAAGGCTAGATAAAACTCCCTTATTTGTATGATCAGTTATAATTACAGC
TATTTTATTACTTTTATCTTTACCTGTATTAGCAGGAGCCATCACTATACTTTTAACAGACCGAAATATTAATAC
CTCATTTTTTGATCCTACAGGAGG

>*Dalbulus_maidis*_1 (578 bp)
TGATGGATCAAAGAATGATGTATTTAAATTTCGATCTGTTAATAATATGGTAATTGCTCCTGCTAATACCGGTAG
TGAGAGTAATAGTAGTACTGCAGTAATTAGGACAGACCATACAAATAAAGGAGTTTTATCTAGGCTTATCCCTAC
GGGTCGTATATTTAAAATTGTAGTAATGAAATTCACTGCTCCTAAAATTGATGAAATTCCTGCCAAATGAAGTGA
AAAAATTGATATATCAACTCTGGGGCCTGCGTGAGCAATATTTGATGAAAGGGGGGGGTAGACAGTTCATCCTGT
TCCTGTCCCTGTCTCAATCAATGATGATATAATTAGCAATGTGAGTGAGGGAGGTAATAATCAAAATCTTATGTT
ATTTAATCGAGGGAAAGCTATGTCTGGAGCCCCAATTATAATTGGTAGTAATCAATTACCGAATCCACCAATTAT
AATTGGTATGACCATAAAAAAAATCATAATAAATGCATGTGAAGTTACAATTACGTTGTAAGTTTGATCATTATT
AATAAACGACCCAGGTTGAGCTAGTTCAATTCGGATAATTATTCTTATTATTA

>*Dalbulus_maidis*_2 (566 bp)
AGAATGATGTATTTAAATTTCGATCTGTTAATAATATGGTAATTGCTCCTGCTAATACCGGTAGTGAGAGTAATA
GTAGTACTGCAGTAATTAGGACAGACCATACAAATAAAGGAGTTTTATCTAGGCTTATCCCTACGGGTCGTATAT
TTAAAATTGTAGTAATGAAATTCACTGCTCCTAAAATTGATGAAATTCCTGCCAAATGAAGTGAAAAAATTGATA
TATCAACTCTGGGGCCTGCGTGAGCAATATTTGATGAAAGGGGGGGGTAGACAGTTCATCCTGTTCCTGTCCCTG
TCTCAATCAATGATGATATAATTAGCAATGTGAGTGAGGGAGGTAATAATCAAAATCTTATGTTATTTAATCGAG
GGAAAGCTATGTCTGGAGCCCCAATTATAATTGGTAGTAATCAATTACCGAATCCACCAATTATAATTGGTATGA
CCATAAAAAAAATCATAATAAATGCATGTGAAGTTACAATTACGTTGTAAGTTTGATCATTATTAATAAACGACC
CAGGTTGAGCTAGTTCAATTCGGATAATTATTCTTATTATT

>*Diabrotica speciosa*_1 (658 bp)
TACTTTATATTTTATTTTTGGAGTATGGGCAGGTATAGTAGGGACATCCCTAAGAATCCTGGTCCGAGCAGAATT
AGGAAGCCCAGGTTCTTTGATTGGAAATGATCAAATTTATAATGTAATTGTTACAGCTCATGCTTTTATCATAAT
TTTTTTTATAGTAATACCTATTATGATTGGCGGATTTGGTAATTGATTAGTACCCCTAATAATTGGAGCTCCTGA
TATAGCATTCCCTCGAATAAATAATATAAGATTTTGATTACTACCACCCTCTTTACTATTACTATTATTAAGAAG
AATAGTAGAAAGAGGGGTAGGGACTGGTTGAACAGTTTATCCTCCTTTATCATCTAATATTGCCCATGGAGGTTC
ATCTGTAGATTTAGCTATTTTTAGATTACATTTAGCTGGTATTTCTTCAATTTTAGGTGCAATTAATTTTATTAC
TACAGTAATTAATATACGACCAATAGGAATAACTTTTGACCGAATACCATTATTTGTTTGAGCTGTTGTGATTAC
TGCAGTTTTATTATTATTATCTTTACCTGTATTAGCTGGGGCAATTACAATACTATTAACAGACCGAAACCTAAA
TACTTCATTTTTTGATCCGGCAGGAGGGGGCGATCCAATTTTATACCAGCACTTATTT

>*Diabrotica speciosa*_2 (658 bp)
TACTTTATATTTTATTTTTGGAGTATGGGCAGGTATAGTAGGGACATCCCTAAGAATCCTGGTCCGAGCAGAATT
AGGAAGCCCAGGTTCTTTGATTGGAAATGATCAAATTTATAATGTAATTGTTACAGCTCATGCTTTTATCATAAT
TTTTTTTATAGTAATACCTATTATGATTGGCGGATTTGGTAATTGATTAGTACCCCTAATAATTGGAGCTCCTGA
TATAGCATTCCCTCGAATAAATAATATAAGATTTTGATTACTACCACCCTCTTTACTATTACTATTATTAAGAAG
AATAGTAGAAAGAGGGGTAGGGACTGGTTGAACAGTTTATCCTCCTTTATCATCTAATATTGCCCATGGAGGTTC
ATCTGTAGATTTAGCTATTTTTAGATTACATTTAGCTGGTATTTCTTCAATTTTAGGTGCAATTAATTTTATTAC
TACAGTAATTAATATACGACCAATAGGAATAACTTTTGACCGAATACCATTATTTGTTTGAGCTGTTGTGATTAC
TGCAGTTTTATTATTATTATCTTTACCTGTATTAGCTGGGGCAATTACAATACTATTAACAGACCGAAACCTAAA
TACTTCATTTTTTGATCCGGCAGGAGGGGGCGATCCAATTTTATACCAGCACTTATTT

>Diabrotica speciosa_3 (658 bp)
TACTTTATATTTTATTTTTGGAGTATGAGCAGGTATAGTAGGGACATCCCTGAGAATCCTGGTCCGAGCAGAATT
AGGAAGCCCAGGTTCTTTGATTGGAAATGATCAAATTTATAATGTAATTGTTACAGCTCATGCTTTTATCATAAT
TTTTTTTATAGTAATACCTATTATGATTGGTGGATTTGGTAATTGATTAGTACCCCTAATAATCGGAGCTCCTGA
TATAGCATTCCCTCGAATAAATAATATAAGATTTTGATTACTACCACCCTCTTTACTATTACTATTATTAAGAAG
AATGGTAGAAAGAGGGGTGGGGACTGGTTGGACAGTTTATCCTCCTTTATCATCTAATATTGCCCATGGAGGCTC
ATCTGTAGATTTAGCTATTTTTAGATTACATTTAGCTGGTATTTCTTCAATTTTAGGTGCAATTAATTTTATTAC
TACAGTAATTAATATACGACCAATAGGAATAACTTTTGACCGAATACCATTATTTGTTTGAGCTGTTGTGATTAC
TGCAGTTTTATTATTATTATCTTTACCTGTATTAGCTGGGGCAATTACAATACTATTAACAGACCGAAACCTAAA
TACTTCATTTTTTGATCCGGCGGGAGGGGGTGATCCAATTTTATACCAGCACTTATTT

>*Doru luteipes*_1(662 bp)
AACAAATGTTGATACAAGATGGGGTCCCCCCCTCCCGCAGGATCAAAAAATGACGTATTCAAATTTCGATCCGTC
AATAGCATCGTAATGGCCCCTGCCAAAACGGGTAACGACAACAACAATAACAAGGCCGTGATTGCCACAGACCAC
ACGAACAACGGCACCCGTTCCGGGCTTAGTCCAGAAGGCCGCATGTTTATCACAGTAGTGATGAAATTGATAGCA
CCTAAAATTGACGAAACCCCTGCTAAATGCAATGAAAAAATTCTCAAGTCCACGGAAGCCCCTGCATGGGCAATG
GCCCCTGACAAAGGGGGGTAAACCGTTCACCCTGTCCCAGCACCTCTATCTACCATGCTTCCGGAAAGCAACAAC
AACAAGGAAGGGGGCAAAAGTCAAAAACTCATGTTGTTTATTCGAGGAAAAGCCATGTCTGGAGCTCTGAGCATC
AAAGGAACCAACCAGTTTCCAAACCCCCCAATCAAAATAGGTATAACTATAAAAAAAATCATCACGAATGCATGG
GCCGTTACAATCACGTTATAAACTTGATCATCCCCAATCAAAGCCCCAGGGTGACCTAATTCTGCACGAATCAAC
AAACTCATCGAAGTCCCCACCATTCCTGCCCAAACCCCGAACACGAAATACAAAGTCCCAAT

>*Doru luteipes*_2(662 bp)
AACAAATGTTGATACAAGATGGGGTCCCCCCCTCCCGCAGGATCAAAAAATGACGTATTCAAATTTCGATCCGTC
AATAGCATCGTAATGGCCCCTGCCAAAACGGGTAACGACAACAACAATAACAAGGCCGTGATTGCCACAGACCAC
ACGAACAACGGCACCCGTTCCGGGCTTAGTCCAGAAGGCCGCATGTTTATCACAGTAGTGATGAAATTGATAGCA
CCTAAAATTGACGAAACCCCTGCTAAATGCAATGAAAAAATTCTCAAGTCCACGGAAGCCCCTGCATGGGCAATG
GCCCCTGACAAAGGGGGGTAAACCGTTCACCCTGTCCCAGCACCTCTATCTACCATGCTTCCGGAAAGCAACAAC
AACAAGGAAGGGGGCAAAAGTCAAAAACTCATGTTGTTTATTCGAGGAAAAGCCATGTCTGGAGCTCTGAGCATC
AAAGGAACCAACCAGTTTCCAAACCCCCCAATCAAAATAGGTATAACTATAAAAAAAATCATCACGAATGCATGG
GCCGTTACAATCACGTTATAAACTTGATCATCCCCAATCAAAGCCCCAGGGTGACCTAATTCTGCACGAATCAAC
AAACTCATCGAAGTCCCCACCATTCCTGCCCAAACCCCGAACACGAAATACAAAGTCCCAAT

>*Eriopis connexa*_1 (653 bp)
TATATTTTTTATTTGGAATATGATCTGGAATAGTGGGAACATCCTTAAGAATTTTAATTCGAATAGAATTAGGAA
CAACTAATAGATTAATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCACATGCTTTTATTATAATTTTTT
TTATAGTAATACCCATTATAATTGGGGGATTTGGAAATTGATTAGTACCACTAATAATTGGTGCTCCAGATATAG
CTTTTCCTCGACTAAATAATATAAGATTTTGATTGTTACCCCCTGCCTTAACTTTATTAATTTTTAGAAGATTAG
TAGAAATAGGAGCAGGAACAGGTTGAACAGTATACCCCCCTTTATCATCTAATTTAGCTCATAATGGTCCTTCAG
TAGATTTAGTAATTTTTAGACTTCATTTAGCAGGAATTTCTTCAATTTTAGGAGCAGTAAATTTTATTTCTACTA
TTATAAATATACGACCAATAGGAATAAATCTTGATAAAACTCCTTTATTTGTATGATCAGTTTTAATTACAGCTA
TTTTATTATTATTATCATTACCTGTATTAGCAGGTGCAATCACAATACTTTTAACTGACCGAAATATTAATACTT
CATTTTTTGACCCCTCAGGAGGAGGAGACCCAATTTTATACCAGCATTTATTC

>*Eriopis connexa*_2 (639 bp)
GGAATATGATCTGGAATAGTAGGAACATCCTTAAGAATTTTAATTCGAATAGAATTAGGAACAACTAATAGATTA
ATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCACATGCTTTTATTATAATTTTTTTTATAGTAATACCT
ATCATAATTGGGGGATTTGGAAATTGATTAGTACCACTAATAATTGGTGCCCCAGATATAGCTTTTCCTCGACTA
AATAATATAAGATTTTGATTGTTACCTCCTGCCTTAACTTTATTAATTTTTAGAAGATTAGTAGAAATAGGAGCA
GGAACAGGTTGAACAGTATATCCCCCTTTATCGTCTAATTTAGCTCATAATGGTCCTTCAGTAGATTTAGTAATT
TTTAGACTTCACTTAGCAGGAATTTCTTCAATTTTAGGAGCAGTAAATTTTATTTCTACTATTATAAATATACGA
CCAATAGGAATAAATCTTGATAAAACTCCTTTATTTGTATGATCAGTTTTAATTACAGCTATTTTATTATTATTA
TCATTACCTGTGTTAGCAGGTGCAATCACAATACTTTTAACTGACCGAAATATTAACACTTCATTTTTTGACCCT
TCAGGAGGAGGAGATCCAATTTTATACCAGCATTTATTC

>*Harmonia axyridis* (617 bp)
TTATACTTTTTATTTGGAATATGGGCAGGAATAGTAGGAACATCGTTAAGTATTTTAATTCGGTTAGAATTAGGA
ACTAGAGGAAGATTAATTGGAAACGACCAAATTTATAATATAATTGTTACAGCTCATGCTTTCATTATAATTTTC
TTTATAGTAATACCTATTATAATTGGGGGTTTTGGAAATTGGTTAGTTCCTTTAATAATTGGAGCTCCTGATATA
GCATTTCCACGATTAAATAACATAAGATTTTGACTTTTACCCCCTGCTTTAACTCTT:TTAATTTTAAGAACAAT
CGTAGAAATAGGGGCAGGAACAGGATGAACTGTTTACCCTCCTCTTTCTTCTAATTTAACACATAATGGGCCTTC
AGTAGATTTAGTGATTTTTAGTTTACATTTAGCAGGAATTTCCTCAATTTTAGGTGCAGTAAATTTCATTTCAAC
TATTATAAATATACGTCCATTTGGTATAATACTTGATAAAACTCCTTTATTTGTATGATCTGTTCTTATTACAGC
AATTCTTTTATTACTATCACTACCAGTTCTTGCAGGAGCAATTACTATACTATTAACTGACCGAAACTTAAATTC
TTCTTTTTTTGACCCAA

>*Hippodamia convergens* (624 bp)
TTATATTTTTTATTTGGAATATGAGCTGGAATAGTTGGAACTTCACTTAGAATTTTAATTCGACTAGAATTAGGA
ACTACAGGAAGTTTAATTGGAAATGACCAAATTTATAACGTAATTGTTACTGCTCATGCTTTTATTATAATTTTT
TTTATAGTTATACCTATTATAATTGGGGGTTTTGGAAATTGATTGGTACCTTTAATAATTGGAGCCCCTGATATA
GCATTTCCTCGATTAAATAATATGAGATTTTGGTTATTACCCCCTGCTTTAACTCTT:CTTTTATTTAGAAGAAT
AGTAGAAATAGGAGCTGGAACTGGGTGAACTGTTTACCCTCCTTTATCTTCAAATTTAGCCCATAATGGACCTTC
TGTTGATTTAGTTATTTTTAGCCTCCATCTAGCTGGAATCTCTTCTATCTTAGGGGCTGTGAATTTTATTTCAAC
AATTATAAATATACGCCCCTATGGTATGAGATTAGATAAAACCCCATTATTTGTATGATCCGTCTTAATTACAGC
TATCCTTTTACTGTTATCCTTACCCGTTCTTGCTGGAGCAATTACTATATTATTAACTGATCGAAATTTAAATAC
TTCTTTTTTTGACCCTACAGGAGG

>*Lagria villosa*_1 (594 bp)
AAGAATAATTATTCGAACTGAATTAAGAGTGTCAGGTTCAATAATTGGAGATGATCAAATCTATAATGTTATTGT
TACTAGACACGCTTTTATCATAATTTTCTTTATGGTGATGCCTATTATAATCGGTGGATTTGGTAATTGGTTAGT
GCCTTTAATATTAGGTGCTCCAGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTATTACCTCCTTC
TCTTTCACTATTATTAATAAGAAGAATGGTTGAAAGAGGAGTAGGGACAGGTTGAACTGTTTATCCTCCTTTATC
ATCAAATTTAGCCCACAGAGGATCATCTGTAGATTTAGCAATTTTTAGGCTTCATTTAGCCGGGATCTCTTCGAT
CCTAGGCGCTGTAAATTTTATTACAACAGTGTTAAATATACGGCCAAGAGGAATAAGAATTGATCGTATATCTTT
GTTTGTTTGATCAATTATTATTACAGCATTTTTATTATTATTGTCTTTACCTGTTTTAGCAGGAGCCATTACTAT
ATTACTAACTGATCGTAATATTAATACGTCATTTTTTGACCCTTCAGGGGGAGGTGATCCTATTCTTTA

>*Lagria villosa*_2 (686 bp)
CATAAAGATATTGGTACTTTATATTTTATTTTTGGCGCTTGATCTAGAATATTAGGAACTTCTTTAAGAATAATT
ATTCGAACTGAATTAAGAGTGTCAGGTTCAATAATTGGAGATGATCAAATCTATAATGTTATTGTTACTAGACAC
GCTTTTATCATAATTTTCTTTATGGTGATGCCTATTATAATCGGTGGATTTGGTAATTGGTTAGTGCCTTTAATA
TTAGGTGCTCCAGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTATTACCTCCTTCTCTTTCACTA
TTATTAATAAGAAGAATGGTTGAAAGAGGAGTAGGGACAGGTTGAACTGTTTATCCTCCTTTATCATCAAATTTA
GCCCACAGAGGATCATCTGTAGATTTAGCAATTTTTAGGCTTCATTTAGCCGGGATCTCTTCGATCCTAGGCGCT
GTAAATTTTATTACAACAGTGTTAAATATACGGCCAAGAGGAATAAGAATTGATCGTATATCTTTGTTTGTTTGA
TCAATTATTATTACAGCATTTTTATTATTATTGTCTTTACCTGTTTTAGCAGGAGCCATTACTATATTACTAACT
GATCGTAATATTAATACGTCATTTTTTGACCCTTCAGGGGGAGGTGATCCTATTCTTTACCAACATTTATTTTGA
TTTTTTGGTCA

>*Spodoptera albula* (655 bp)
TCAAAATAAATGTTGATAGAGAATAGGATCACCTCCTCCAGCAGGATCAAAAAATGATGTATTTAAATTTCGATC
AGTGAGTAATATAGTAATAGCTCCAGCTAGAACAGGTAAAGATAATAATAAAAGGAATGCAGTAATACCTACAGC
TCAAATAAATAGAGGTATTTGATCGAATGATAAATTATTTAATCGTATATTAATAATAGTGGTAATAAAATTAAT
AGCTCCTAAAATAGATGAAATTCCAGCTAAGTGAAGAGAAAAAATAGCTAAATCAACTGAACTTCCACCATGAGC
AATATTAGAGGAGAGGGGGGGGTAAACTGTTCATCCAGTTCCTGCTCCATTTTCTACAATTCTTCTTGAAATTAA
TAAGGTTAAAGAGGGGGGTAAAAGTCAAAAACTTATATTATTTATACGAGGAAAAGCTATATCAGGGGCCCCTAA
TATTAATGGAACAAGTCAATTTCCAAATCCTCCAATTATAATAGGTATAACTATAAAAAAAATTATAATGAAAGC
ATGAGCTGTCACAATAGTATTATAAATTTGATCATCTCCAATTAAAGATCCAGGGGTTCCGTAATTCTGCTCGAA
TTAATAAACTTTAAAGGAAGTTCCCACTATTCCAGCTCAAATACCAAAAATAAAA

>*Spodoptera cosmioides* (661 bp)
AATCAAAATAAATGTTGGTAAAGAATAGGGTCACCTCCTCCTGCAGGATCAAAAAATGATGTATTTAAGTTTCGA
TCAGTAAGTAATATTGTAATAGCTCCAGCTAAAACAGGTAAAGATAATAATAATAAAAATGCAGTAATACCTACA
GCTCAAATAAATAAAGGTATTTGATCAAAGGATAAATTATTTAATCGTATATTAATAATAGTAGTAATAAAATTA
ATAGCTCCTAAAATAGATGAAATTCCAGCTAAATGAAGAGAAAAAATAGCTAAATCTACAGATCTTCCACCATGA
GCAATATTAGAGGAGAGGGGGGGGTAAACTGTTCATCCAGTTCCTGCTCCATTTTCTACAATTCTACTTGAAATT
AATAAGGTTAAAGAGGGTGGTAAAAGTCAAAAACTTATATTATTTATACGTGGAAAAGCTATATCAGGGGCTCCT
AATATTAAAGGTACAAGTCAATTTCCAAAACCTCCGATTATAATAGGTATAACTATAAAAAAAATTATAATAAAA
GCATGAGCTGTTACGATAGTATTATAAATTTGATCATCTCCAATTAAAGATCCTGGAGTTCCTAATTCAGCTCGA
ATTAATAAACTTAAAGAAGTTCCTACTATTCCAGCTCAAATTCCAAAAATAAAATATAATG

>*Spodoptera eridania* (649 bp)
GACCAAAAAATCAAAATAAATGTTGATAGAGAATAGGATCTCCTCCTCCTGCAGGGTCAAAAAATGATGTATTTA
GGTTTCGATCAGTTAATAATATAGTAATAGCACCGGCTAAGACGGGTAAAGATAATAATAAAAGAAATGCAGTAA
TACCAACAGCTCAAATAAATAAAGGTATTTGATCAAATGATAAGTTATTTAATCGTATATTAATAATAGTTGTAA
TAAAGTTAATAGCTCCTAAAATAGATGAAATTCCAGCTAAATGAAGGGAAAAAATAGCTAAATCCACTGAGCTAC
CACCATGGGCAATATTAGAGGAGAGGGGGGGGTAAACTGTTCAACCAGTTCCTGCTCCATTTTCTACAATTCTAC
TTGAAATTAATAAAGTTAGTGATGGGGGTAAAAGTCAAAAACTTATATTATTTATTCGGGGGAAAGCTATATCTG
GGGCTCCTAATATTAATGGTACAAGTCAATTACCAAATCCTCCAATTATAATAGGTATAACTATGAAAAAAATTA
TAATAAAAGCATGAGCTGTTACAATAGTATTATAAATTTGATCATCTCCAATTAAAGATCCTGGGGTTCCTAATT
CTGCTCGAATTAATAAACTTAATGAAGTTCCGACCATTCCAGCTCAAAT

>*Spodoptera frugiperda* (674 bp)
GACCAAAAAATCAAAATAAATGTTGATAAAGAATAGGATCACCTCCACCTGCAGGATCGAAAAATGATGTATTTA
AATTTCGATCAGTAAGTAATATAGTAATAGCTCCGGCTAAAACAGGTAAAGATAATAATAATAAGAATGCAGTAA
TACCTACAGCTCAAATAAATAAAGGTATTTGATCAAATGATAAATTATTTAATCGTATATTAATAATAGTAGTAA
TAAAGTTAATAGCTCCTAAAATAGATGAAATTCCAGCTAAATGAAGTGAGAAAATAGCTAAATCTACTGAACTAC
CACCATGAGCAATATTAGAGGAGAGGGGGGGGGTAAACTGTTCATCCAGTTCCTGCTCCATTTTCTACAATGCTAC
TAGAAATTAATAAAGTTAAAGATGGGGGTAAAAGTCAAAAACTTATATTATTTATACGTGGGAAAGCTATATCAG
GGGCTCCTAATATTAGAGGTACAAGTCAATTTCCAAATCCTCCAATTATAATAGGTATAACTATAAAAAAAATTA
TAATAAAAGCATGAGCTGTTACAATAGTATTATAAATTTGATCATCTCCAATTAAAGATCCTGGAGTCCCGTGAA
TTCAGCTCGAATTAATAAACTTAAAAGAAGTACCTACTATCCCTGCTCAAATTCCAAAAATAAAATATAATGTT

APPENDIX B

DNA barcode from 18 morphotypes of ground beetles sampled in organic and conventional maize produced in chapter 4.

>Morphotype1 - *Selenophorus* sp1

ATCTTCCGAATGCAACTTATGCACATTAATATTCTTGGTGATAATTACCAATTATATAATGTAATAGTCACAAGT
CATGCTTTGGTTATGGTATTTTTTATGATTATGCCAGCTTTAATGGGTGGTTTTGGTAATTGGTTTGTTCCCCTT
ATGATAGGTGCACCGGATATGGCCTTTCCGCGCATGAATAATTTGAGCTTTTGGCTTCTTGTATCATCCTTTATC
TTGCTCATTATGTCTCTGTTTGTTGGTGAAGGAGCGGGTACTGGTTGGACGTTATACCCACCACTATCACAAGTT
AACTCCCATCCTAGTGCAGCTGTTGATCTGACAATATTTGCACTTCATGTTGCTGGAATCTCTTCAATTGTTGGT
GCTATTAATTTTATCGTTACTATATTTAATATGCGTACTCCTGGGATGACCCTGCATAAGATGCCTTTATTCGTT
TGGTCTATTTTACTTACAGCTTTTATGATCATAGTTGCTTTGCCAGTATTAGCTGGAGCTATCACTATGCTGATT
ACCGACCGCAATTTGGGCACTGCA

>Morphotype2 - *Pterostichus* sp1

AACATTATATTTTATTTTTGGTGCATGATCAGGAATAGTAGGAACATCCTTAAGCATACTTATTCGAGCTGAATT
AGGAAACCCTGGGTCATTAATTGGTGACGATCAAATTTATAATGTTATTGTTACTGCTCATGCATTTGTTATAAT
TTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGTAATTGATTAGTCCCACTAATATTAGGAGCCCCTGA
TATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGACTTCTTCCTCCTTCTTTAAGACTTTTATTAATAAGCAG
TTTGGTTGAAAGTGGAGCTGGTACTGGATGAACAGTTTACCCACCCCTATCATCAGGAATTGCACATGCAGGAGC
TTCAGTTGATTTAGCTATTTTTAGTTTACATTTAGCTGGAGTTTCATCTATTTTAGGAGCTGTAAATTTTATTAC
TACTATTATTAATATACGATCAGTTGGAATAACTTTTGATCGAATACCTTTATTTGTTTGATCAGTAGGAATTAC
TGCTTTATTACTTTTACTTTCATTACCTGTATTAGCTGGGGCTATTACAATACTTTTAACAGATCGAAATTTAAA
TACTTCATTTTTTGACCCAGCAGGAGGAGGAGACCCAATTCTTTACCAACACTTATTT

>Morphotype3 - *Acupalpus* sp1

AACATTATATTTTATTTTTGGAGCATGATCAGGAATAGTAGGTACTTCATTAAGAATATTAATTCGAGCTGAATT
AGGAACTCCTGGAGCATTAATTGGTGATGATCAAATTTATAATGTTATTGTTACTGCTCATGCTTTTATTATAAT
TTTTTTTATAGTAATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCTCCTGA
TATAGCATTCCCTCGAATAAATAATATAAGTTTTTGATTACTTCCACCTTCTTTAACTCTTCTTTTAATGAGAAG
AATAGTTGAAAGAGGAGCAGGTACTGGTTGAACAGTTTACCCACCTTTATCATCAGGTATTGCTCATAGAGGAGC
TTCAGTAGATTTAGCAATTTTTAGACTTCACTTAGCTGGAGTATCTTCTATTTTAGGAGCTGTAAATTTTATTAC
TACTATTATTAATATACGATCAATTGGGATAACATTTGATCGAATACCTTTATTTGTTTGATCAGTAGGAATTAC
TGCTTTATTATTATTATTATCATTACCTGTTTTAGCAGGAGCTATTACTATACTATTAACAGATCGAAATTTAAA
TACTTCATTTTTTGATCCTGCGGGAGGAGGGGATCCAATTTTATATCAACATTTATTT

>Morphotype4 - *Pterostichus* sp 2

TTTATACTTTATTTTTGGTGCATGAGCAGGAATAGTAGGGACTTCTTTAAGTATACTAATTCGAGCAGAATTAGG
GAATCCTGGTTCATTAATTGGTGACGATCAAATTTATAATGTTATTGTTACTGCTCATGCTTTTATTATAATTTT
CTTTATAGTTATACCTATTATAATTGGAGGTTTTGGAAATTGACTTGTACCTTTAATATTAGGAGCCCCTGATAT
AGCCTTTCCTCGAATAAATAATATAAGTTTTTGACTTCTACCCCCTTCTTTAACCCTTCTTTTAATGAGAAGAAT
AGTTGAAAGAGGAGCAGGGACAGGATGAACAGTTTACCCTCCCTTATCTTCAGGTATTGCTCATAGAGGAGCTTC
AGTAGATTTAGCAATTTTTAGATTACATCTAGCAGGAGTTTCATCAATTTTAGGGGCAGTAAATTTTATTACAAC
AATTATTAATATGCGATCAGTAGGAATAACATTTGATCGAATACCTTTATTTGTATGATCCGTAGGAATTACTGC
TTTATTACTTCTTTTATCACTACCAGTATTAGCAGGAGCTATTACAATATTATTAACAGACCGAAATTTAAATAC
TTCTTTTTTTGATCCAGCAGGAGGGGGAGATCCTATTCTATACCAACATTTATTT

>Morphotype6 - *Lebia* sp1

GACTTTATATTTTATCTTTGGAGCATGAGCAGGAATAGTAGGAACTTCATTAAGTATATTAATCCGTGCAGAATT
AGGAAATCCAGGAGCTTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCTCATGCTTTTATTATAAT
TTTTTTCATAGTAATACCTATTATAATTGGAGGATTTGGTAATTGATTAGTTCCTTTAATATTAGGAGCACCCGA
TATAGCTTTTCCTCGAATAAATAATATAAGATTTTGACTTTTACCTCCATCTTTAACTCTATTACTAATAAGAAG
TCTAGTGGAACAGGGAGCTGGTACAGGATGAACTGTGTACCCCCCACTCTCTTCAGGAATTGCTCATGCTGGAGC
TTCAGTAGATTTAGCTATTTTTAGTTTACATTTAGCAGGAATTTCTTCAATTTTAGGAGCAGTAAATTTTATTAC
TACAATCATTAATATACGATCTATTGGTATAACCTTTGATCGAATACCTTTATTTGTTTGATCAGTAGGTATTAC
TGCTTTATTATTATTACTTTCATTACCTGTATTAGCTGGAGCTATTACTATATTGTTAACTGATCGAAATTTAAA
TACCTCTTTTTTTGATCCTGCAGGAGGAGGAGATCCTATTTTATATCAACATTTATTT

>Morphotype7 - *Trichotichnus* sp1

TACTTTATATGTTATTTTTTCCATTTTTGCTGGAGTGATAGGTGGTGTTTTGTCTTTGATCTTCCGTATGCAGCT
TATGCACATTAATGTTCTTGGTGATAATTATCAATTATATAATGTAATAGTCACAAGTCATGCTTTGGTTATGGT
ATTTTTTATGATTATGCCAGCTTTGATGGGTGGTTTTGGTAATTGGTTCGTCCCTCTTATGATAGGTGCACCAGA
TATGGCCTTTCCGCGCATGAATAATTTGAGCTTTTGGCTTCTTGTATCATCTTTTATTTTGCTTATAATGTCTTT
GTTTGTTGGTGAGGGGGCAGGCACTGGTTGGACGTTGTACCCACCATTATCGCAGATTAATTCTCATCCTAGCGC
AGCTGTTGATCTAGCAATATTTGCACTTCATGTTGCTGGAATCTCCTCAATTGTTGGTGCTATTAATTTTATCGT
TACTATATTTAATATGCGCACTCCTGGGATGACCTTGCATAAAATGCCTTTATTCGTTTGGTCTATTTTACTTAC
AGCTTTTATGCTCATAATTGCTTTGCCAGTATTGGCTGGAGCTATAACTATGCTGCTTACCGACCGCAACTTGGG
GACTGCATTTTTTGAGCCAGCAGGTGGCGGTGATCCGGTGTTATTTCAGCATTTATTT

>Morphotype8 - Harpalini sp1

AACATTATACTTTATTTTTGGAGCATGAGCAGGAATAGTAGGGACTTCTTTAAGTATATTAATTCGAGCCGAATT
AGGAACTCCCGGTGCATTGATTGGTGATGATCAAATTTATAATGTTATTGTTACTGCACATGCTTTTATTATAAT
TTTCTTTATAGTAATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCTCCTGA
TATAGCCTTTCCTCGAATAAATAATATAAGTTTTTGACTACTTCCCCCATCTTTAACTCTTCTTTTAATGAGAAG
AATAGTAGAAAGTGGAGCAGGTACTGGATGAACAGTTTACCCACCTTTATCATCAGGAATTGCCCATAGAGGAGC
TTCAGTAGATTTAGCTATTTTTAGTCTTCATTTAGCAGGAATTTCATCAATTTTAGGAGCTGTAAATTTTATTAC
AACAATTATTAATATACGATCAATTGGAATAACTTTTGATCGAATACCATTATTTGTATGATCAGTAGGAATTAC
TGCTTTATTATTACTATTATCATTACCAGTTTTAGCAGGAGCAATTACTATACTTTTAACAGATCGAAATTTAAA
TACTTCATTTTTTGATCCTGCGGGAGGAGGAGACCCTATTTTATATCAACATTTATTT

>Morphotype9 - *Selenophorus alternans ()*

AACTTTATATTTTATTTTTGGAGCATGAGCAGGAATAGTAGGGACTTCATTAAGTATATTAATTCGAGCTGAATT
AGGAACTCCCGGTGCATTAATTGGTGATGATCAAATTTATAATGTTATTGTTACAGCACATGCTTTTATTATAAT
TTTCTTTATAGTAATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCTCCTGA
TATAGCCTTTCCTCGAATAAATAATATAAGATTTTGACTACTTCCTCCATCTTTAACTCTTCTTTTAATGAGAAG
AATAGTAGAAAGAGGGGCTGGAACTGGATGAACAGTTTATCCACCTTTATCATCTGGAATTGCCCATGGAGGTGC
TTCAGTAGATCTAGCTATTTTTAGTCTTCATCTTGCTGGAGTTTCTTCAATTTTAGGAGCAGTAAATTTTATTAC
AACAATTATTAATATACGATCTGTAGGAATAACTTTTGATCGAATACCATTATTTGTTTGATCAGTAGGTATTAC
TGCATTATTATTATTATTATCACTACCAGTATTAGCAGGAGCAATTACTATATTATTAACAGATCGAAATTTAAA
TACTTCTTTTTTTGATCCTGCTGGAGGAGGTGATCCAATTTTATATCAACATTTATTT

>Morphotype10 - *Notiobia* sp1

AACATTATATTTTATTTTTGGAGCATGATCAGGAATAGTAGGGACTTCATTAAGTATACTAATTCGAGCAGAGTT
AGGAGCTCCTGGTGCATTAATTGGTGATGATCAAATTTATAATGTTATTGTTACTGCACATGCTTTTATTATAAT
TTTCTTTATAGTAATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTACCTTTAATATTAGGTGCTCCTGA
TATAGCCTTTCCTCGTATAAATAATATAAGTTTTTGATTACTTCCTCCTTCATTAACTCTTCTTTTAATGAGAAG
TATGGTTGAAAGAGGAGCAGGTACAGGATGAACAGTTTACCCCCCATTATCATCAGGTATTGCCCATGGAGGTGC
CTCTGTTGATTTAGCTATTTTTAGATTACATCTTGCAGGAGTTTCATCTATTTTAGGTGCTGTAAATTTTATTAC
TACAATTATTAATATACGATCAGTAGGAATAACATTTGATCGAATACCTTTATTTGTTTGATCAGTAGGAATTAC
TGCTTTATTATTATTATTATCATTACCTGTATTAGCTGGAGCTATTACTATATTATTAACAGATCGAAATTTAAA
TACTTCATTTTTTGACCCTGCTGGAGGAGGAGATCCTATTTTATATCAACATTTATTT

>Morphotype11 - *Tetragonoderus laevigatus*

AACATTATATTTTATTTTTGGTGCATGAGCTGGAATAGTAGGAACTTCTTTAAGTATACTAATTCGAGCAGAATT
GGGAAATCCTGGAGCATTAATTGGAGACGATCAAATTTATAATGTAATTGTTACTGCTCATGCATTTATTATAAT
TTTTTTTATAGTAATACCTATTATAATTGGAGGATTTGGAAATTGGTTAGTTCCTTTAATACTAGGAGCTCCTGA
TATAGCATTTCCTCGAATAAATAATATAAGATTTTGACTACTTCCACCTTCATTAACCTTACTTTTAATGAGAAG
TATGGTTGAAAGAGGAGCAGGAACAGGATGAACAGTATACCCCCCTTTATCATCTGGTATTGCTCATGCAGGAGC
TTCTGTAGATTTAGCTATTTTTAGATTACATTTAGCAGGAATTTCTTCAATTTTAGGAGCTGTAAATTTTATTAC
AACAATTATTAATATACGATCTGTAGGAATAACATTTGATCGAATACCATTATTTGTTTGATCAGTAGGAATTAC
TGCTTTATTACTACTATTATCTTTACCTGTATTAGCTGGAGCTATTACAATATTATTAACAGATCGAAATTTAAA
TACTTCATTTTTTGATCCAGCAGGAGGGGGAGATCCAATTTTATATCAACATTTATTT

>Morphotype12 - *Pterostichus* sp3

TAAATGTTGGTATAAAATTGGATCACCTCCTCCCGCAGGATCAAAGAAAGAAGTATTTAAATTTCGATCTGTTAA
AAGTATTGTAATAGCTCCAGCTAAAACTGGTAATGATAAAAGTAAAAGTAAAGCAGTAATTCCAACTGATCATAC
AAATAAAGGTATTCGATCAAAAGTTATTCCTACTGATCGTATATTAATAATTGTTGTAATAAAATTTACAGCTCC
TAAAATTGATGATACTCCTGCTAAATGTAATCTGAAAATAGCTAAGTCTACAGAAGCTCCTCTATGGGCAATTCC
TGATGATAAAGGAGGGTAAACTGTTCATCCTGTACCAGCTCCTCTTTCTACTATTCTACTTATTAAAAGAAGAGT
TAATGAAGGGGGAAGAAGTCAGAATCTTATATTATTTATTCGAGGAAAAGCTATATCAGGAGCTCCTAATATTAA
AGGAACTAATCAATTTCCAAATCCTCCAATTATAATAGGTATTACTATAAAGAAAATTATAATGAAAGCATGTGC
AGTTACAATAACGTTATAAATTTGATCATCACCAATTAGTGAACCAGGATTTCCT

>Morphotype13 - *Tetragonoderus intersectus*

TAAATGTTGATATAAAATAGGATCTCCTCCTCCAGCTGGATCAAAAAATGTAGTATTTAAATTTCGATCAGTTAA
TAGTATTGTAATAGCTCCTGCTAATACAGGTAAAGATAGTAATAATAATAAAGCAGTAATTCCTACTGATCATAC
AAATAAAGGTATTCGATCAAATGTTATTCCAATTGATCGTATATTAATAATTGTTGTAATAAAATTTACAGCCCC
TAAAATTGAAGAAATTCCAGCTAAATGTAATCTAAAAATAGCTAAATCTACAGAAGCTCCAGCATGAGCAATTCC
AGATGATAAAGGTGGATATACTGTTCATCCAGTTCCTGCTCCTCTTTCAACTATACTACTCATTAGAAGTAAAGT
TAATGAAGGAGGAAGAAGTCAAAATCTTATATTATTTATTCGAGGAAAGGCTATATCAGGTGCACCTAATATTAA
AGGAACTAATCAATTTCCAAACCCTCCAATTATAATAGGTATTACTATAAAAAAAATTATAATAAATGCATGTGC
AGTAACAATTACATTATAAATTTGATCATCTCCAATTAATGCTCCAGGATTACCTAATTCTGCTCGAATTAGTAT
ACTTAATGAAGTCCCTACTATTCCAGCTCATGCACC

>Morphotype14 - *Bradycellus* sp1

AACACTATATTTTATCTTTGGAGCATGATCTGGGATAGTAGGAACCTCATTAAGTATATTAATTCGAGCTGAATT
AGGTACACCTGGAGCATTAATTGGTGATGATCAAATTTATAATGTTATTGTCACTGCTCATGCTTTTGTAATAAT
TTTTTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAATTGATTAGTCCCATTAATATTAGGTGCTCCTGA
TATAGCATTTCCTCGTATAAATAACATAAGTTTTTGACTATTGCCCCCTTCTTTAAGACTTTTATTAATGAGAAG
TTTAGTTGAAAGAGGAGCTGGCACTGGATGAACAGTTTACCCTCCCCTATCATCTGGTATTGCCCATAGAGGAGC
ATCAGTTGATCTTGCGATTTTTAGATTACATTTAGCAGGAGTGTCCTCTATTTTAGGAGCAGTAAACTTTATTAC
AACAATTATTAATATACGATCAATTGGAATAACATTCGATCGAATACCTCTATTTGTATGATCAGTTGGAATTAC
AGCTCTGTTATTATTATTATCATTACCTGTATTAGCTGGTGCTATTACAATATTATTAACAGATCGAAATTTAAA
TACTTCTTTCTTTGATCCTGCAGGAGGGGGAGACCCAATTCTTTATCAACATTTATTT

>Morphotype15 - *Colliuris pilatei*

AACATTATATTTTATTTTTGGTGCATGAGCAGGAATAGTAGGAACTTCTCTTAGTATATTAATTCGAGCTGAATT
AGGGAATCCTGGAGCATTAATTGGAGATGATCAAATTTATAATGTTATTGTAACTGCACATGCTTTTATTATAAT
TTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATACTAGGAGCTCCTGA
TATAGCATTTCCTCGAATAAATAATATAAGTTTTTGATTATTACCTCCTTCTCTTACTTTGCTCCTTGTTAGTAG
AGTAGTTGAAAGAGGTGCTGGTACAGGATGAACAGTATACCCTCCCCTTTCTTCAGGAATTGCYCATGCAGGAGC
ATCAGTAGATTTAGCAATTTTTAGATTACATTTAGCAGGAGTTTCATCAATTTTAGGAGCAGTAAATTTTATTAC
CACTATTATTAATATACGATCTGTTGGAATATCATTTGACCGTATACCTTTATTTGTTTGATCTGTAGGAATTAC
TGCTCTACTTTTACTTTTATCATTACCTGTATTAGCTGGAGCAATTACAATACTTTTAACAGATCGAAATTTAAA
TACTTCCTTTTTTGATCCTGCAGGAGGGGGAGACCCAATTCTTTATCAACATTTATTT

>Morphotype16 - *Cymindis* sp1

AACTTTATACTTTATTTTTGGTGCATGAGCAGGAATAGTAGGGACTTCTTTAAGTATACTAATTCGAGCAGAATT
AGGGAATCCTGGTTCATTAATTGGTGACGATCAAATTTATAATGTTATTGTTACTGCTCATGCTTTTATTATAAT
TTTCTTTATAGTTATACCTATTATAATTGGAGGTTTTGGAAATTGACTTGTACCTTTAATATTAGGAGCCCCTGA
TATAGCCTTTCCTCGAATAAATAATATAAGTTTTTGACTTCTACCCCCTTCTTTAACCCTTCTTTTAATGAGAAG
AATAGTTGAAAGAGGAGCAGGGACAGGATGAACAGTTTACCCTCCCTTATCTTCAGGAATTGCTCATAGAGGAGC
TTCAGTAGATTTAGCAATTTTTAGATTACATCTAGCAGGAGTTTCATCAATTTTAGGAGCAGTAAATTTTATTAC
AACAATTATTAATATACGATCAGTAGGAATAACATTTGATCGAATACCTTTATTTGTATGATCCGTAGGAATTAC
TGCTTTATTACTTCTTTTATCACTACCAGTATTAGCAGGAGCTATTACAATATTATTAACAGACCGAAATTTAAA
TACTTCTTTTTTTGATCCAGCAGGAGGGGGAGATCCTATTCTATATCAACATTTATTT

>Morphotype17 - *Helluomorphoides* sp1

AACATTATACTTTATTTTCGGAATCTGAGCTGGTATAGTAGGAACTTCCTTAAGAGTCTTAATTCGAGCAGAATT
AGGAACTCCAGGAGCATTAATTGGAGACGATCAAATTTTTAATGTAGTAGTTACAGCTCATGCATTCATTATAAT
TTTTTTTATAGTAATACCTATTATAATTGGAGGTTTTGGAAATTGATTAGTTCCTTTAATGCTAGGTGCACCTGA
TATAGCCTTTCCCCGAATAAATAATATAAGATTTTGACTTCTCCCYCCATCTTTAAGCCTTTTACTAATAAGTAG
ACTTGTCGAAAARGGTGCAGGTACAGGATGAACTGTTTACCCTCCTTTATCAARTGCTATTGCCCACAGAGGAGC
TTCAGTAGACCTAGCAATTTTTAGWTTACATCTAGCAGGAATTTCATCTATTTTAGGAGCTGTAAATTTTATYAC
AACAATAATTAATATACGACCAGCAGGAATAACTATTCTCCAAATACCCCTATTTGTTTGATCAGTAGGAATTAC
TGCTCTTTTACTTCTTCTTTCTTTACCAGTATTAGCAGGAGCTATTACTATATTATTAACAGACCGAAATTTAAA
TACTTCATTTTTTGATCCAGCAGGAGGAGGAG

>Morphotype18 - *Calosoma alternans*

AACTTTATATTTTATTTTTGGTGCTTGATCAGGAATAGTGGGAACTTCATTAAGAATACTAATTCGAGCTGAATT
AGGAAACCCTGGCTCTTTAATTGGAGATGATCAAATTTACAATGTTATTGTAACTGCTCATGCTTTTGTAATAAT
TTTCTTTATAGTAATACCTATTATAATCGGGGGATTTGGAAATTGATTAGTCCCACTAATATTAGGAGCCCCTGA
TATGGCCTTTCCACGAATAAATAATATGAGTTTTTGACTTCTTCCTCCTTCTTTGACCCTCCTATTGATGAGCAG
TATGGTTGAAAGAGGGGCAGGTACAGGATGAACAGTATACCCCCCTCTCTCTTCAGTTATTGCCCACAGCGGGGC
TTCTGTTGATTTAGCAATTTTTAGTTTACATTTAGCCGGGATTTCTTCTATTTTAGGTGCAGTAAATTTTATTAC
AACAATTATTAATATACGATCAGTGGGAATAACATTCGATCGAATACCATTATTTGTATGATCAGTAGGAATTAC
AGCGTTATTGCTCTTATTATCACTACCAGTACTAGCCGGAGCTATCACAATACTATTAACTGACCGAAACTTAAA
CACATCATTTTTTGACCCTGCAGGAGGGGGAGACCCTATTTTATACCAACATCTTTTC

>Morphotype19 - *Galerita brasiliensis*

AACATTGTATTTTATTTTTGGTGTGTGAGCCGGGATAGTAGGGACTTCCTTGAGTGTACTAATTCGAGCAGAATT
AGGGACTCCAGGTGCATTAATTGGAGACGATCAGATTTTTAATGTTGTAGTTACTGCCCATGCTTTTATTATAAT
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