The Community Responses of Orthoptera to Tropical Forest Degradation with Applications of DNA Barcoding for Food Web Analysis

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The Community Responses of Orthoptera to Tropical Forest Degradation with Applications of DNA Barcoding for Food Web Analysis

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Synopsis

As plants and insect herbivores represent major components of eukaryote biodiversity, they form the basis of virtually all terrestrial food webs. Despite this, host plants, dietary breadth and species interactions of many insects are unknown or poorly understood. Identifying and quantifying these complex interactions among insects and their hosts is challenging but is fundamental to our understanding of ecosystem structure and function. DNA barcoding is becoming an accessible tool for ecologists and has the potential to revolutionise our understanding of food webs. There is an urgent need to address species interactions, particularly with the rapid habitat degradation occurring in some of the most biodiverse habitats on Earth.

This thesis investigates the ecology of a large and relatively under-studied insect Order, the Orthoptera. The challenge of studying the interactions of these insects and their host plants in natural environments is addressed by examining the use of DNA barcoding and metabarcoding. Orthoptera were sampled in a range of habitat types in sub-tropical southeast Queensland, Australia, and tropical Sabah, Malaysian Borneo, so that community and dietary analyses could be carried out. I addressed the following aims: 1) to compare the utility of simple DNA barcoding and metabarcoding for herbivorous insect food web analysis, 2) to assess dietary breadth and overlap among different orthopteran species sharing the same habitat type, in an Australian subtropical eucalypt woodland, 3) to describe the effects on the composition of orthopteran assemblages of landscape-level habitat change (logging of primary forests and conversion to oil palm (*Elaeis guineensis*) plantations) in the southeast Asian tropics, 4) to compare the diet of one group of orthopterans (the Tetrigidae) across different habitat components within anthropogenically modified Malaysian tropical rainforest.

DNA barcoding using Sanger sequencing and DNA metabarcoding using an Illumina Miseq were trialled to determine how appropriate they were for the use of gut content analysis using the orthopteran specimens collected from southeast Queensland. The use of two plant gene markers was also assessed. From these results, the diets of two abundant species *Merrinella* sp. and *Methiolopsis* sp., occupying the same habitat, have been described and their dietary overlap measured. Orthoptera were also collected from primary rainforest, logged forest and oil palm plantations in Sabah, Malaysia, in order to compare the assemblages across the landscape. Additionally, dietary analysis using DNA metabarcoding was carried out on one family, Tetrigidae, to assess dietary differences with anthropogenic habitat change.

My research presents detailed methodology for the use of two DNA barcoding methods for measuring herbivorous insect diets. Our results found that although the Sanger sequencing method is useful for single species (unmixed) samples, the metabarcoding approach is more appropriate when sampling species with unknown dietary breadths. There remain limitations within this field in terms of accuracy of taxonomic identifications, but nevertheless, DNA metabarcoding is a useful method that is changing our understanding of food web ecology.

Using DNA metabarcoding, we confirmed that dietary overlap was low across *Methiolopsis* sp. and *Merrinella* sp. but also that they both had very broad diets. These results suggest that competition for resources between these two species is low. This could change, however, if plant availability is altered in response to environmental change.

Orthoptera assemblages were affected by habitat degradation (through logging) and the conversion of forest to oil palm across a landscape in Sabah, Malaysia. The greatest difference in species composition was between plantations and primary forests. Logged forests had the lowest species richness of the three habitats. The assemblage of species in

palm plantations was clearly different from those elsewhere. These findings support other studies on different arthropod taxa and conclude that the plantation habitats are unsuitable for many forest-dwelling species. Additionally, diets of the Tetrigidae differed across these habitat types, with species in plantations feeding on a much higher proportion of mosses than those in forests.

Overall, this thesis describes and tests molecular methodology that can be used in future food web studies to measure dietary composition, breadth and overlap of insects. I show how habitat degradation can impact orthopteran community composition and highlight that Tetrigidae are an important family in tropical habitats. My research has addressed a gap in our understanding of the community and feeding ecology of Orthoptera and can be applied to other taxa to monitor responses of biodiversity in a changing world.

Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Jane Louise Hardwick

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Chapters 3, 4, 5 and 6 in this thesis are co-authored manuscripts. My contribution, and each co-authors contribution to each of these chapters are outlined at the beginning of each chapter. Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the acknowledgments section at the end of each chapter.

The bibliographic details for these papers are:

Chapter 3: Hardwick, J. L.[1], Shapcott, A.[2], Maunsell, S. C.[1,3], Stork, N. E.[1], & Kitching, R. L.[1] (prepared manuscript). DNA barcoding for ecology: a comparative study on the use of Sanger sequencing and metabarcoding for herbivore dietary analysis.

Chapter 4: Hardwick, J. L.[1], Shapcott, A.[2], Maunsell, S. C.[1,3], Stork, N. E.[1], & Kitching, R. L.[1] (prepared manuscript). What's eating what? A diet assessment of Orthoptera using rbcL and trnH-psbA.

Chapter 5: Hardwick, J. L.[1], Maunsell, S. C.[1,2], Stork, N. E.[1], Yusah, K. M.[3], & Kitching, R. L.[1] (prepared manuscript) Assemblages of Orthoptera and forest conversion in Borneo.

Chapter 6: Hardwick, J. L.[1], Shapcott, A.[2], Maunsell, S. C.[1,3], Stork, N. E.[1], Yusah, K. M., & Kitching, R. L.[1] An insight into the diets of Tetrigidae (Orthoptera) across an altered landscape in Sabah, Malaysia, using DNA metabarcoding.

(Signed) _____ (Date) 16.05.18

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Primary Supervisor: Professor Roger Kitching

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Chapter 1: Introduction, aims, and thesis outline

1.1 Key thesis topics

Food webs, in simple terms, are maps that describe which kinds of organisms in a community eat which other kinds (Pimm & Cohen 1991) and are the defining objects of community ecology (Kitching 2004). Understanding a food web can help address how a community works. Interactions among insect herbivores and their host plants dominate terrestrial ecology on Earth (Borror, Triplehorn & Johnson 2004) and it is estimated that the survival of more than one million phytophagous insect species depends on plants as food resources (Jander & Howe 2008). The diversity of herbivorous insects is greatest within sub-tropical and tropical rainforests (Lamarre et al. 2016), but many species remain undescribed and the specifics of their interactions are unknown. Gaining a better understanding of species and their interactions is key to understanding ecosystem dynamics. Before the recent introduction of molecular technologies, information on the feeding interactions of arthropods has been uncertain and often difficult to obtain.

DNA barcoding has the potential to revolutionise food web ecology. It is already changing the way we understand food webs and is leading to the discovery of previously unknown interactions (Roslin & Majaneva 2016). The application of DNA barcoding to food webs is not without its limitations. A major challenge is the analysis of highly degraded gut content material. Earlier studies examined a range of, principally predatory, arthropod taxa (Juen & Traugott 2006; Symondson, Erickson & Liddell 1997). More recently the methodology has been extended to insect herbivores (Garcia-Robledo et al. 2013; Kajtoch 2014). In general, these methods have been deemed successful, particularly if certain technical criteria are met (see Chapters 3 and 4). The use of DNA barcoding can increase our understanding of the

function of herbivorous insects in terms of species' dietary breadth and composition and, in consequence, how feeding ecology may be affected by habitat change.

In the tropics, habitat change at the landscape scale is occurring through both timber extraction and plantation establishment. In south-east Asia this latter transformation is largely due to the establishment of often vast, oil palm (*Elaeis guineensis*) plantings. These changes may well be detrimental to biodiversity (Gibson et al. 2011). Very little is documented, however, on the effects of habitat change on insect communities (Turner, EC et al. 2008). As the most dominant and, arguably, the most important, metazoan components of terrestrial food webs, a better appreciation of how insects are impacted by habitat change is essential for our broader understanding of ecosystem functioning in anthropogenic landscapes. Orthoptera, in particular, are a large insect Order (including >24,000 described and many more undescribed species). This includes abundant herbivores, detritivores and omnivores across almost all habitat types on Earth (Rentz 1996). Although studies have investigated species dynamics in grassland and some agricultural landscapes (principally, to assess the role of Orthoptera as pests), very few have assessed their role in natural environments such as tropical rainforests. The very few studies that do exist have been of Neotropical (Amedegnato 2003) and Papuasian (Novotny, V. et al. 2002; Novotny, V. et al. 2010), systems.

1.2 Aims

The work in this thesis centres on the insect Order Orthoptera, with a primary focus on phytophagous species that consume either living or decomposing plant material. I address the challenges of studying the interactions of insects and their host plants in natural environments and investigate the use of DNA barcoding and metabarcoding as effective tools in trophic ecology. I have sampled Orthoptera from sub-tropical habitats in southeast Queensland,

Australia, and tropical habitats in Sabah, Malaysia. Community and dietary analyses have been carried out to fulfil my general research aims.

Overall, I have worked to gain a greater understanding of the ecology of Orthoptera and of the dietary differences within Orthoptera across habitats and taxa, within subtropical and tropical forests.

The following four specific topics are addressed.

1. I compare the utility of simple DNA barcoding and metabarcoding for insect herbivore food web analysis (Chapter 3).

2. I assess dietary breadth and overlap among different orthopteran species sharing the same habitat type, in an open eucalypt woodland in southeast Queensland, Australia (Chapter 4).

3. I describe the effects on the composition of orthopteran assemblages of landscape-level habitat change (logging of primary forests and conversion to oil palm plantations) in the southeast Asian tropics (Chapter 5).

4. I compare the diet of one group of orthopterans (the Tetrigidae) across different habitat components within anthropogenically modified Malaysian tropical rainforest (Chapter 6).

1.3 Thesis structure

To address the aims listed above, this thesis is organised as a series of chapters each prepared as a manuscript for submission to a journal (except for this Chapter, Chapter 2 and Chapter 7).

Chapter 2 is a literature review entitled '*Orthoptera in degraded habitats and the use of DNA barcoding for food web ecology'*. This chapter reviews how habitat change affects insects with a focus on Orthoptera. It specifically addresses the impacts of the oil palm industry on different taxa and why monitoring change is so important. Chapter 2 also introduces the use of DNA barcoding in ecological studies.

Chapter 3 is a methodological research paper, prepared for publication entitled *DNA barcoding for ecology: a comparative study on the use of Sanger sequencing and metabarcoding for herbivore dietary analysis*. This study compares two common DNA sequencing methods and gene markers for the diet analysis of insect herbivores.

Chapter 4 is a research paper, prepared for publication entitled *What's eating what? A diet assessment of Orthoptera using* rbcL *and* trnH-psbA. This study uses the same data set and methodology from Chapters 3 but includes a detailed assessment of the use of two different gene markers (rbcL and trnH-psbA) and discusses detailed diets of two genera of Orthoptera.

Chapter 5 is a research paper, prepared for publication entitled *Assemblages of Orthoptera and forest conversion in Borneo.* This study assesses the community composition of Orthoptera across primary forest, logged forest and oil palm plantations in Sabah, Malaysia, where land-use change is occurring at a rapid rate.

Chapter 6 is a research paper, prepared for publication, entitled *An insight into the diets of Tetrigidae (Orthoptera) across an altered landscape in Sabah, Malaysia, using DNA metabarcoding*. This chapter uses methods described in Chapters 1 and 2 to investigate the diets of Tetrigidae, a family of Orthoptera, along the gradient of habitats described in Chapter 5, in Sabah, Malaysia.

Chapter 7 is the final chapter entitled *General discussion and conclusions*. This chapter concludes the thesis by discussing the wider implications of the study.

Relevant 'Supplementary Material' is provided following each chapter where necessary. To avoid duplication, a combined reference list is provided at the end of the thesis.

Chapter 2: Literature review: Orthoptera in degraded habitats and the use of DNA barcoding for food web ecology

2.1 Orthopteran ecology

The Orthoptera are a large Order of exopterygote insects characterised by their generally unspecialised biting mouthparts and fan-like hindwing structure. They include many herbivorous species of great economic importance (Grimaldi & Engel 2005; Rentz 1991). The ~ 25,000 described Orthoptera species worldwide show considerable variation in biology, abundance, population variability and geographic range (Bidau 2014).

Within Insecta, Orthoptera are grouped with the early divergent and hemimetabolous insects, in the subdivision Polyneoptera along with 10 other higher taxa. These include Mantodea (mantids), Phasmatodea (stick insects), Dermaptera (earwigs), Grylloblattodea (rock crawlers), Zoraptera (zorapterans), Plecoptera (stoneflies), Blattodea (cockroaches), Isoptera (termites), Embioptera (webspinners), and the recently discovered insect Order Mantophasmatodea (heelwalkers) (Song, N et al. 2016). There are two main evolutionary branches in the Order Orthoptera which separate the two Suborders Caelifera (grasshoppers) and Ensifera (crickets and katydids) (Figure 2.1). There is evidence that both of the Suborders are monophyletic (Song, H et al. 2015). The three most diverse families are: Acrididae (short-horned grasshoppers) which correspond with grassland radiation, Tettigoniidae (katydids) which diversified around the same time as flowering plants, and Gryllidae (bush crickets) which diverged throughout the Triassic and Jurassic periods (~250 – 145 mya) (Song, H et al. 2015) (Figure 2.1).

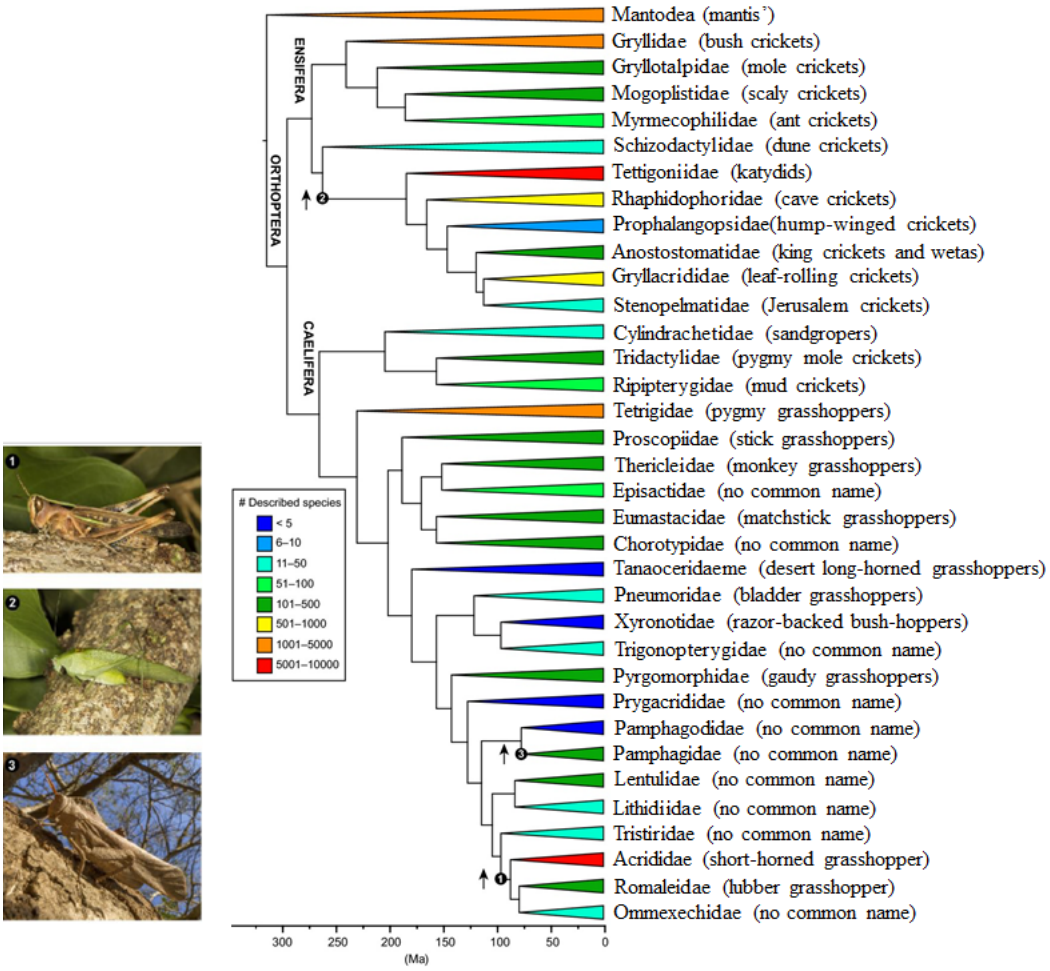

Figure 2.1: Adapted from Song et al. (2015). Diversification patterns of major lineages of Orthoptera as deduced from MEDUSA analysis by Song et al. (2015). Each terminal represents a monophyletic family. Terminals are colour coded to show the species diversity. The up arrows indicate an increase in diversification rates. The images on the left show the representatives of the clades that experienced the shifts in diversification rates. Mantodea shown at the top of the tree is an outgroup.

Most species within this diverse order are herbivores, but there are also many species of omnivores and even scavengers and predators, particularly within the katydids (Tettigoniidae) (Rentz 1996). Most studies of Orthoptera have focused on their importance within grassland habitats, where they are often among the most conspicuous and dominant arthropods in terms of both abundance and diversity (Gardiner, Hill & Chesmore 2005; More

& Nikam 2016). In addition, orthopterans (mainly 'locusts' from the family Acrididae) are well documented as agricultural pests (Barbosa, Letourneau & Agrawai 2012; Samways & Lockwood 1998; Schell & Lockwood 1997; Usmani, Nayeem & Akhtar 2012). Their ecology in forest ecosystems remains largely unknown and poorly investigated. In the few studies of Orthoptera in tropical rainforests, it is known that differences in grasshopper density and abundance, but not species richness, are influenced by microhabitats (Amedegnato 2003). Orthoptera have been considered as one of the major groups of herbivorous insects, alongside Coleoptera (beetles) and Lepidoptera (butterflies and moths) (Novotny, V. et al. 2002; Novotny, V. et al. 2010). In comparison with these other groups they have been found to have the least specialised diets, with no monophages or plant genus specialists among >100 Orthoptera collected from a New Guinea rainforest. In contrast, numerous species of short-horned grasshoppers (Acrididae) with specialised diets have been reported from a Costa Rican rainforest (Marquis & Braker 1993). This has also been reported as a general finding – that species of grasshopper from the family Acrididae tend to have more specialised diets than species of crickets within the Suborder Ensifera (Rentz 1996). Dietary breadth from these studies, however, was measured experimentally after collecting and rearing specimens and offering them a number of different food types, and therefore may not necessarily reflect natural preferences.

Many species of Orthoptera have been identified as being ecologically important for a number of reasons. They may be keystone species structuring food webs based on their considerable roles as herbivores on the one hand, and readily available prey items for larger animals (Ingrisch & Kohler 1998). From the point of view of consumer species, they are often the most readily available and abundant invertebrates in grassland communities (Curry 1994). They have been classified as the primary prey species for vertebrates, such as the red-backed shrike (*Lanius collurio*) (Morelli et al. 2015). A species of tettigoniid may contain

noxious chemicals which the slow loris (*Nycticebus javanicus*) sequesters and which may contribute to the production of the primate's venom (Rode-Margona et al. 2015). Orthoptera are also important regulators of ecosystem processes. Mole crickets (Gryllotalpidae), for example, regulate and reduce run-off by altering soil infiltration within the soil/leaf litter (Bailey et al. 2015). They are ecological indicators of succession in grassland communities (Fartmann et al. 2012) and have been used as indicators of ecosystem health in deciduous forests (Joshi et al. 1999).

Orthopteran insects are often recognised by their ability to produce sound and this has received some interest in terms of behavioural (Helversen & Helversen 1994) and physiological (Bennet-Clark 1989) research. It has also been suggested that acoustic monitoring of the 'singing orthopterans' can provide specific information which can be used for inventorying and monitoring both individual species and communities (Lehmann et al. 2014).

Habitat degradation and land-use change for agriculture and urbanisation are threats to orthopteran diversity. In grassland communities, the genetic diversity of short-horned grasshoppers (Acrididae) was reduced due to fragmentation (Ortego et al. 2015). Anthropogenic disturbances reduced total abundance, species richness and diversity of orthopterans in dry (Saha, Sarkar & Haldar 2011) and moist (Joshi et al. 1999) deciduous forest in India and urbanisation in France was found to have a negative effect on total species richness, abundance and community specialisation (Penone et al. 2012).

In tropical environments, animal communities may be particularly sensitive to habitat change due to many species showing a more localised occurrence than in temperate areas (Riede 1998). Different taxa, however, react differently to disturbances, with some adapting better than others and therefore it is important to assess as wide a range of taxa as possible (Lawton

et al. 1998). The responses of Orthoptera to tropical habitat degradation and land-use change from primary forest to oil palm plantations in the tropics remains, hitherto, unexplored.

2.2 Oil palm agriculture and deforestation in Malaysia

The preservation of tropical forests is of extreme importance for conserving biodiversity; globally they contain more than two thirds of all terrestrial flora and fauna (Gardner et al. 2009). Degradation and loss of these habitats are arguably the greatest threats to biodiversity today (Fahrig 2003; Laurance & Bierregaard 1997; Travis 2003; Wilcove, DS et al. 1998; Wilcove, DSM, C. H. & Dobson 1986). From 2000 to 2010 the average net loss of forest reached approximately 5.2 million hectares per annum and the trajectory of global deforestation has more or less followed the rate of human population growth (FAO 2010b). Before this, from 1981 to 1990, 154 million hectares of tropical forests alone had been lost (Whitmore 1997). As tropical habitats contain very high levels of biodiversity, there are serious concerns about landscape modification within these regions (Basset, Charles, et al. 2001). Collectively, Malaysia and Indonesia produce over 80% of the world's palm oil (*Elaeis guineensis*) (Lam, Lee & Mohamed 2009) and it is also these regions that contain some of the planet's greatest levels of endemism and biodiversity (Koh & Wilcove 2007). There are two 'biodiversity hotspots' within the Malay Archipelago. First, Sundaland, covers the western half of the Indo-Malay Archipelago and includes the large islands of Borneo and Sumatra. Second, the Islands of Wallacea lie between Sundaland to the west and Near Oceania, and include the islands of Sulawesi, the Moluccas and the Lesser Sundas to the east (McGinley 2010). Meta-analyses of geological, climatic and biological data sets from within these regions indicate that Borneo and Indochina are major evolutionary hotspots for biodiversity, dating back to the Miocene (de Bruyn et al. 2014). Species in these regions are now facing serious threats with the rapid expansion of oil palm cultivation (Donald 2004; Koh 2008; Koh & Wilcove 2007). Less than 10% of Malaysian tropical forest is protected by

the Malaysian Government (Iremongerm, Ravilious & Quinton 1997) and outside of the conserved areas most of the remaining forests are within logging concessions (Sheil & Meijaard 2008). In tropical dipterocarp forests in Borneo, damage to residual stands by logging operations is known to affect up to 50% of remaining trees (Bertault & Sist 1997). Once already logged, there is then strong pressure for conversion of the land to oil palm plantations or other uses (Wilcove, DS & Koh 2010).

Currently, there is an ever-increasing demand for palm oil as it is the highest yielding oil crop. In a recent report, palm oil remains the lowest priced oil and many countries are raising their import quantities (FAO 2014). For example, India has recently increased its palm oil imports to 9.9 million tonnes per year (USDA 2015). With this high demand, it is the world's most traded oil (Carter et al. 2007; FAO 2014). As well as being very efficient to produce, the palm oil industry provides direct employment in Malaysia to almost 500,000 people (USDA 2015) and the oil itself has multiple purposes, ranging from use in food products, cosmetics, lubricants and as a source of biofuel (Tan, KT et al. 2009).

Despite being a highly efficient crop, there are many unfavourable realities about the palm oil industry. The crop requires a tropical environment with high rainfall, and as a result now occupies more than 13.5 million hectares of land in the world's most biodiverse terrestrial biome (Fitzherbert et al. 2008). Palm oil production is strongly contested as one of the greatest drivers towards loss of biodiversity in tropical regions as it results in the loss of lowland forest (Koh 2008; Lucey & Hill 2011; Sodhi et al. 2006; Sodhi et al. 2004) through the total clearance of vegetation before planting (MacKinnon et al. 1996).

In response to global concerns, the Roundtable on Sustainable Palm Oil (RSPO), a not-for-profit organisation, was established in 2004 to promote the growth and use of sustainable palm oil. The RSPO set a list of principles and criteria to ensure that palm oil production is

environmentally and economically viable and encourages oil producers, traders and retailers to join as members (Roundtable on Sustainable Palm Oil 2018).

Research on the impacts of the various stages of forest conversion to oil palm plantation are also being monitored by a large-scale ecological experiment called the Stability of Altered Forest Ecosystems (SAFE) project, located in the lowland tropical forests of Borneo (Sabah, Malaysia). This project was developed as an opportunity for ecologists of many specialities to generate a broad understanding of the effects of landscape-scale habitat change on ecological patterns and processes (Ewers et al. 2011). The area in which the project operates is gradually being converted from salvage-logged forestry estate to oil palm plantations and The Sabah Foundation, in collaboration with its subsidiary company, Benta Wawasan, has allowed an opportunity for scientists to collect data throughout the process (Ewers et al. 2011). This is among the world's largest controlled ecological manipulations, and, although it will not prevent the land-use change within this area, it does present a unique opportunity for the ecological changes with habitat degradation to be examined in greater detail than ever before (Ewers et al. 2011).

2.3 Insect biodiversity and habitat degradation

Arthropods constitute the largest component of animal eukaryote biomass and species in tropical habitats. Past estimates for global species richness of arthropods have been as high as 30- 100 million species (Erwin 1982, 1988) but more reliable techniques have suggested this is a huge over-estimation (Hamilton et al. 2010; Stork 2018). The most recent estimates suggest that there are between 5.9-7.4 million species (Stork et al. 2015). A strong latitudinal gradient is clear, with most arthropod groups increasing in number of species towards the equator (Hamilton et al. 2010). Due to the sheer diversity of arthropods in tropical habitats, there remains much to be discovered about most taxa.

Within the Insecta, the roles of species in ecosystems vary but, as a whole, they are essential for pollination, regulating nutrients and transfer of energy from plant materials, and decomposition (Janzen 1987). Insects link components within complex food webs and are an important food source for many other organisms including other arthropods and vertebrates. They are arguably the dominant elements within the ecosystems they occupy (Janzen 1987). The impact of land-use change on arthropods is far from straightforward (Turner, EC & Foster 2009) and the effects of logging and converting primary forest to oil palm on invertebrate groups are poorly understood. Selective logging is the most common approach for commercial timber production in southeast Asia and as it modifies the structure of the forests, its impacts on regeneration dynamics and community composition are large (Kitching et al. 2013; Okuda et al. 2003). Logging causes a reduced abundance of key functional arthropod groups within tropical forests and this leads to a decreased contribution towards essential ecosystem processes. Although some ecosystem processes are relatively resilient, increasing anthropogenic pressures are likely to leave logged ecosystems vulnerable to future change (Ewers et al. 2011).

Based on published literature, ants are the most thoroughly studied group in terms of the effects of forest conversion to oil palm on arthropods (Brühl & Eltz 2010; Lucey & Hill 2011; Pfeiffer, Tuck & Lay 2008; Wang, WY & Foster 2015). In Malaysia, species richness of ground ants was severely reduced in plantations when compared with primary forest (Brühl & Eltz 2010; Fayle et al. 2010). Community composition was also severely altered with as little as 5% of ground-dwelling ant species from the forest interior surviving in oil palm plantations (Brühl & Eltz 2010). Species richness of ants occupying the canopy is also lower in oil palm plantations compared with old growth forest, and non-native species are more abundant in the canopy of plantations where few of the natural forest species survive (Fayle et al. 2010). Other invertebrate research in Malaysia has indicated significant changes

in response to conversion of forest to oil palm to moth (Chey 2006; Chey, Holloway & Speight 1997), butterfly (Koh 2008; Lucey & Hill 2011; Tscharntke et al. 2002), beetle (Chung et al. 2000; Gray et al. 2014), aquatic Heteroptera (Cunha, Montag & Juen 2015) and bee (Liow, Sodhi & Elmqvist 2001) communities.

Research on vertebrates has also suggested negative impacts, for example, for birds, significant declines in species richness have been identified within oil palm plantations when compared with primary forest, logged or fragmented forests in many different biogeographic regions. These include southern Thailand (primary forest comparison, 60% reduction; Aratrakorn, Thunhikorn & Donald (2006), peninsular Malaysia (logged forest comparison, 48-60% reduction; Azhar et al. (2011), Guatemala (fragmented forest comparison, 82.5% reduction; Cajas-Castillo et al. (2015) and the Amazon (primary forest comparison, 67.5% reduction; Lees et al. (2015)). For mammals, there has been a focus on orang-utans (*Pongo* spp.) as oil palm plantations have been suggested to be hastening their extinction (Nantha & Tisdell 2009). Predictive models have shown that orang-utans could lose up to 74% of suitable refuge habitats by 2080, as a result of the combination of land-use and climate change, indicating that many other species that occupy similar niches are also at risk (Struebig et al. 2015). A lower diversity of terrestrial mammals has also been recorded within plantations in central Sumatra, Indonesia, when compared with protected areas, confirming that oil palm plantations are poor habitats for wildlife (Jennings et al. 2015).

To gain a better understanding of the impacts of logging and oil palm agriculture on biodiversity, there is an urgent need for more studies to be conducted of different taxonomic groups as these are likely to be affected differentially in terms of changes to community structures and ecological functions (Koh 2008; Turner, EC et al. 2008).

2.4 Food webs and herbivore ecology

Food webs are a complex and fundamental component of both theoretical and applied ecology (Post 2002). Plant-herbivore food webs comprise at least 40% of global terrestrial biodiversity and these are of considerable importance to our understanding of tropical ecology (Novotny, V. et al. 2010). Many plant species have evolved chemical defences against herbivores, and in turn, herbivores have developed strategies to overcome plant defences (Lowman & Rinker 2004; Mello & Silva-Filho 2002). This theory of 'coevolution', first proposed by Ehrlich and Raven (1964), has been difficult to confirm unequivocally (Suchan & Alvarez 2015), but many believe it is central to driving patterns of species diversification and radiation. Accordingly, understanding herbivore feeding preferences across communities is critical for understanding speciation, plant metabolites as species-specific antifeedants, host plant resistance, host plant selection and resource demands within herbivores across different habitats (Duffy et al. 2007; Jurado-Rivera et al. 2009).

An assumption commonly made is that insects that feed in similar ways should exhibit similar ecologies and responses to change (Root 1973). Separating insect herbivores into feeding guilds, leads to some generalities. Sap-sucking insects, for example, may be less vulnerable to chemical defences used by plants than leaf-chewing insects (Hunter 1997). The proposition that guilds can be applied in this general manner, however, is a debated topic, particularly by taxonomists. Classical niche theory suggests that assemblages of herbivores on a particular plant species will be dominated by species from host-specific guilds with narrow niches (Novotny, V. et al. 2010). In tropical forests, however, the opposite has been observed where assemblages on a particular plant species were dominated by insects from the least specialised mobile leaf-chewing guild, rather than the highly specialised leaf miners and mesophyll suckers (Novotny, V. et al. 2012). Orthoptera are leaf-chewers, together with Phasmatodea (stick insects), Lepidoptera and most phytophagous Coleoptera (beetles). This

guild is generally considered to be a generalist group and is used in comparisons against highly specialised guilds such as leaf-miners (Novotny, V. et al. 2010). Orthoptera, however, vary greatly in their degree of specialisation (Joern, A. 1983), and little is known about the diet breadth and feeding behaviours of tropical species.

In grasslands, studies have compared acridid species as a way of testing theories on co-existence and niche overlap of herbivorous species (Behmer & Joern 2008; Ueckert & Hansen 1971). A principal postulate is that coexisting species use different resources and therefore can overlap spatially without competition (Chesson 2000). The combination of top-down (e.g. pressures from predators) and bottom-up (e.g. availability of food resources) forces, however, are also strong driving factors leading to herbivore coexistence (Owen-Smith 2015). Active partitioning of macronutrients has been recorded in coexisting acridids, placing them in clearly defined species-specific nutritional niches which could moderate the effects of interspecific competition (Behmer & Joern 2008). Other studies have found that as food niche dimensions (e.g. amount of plant resource) increase, dietary overlap decreases (Ueckert & Hansen 1971), supporting a theory that the number of competing species which can coexist is proportional to the total range of the environment divided by the niche breadth of the species (Macarthur & Levins 1967).

Although this relationship has not yet been thoroughly explored across a range of taxa, the theory of niche evolution predicts that more specialised species are more likely to be negatively affected by habitat loss than generalists, as they may be unable to adapt rapidly to a changing environment (Zabel & Tscharntke 1998). This has been confirmed for butterflies: monophagous species of butterflies in agricultural landscapes in Germany were more sensitive to landscape fragmentation than less specialised oligotrophic and polytrophic species and both species richness and abundance of the specialist species were significantly reduced in small fragments (Tscharntke et al. 2002). An investigation on a wider range of

known generalist and specialist insects (from the families Curculionidae (beetle) and Cicadellidae (leafhopper)) in agricultural landscapes in Canada has concluded that the species abundance and richness of generalists increased with increasing landscape heterogeneity. Specialists, however, were not significantly affected by habitat fragmentation. It was suggested that the distance between isolated patches could have been within dispersal distances for the specialist insects sampled or that host plants in road verges may act as suitable refugia and feeding grounds (Jonsen & Fahrig 1997).

These sometimes conflicting results about the effects of habitat degradation on specialist and generalist herbivores remains one of the key reasons that it is important to study feeding interactions across different taxa.

Although the concept of trophic levels has been useful for food web analysis, there are still difficulties when it comes to measuring the degree of specialisation and generalisation for individual species (Jonsen & Fahrig 1997; Novotny, V. et al. 2012; Tscharntke et al. 2002), particularly in challenging ecosystems such as tropical rainforests. Conventional methods of diet analysis have relied on direct observations of feeding behaviour (which are often difficult or impossible to carry out and very time-consuming) and microscopic or carbon isotope analysis of either gut contents or faecal matter (Tieszen et al. 1983; Valentini et al. 2009). Recent developments in DNA sequencing, however, have allowed for accurate identification of host plants and prey species from digested and degraded materials (Jurado-Rivera et al. 2009). With a large enough sample size, this approach can be used to classify species as herbivores, carnivores or omnivores and as specialists or generalists by identifying the number and type of different species of plant or animal in the gut of the consumer.

2.5 DNA Methods

DNA barcoding has been described as "*a novel system designed to provide rapid, accurate and automatable species identifications by using short standardized gene regions as internal species tags*" (Hebert & Gregory 2005). It relies on short, highly variable regions of the genome and can be applied to plants, animals and fungi. Even from degraded specimens, mitochondrial (in the case of animals) and chloroplast (in the case of plants) sequences can be amplified by polymerase chain reaction (PCR). Standardised sequencing of the cytochrome oxidase 1 (CO1) gene has made barcoding for animal taxonomy very efficient and previous research has utilised this approach to analyse predator diets from faeces with considerable success (Deagle, Kirkwood & Jarman 2009; Jarman, Deagle & Gales 2004; Symondson 2002). Animal matter can be identified by targeting mitochondrial DNA for which there is an extensive reference database in GenBank and Barcode of Life Data Systems (BOLD). Using this approach for plant identification, however, is not so straightforward (Bradley et al. 2007) as plants have low substitution rates for mitochondrial DNA (Hebert et al. 2003). As an alternative, a number of different regions can be used to sequence plant DNA including the trnL intron, MatK and rbcL, the non-coding spacer trnH-psbA and the nuclear ITS region. The ideal barcode should be retrievable routinely with a single primer pair, be amenable to bidirectional sequencing and provide maximal discrimination among species. Three commonly used loci that best fit these criteria are trnH-psbA, rbcL and matK (Hollingsworth, PM, Forrest, et al. 2009). None of these alone, however, can discriminate to the species-level with the same success as the CO1 gene in animals and, accordingly, a multi-locus approach has been developed (Hebert et al. 2003; Kress, WJ & Erickson 2007).

When there is only one type of prey or plant species in the gut of an animal, conventional Sanger sequencing can be employed and is often successful (Avanesyan 2014; Jurado-Rivera

et al. 2009; Navarro et al. 2010; Valentini et al. 2009). For generalist species, that are likely to have multiple species in the gut, the PCR product requires cloning before Sanger sequencing, which can be impractical when there are many samples. Metabarcoding uses next-generation sequencing (NGS) technology which enables thousands of PCR products to be sequenced in parallel, within a single reaction (Glenn 2011). This is well-suited for within-species population assessments of diet choice but for dietary analyses on a large number of individual samples across multiple species, each sample needs to be amplified by a uniquely tagged pair of primers (Pompanon et al. 2012). NGS can provide important insights into diet specialisation (McClenaghan et al. 2015) at the family, genus or species level, depending on the taxonomic coverage of reference barcodes available (Jurado-Rivera et al. 2009). NGS has excellent potential for ecological applications as it can give clear and accurate results for digested materials that would usually be unidentifiable (McClenaghan et al. 2015).

Both traditional DNA barcoding and meta-barcoding have been shown to be reliable techniques in laboratory-controlled feeding experiments (Avanesyan 2014) or when the diets are largely already known. As an example of the latter, the host associations for the Australian Chrysomelinae (a subfamily of leaf beetles), feeding on endemic Australian lineages of plants have been investigated using the trnL marker. Sequences of host plants between 313-581 base-pairs in length were successfully identified for 78 beetle specimens (Jurado-Rivera et al. 2009). Although this study proves that this method can be successful, these species have a well-known and narrow plant associations and all of the plants concerned were sequenced as part of the experiment. When host plants are unknown, it is not possible to identify them to species unless the sequences retrieved can be matched to the same sequence within an online database. A number of other studies have focused only on the use of a single marker (Avanesyan 2014; Navarro et al. 2010; Valentini et al. 2009), which can restrict the amplification of certain botanical groups and may lead to inaccurate

identification (Korning et al. 1996). More recently, two markers have been used for more reliable results (Kajtoch 2014; Kartzinel et al. 2015).

2.6 Summary

Anthropogenic habitat degradation is threatening biodiversity, particularly within tropical forests where both species diversity and human pressures are high (Dirzo & Raven 2003). Habitat alterations lead to changes in species composition, but different taxa respond in different ways. For many taxa (such as the insect Order, Orthoptera) only limited data are available. Accordingly, to gain a better understanding of the dynamics of changing ecosystems, investigation of these less-studied groups is required. It is also unclear how habitat modification affects interactions among species (van der Putten et al. 2004). The use of modern molecular tools will produce more accurate food webs to identify species-species links. In turn these constructs have applications across a wide range of ecological problem sets.

Chapter 3

STATEMENT OF CONTRIBUTION TO CO-AUTHORED PUBLISHED PAPER

Chapter 3 is a co-authored paper which has been **prepared** for publication. The bibliographic details of the co-authored paper, including all authors, are:

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My contribution to the paper involved all field work to collect specimens, all laboratory work to sequence the samples and all data processing and analysis. Associate Professor Alison Shapcott supervised the project, trained me in the laboratory both in preparation and during sequencing the samples and provided help with my methods and feedback on the manuscript. Dr. Sarah Maunsell assisted with my field work, laboratory work and supervised this project, assisting with edits to the manuscript. Professor Roger Kitching and Professor Nigel Stork also supervised this project and were responsible for direction and guidance in regard to the design of the project and scope and structure of the manuscript.

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Chapter 3: DNA barcoding for ecology: a comparative study on the use of Sanger sequencing and metabarcoding for herbivore dietary analysis

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3.1 Abstract

Measuring insect herbivore trophic interactions is challenging, but, with its increasing technological advances, the emerging field of ecological genomics is helping to overcome previous limitations. Here, the efficiency of traditional single-species barcoding using Sanger sequencing is compared with metabarcoding using an Illumina Miseq. These two sequencing methods are trialled on the same selection of Orthoptera to analyse their diets when host plant species and diet breadth are largely unknown. The outputs from each barcoding method were compared using two DNA barcode markers (rbcL and trnH-psbA). We describe a hybrid method which was developed for the Illumina Miseq to allow for multiple marker PCR products to be sequenced simultaneously. Overall, we found that although the Illumina Miseq gives much more exhaustive results than the Sanger method in terms of number of sequences obtained and number of successful samples, the Sanger sequences were longer in length and could be assigned taxonomically with greater confidence. One major drawback with Sanger

sequencing for the use of diet analysis is that it often failed to deliver a readable sequence from a sample when there was DNA from multiple species in the gut contents; limitation is overcome by metabarcoding. We obtained a greater number of operational taxonomic units (OTUs) using the rbcL marker, but we found that the two markers are useful for the purpose of completing the network, rather than complementing each other with identical taxonomic identities. Overall, we suggest that the Sanger method is a more appropriate approach for measuring trophic interactions only for known specialist species. Although both methods are valuable across many fields, metabarcoding has a more extensive potential for developing a wider understanding of trophic interactions in ecological systems.

3.2 Introduction

With loss of species in communities occurring globally due to habitat change, there are related consequences for ecosystem function (Cardinale et al. 2012). Food webs provide the link between community ecology and ecosystem functioning and have been described as the defining objects of community ecology (Kitching 2004). Therefore, understanding the interactions between species is important for the structure and function of biodiversity (Thompson et al. 2012). Arthropods constitute the most species-rich (Stork 2018) and abundant non-microbial organisms on Earth (Basset et al. 2012; Scheffers et al. 2012), however, determining precisely what they eat has often proved difficult. Methods for identifying the diets of arthropods have been approached in a variety of ways in the past including direct observation (e.g. Basset 1992; Ødegaard, Diserud, and Østbye 2005), laboratory feeding trials (e.g. Barone 1998), microscopical gut content analysis (e.g. Johnson and Nicolson 2001) and carbon/nitrogen stable isotope analysis (e.g. Blüthgen, Gebauer, and Fiedler 2003). While these measurements have provided the basis for our current understanding of insect-plant interactions, they have been limited to taxonomic groups for

which interactions are easily observed and/or provide only coarse taxonomic resolution of host plants.

DNA barcoding is a term used to describe the use of a standardised DNA region as a tag for species identification as they are relatively short sections of a gene that show interspecific divergence (Hebert & Gregory 2005). Although the term ‘DNA barcoding‘ first appeared in the literature in 1993, it did not receive much attention in the scientific community until 2003 (Hebert et al. 2003) and has since evolved in a number of ways. Within the fields of ecology and conservation, scientists are using DNA barcoding for taxomony (e.g. Hebert et al. 2003), to measure trophic interactions (e.g. de Vere et al. 2017), host–parasitoid relationships (e.g. Santos, Besnard, and Quicke 2011) and to examine environmental samples such as soil/water (e.g. Taberlet et al. 2012). DNA sequencing technologies have improved greatly from manual to automated sequencing, and the technology is now accessible to anyone, with or without sequencing expertise (Valentini, Pompanon & Taberlet 2009). As sequencing tools have become more popular, public databases of DNA sequences are becoming larger. As an example, GenBank, which was founded in the 1980’s, now contains nucleotide sequences for over 400,000 named species (Benson et al. 2018). This database is free and accessible to the public. Caution should be taken, however, when using these databases as the quality of sequences are not always perfect (Harris 2003).

In terms of using DNA barcoding for measuring trophic interactions, researchers have used a variety of different sequencing platforms. Many studies have utilised the traditional Sanger sequencing techniques (Sanger, Nicklen & Coulson 1977). This method is limited in that it can only sequence single species (Shokralla et al. 2012) unless a labour-intensive cloning step is carried out (Traugott et al. 2013). It has, however, proved valuable for determining the feeding ecology of individual species (e.g. Garcia-Robledo et al. 2013; Avanesyan 2014) where insects are known to have only fed on a single species of plant or where they are likely

to be monophagous (e.g. Matheson et al. 2008). Metabarcoding is a method known as Next-Generation Sequencing (NGS) and can be carried out using a variety of related, but distinct platforms including Illumina, Roche454 and Ion Torrent. This method differs from Sanger sequencing as it can sequence thousands to millions of short DNA fragments simultaneously (Kress, J et al. 2015). Mixed species samples can be barcoded (Ji et al. 2013) and, in the case of diet analysis, a wide range of food items can be detected (Pompanon et al. 2012). Although metabarcoding has been used for studies of the diets of mammalian herbivores (Kartzinel et al. 2015), very little has been reported on its use for insect herbivore interactions, although a number of studies have used the technique to identify pollen samples from bees (de Vere et al. 2017; Hawkins et al. 2015; Sickel et al. 2015).

An additional choice to make is which gene region or regions to target when barcoding samples. If targeting animal DNA, this choice is easier than for plant DNA. The cytochrome C oxidase (CO1) gene is highly effective for discriminating between animal species (Hebert et al. 2003). It has, however, been deemed ineffective for species-level discrimination as the mitochondrial genes in plants have a lower rate of sequence change than that of animals (Kress, J et al. 2005; Kress, WJ & Erickson 2007). Consequently, DNA sequencing could not be used for the detection of plant material in animal gut contents until suitable alternatives to the CO1 gene were discovered. The following chloroplast gene regions have been used as appropriate target regions for plant identity, each with varying success levels: trnL intron (Taberlet et al. 2007), rbcL (Matheson et al. 2008), matK and trnH-psbA, and the nuclear ITS region (Hollingsworth, PM, Graham & Little 2011; Kress, J et al. 2005). Although each of these show high levels of interspecific divergence, currently, there remains no consensus on a single locus that can discriminate to the species-level with the same level of success as the CO1 gene in animals (Hebert et al. 2003; Kress, WJ & Erickson 2007). Therefore, it is suggested that the use of

multiple genes is the most successful way to reduce both Type 1 and Type 2 errors in plant species assignments (Kress, WJ & Erickson 2007).

To measure insect–plant interactions, degraded DNA must be amplified from the gut contents. Using shortened gene markers can increase the recovery of degraded DNA (Garcia-Robledo et al. 2013). Additionally, for diet analysis, an appropriate reference library of sequences of potential food items has been identified as a key requirement (Garcia-Robledo et al. 2013; Hollingsworth, PM, Graham & Little 2011; Kress, J et al. 2015). Where local species information is unavailable, sequences can be compared against global databases such as GenBank (Pompanon et al. 2012), although inaccuracies may arise due to a higher chance of sequencing errors and sample misidentifications from uploaded sequences (Harris 2003).

The traditional Sanger method and the more recent metabarcoding methods have both proved useful for identifying trophic interactions, yet only one study to date compares the two methods (Kajtoch 2014). In this study, we use Sanger sequencing and metabarcoding using an Illumina Miseq with trnH-psbA and rbcL gene markers, for the identification of plant DNA sequences from the gut contents of a variety of Orthoptera (as these are a largely herbivorous group) from a range of habitats in southeast Queensland, Australia. We provide detailed methods on the procedures used for DNA sequencing with both Sanger and Illumina platforms. Using a customised plant reference database for the area, we assess, 1) the technical differences in the use of the two sequencing methods by measuring the accuracy and variation in outputs in terms of sequencing lengths and quality, 2) the number of food web links and number of operational taxonomic units (OTUs) that can be identified by each sequencing method and marker and, 3) how much overlap there is across markers to increase confidence of the taxonomic assignments. As this study focuses on the methods and technical outputs of each sequencing platform and gene marker, we do not discuss the ecological details of the species.

3.3 Methods

Sample collection

Orthoptera were collected using sweep-netting from a eucalypt open woodland and sub-tropical rainforest in Lamington National Park (28° 13’ S 153° 08’E), and a dry eucalypt forest in Toohey Forest (27° 32’ S 153° 03 E), in southeast Queensland, Australia. These different habitat types were chosen to increase the variety of plant groups in the diets of the orthopterans. Forty-six individuals were collected from the Suborder Caelifera and sorted into the families Acrididae, Tetrigidae and Pyrgomorphidae, and fifty individuals were collected from the Suborder Ensifera and sorted into the Families Gryllidae, Gryllacrididae and Tettigoniidae (Supplementary Material S3.1). Immediately after collection each specimen was placed into 98% ethanol and stored at −20ºC within 4 hours of collection.

Plant reference libraries

A customised reference database of plant barcodes was created for each of the plant gene markers used. A comprehensive DNA barcode library of southeast Queensland rainforest plants, created by Shapcott et al. (2015) and Howard et al. (2016), was utilised for this purpose. This reference database was supplemented by sequences of additional plant species (based on comprehensive local lists and regional ecosystem maps) for the sample locations (Neldner et al. 2017). These were downloaded from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). For rbcL we obtained sequences for 3606 species in the sampling regions and 1416 species for trnH-psbA. A separate database was created using all sequences available from the NCBI website for each marker (approximately 100,000 sequences) to use for samples that did not successfully match to any sequences to the smaller customised reference library.

Sample preparation and DNA extraction

Before DNA extraction, all Orthoptera specimens were rinsed with 98% ethanol to remove any external plant DNA. Whole genomic DNA was extracted from the entire bodies of all 96 specimens following methods used by Shapcott et al. (2015) and consistent with Ivanova, Fazekas and Hebert (2008) and Kress, J et al. (2009). For a detailed laboratory protocol, see Supplementary Material S3.2. Extracts were tested for the presence of DNA using gel electrophoresis (1%) and then stored at −20°C.

PCR amplification

Two standard plant DNA barcode markers amplifying the chloroplast coding region rbcL and the non-coding trnH-psbA region, were chosen as these had been used successfully previously for barcoding plants in the region (Shapcott et al. 2015) and others had found them successful for herbivore gut content analyses (Galimberti et al. 2016). PCR amplification tests were performed following established methods used by Shapcott et al. (2015) in standard 96-well plate formats. The reaction mix of 20 µl was made up of: 0.1 µl of each forward and reverse primer (100 µM stock), 2 µl Bioline 10x biolase buffer, 1 µl MgCl2 (50 nM stock), 1 µl dNTP's (10 mM stock), 0.2 µl Bioline biolase Taq (5 U/µl) and 14.6 µl molecular grade H_2O. For Sanger sequencing, a standard PCR cycle was followed for each primer with the annealing temperature at 55°C for rbcL (39 cycles) and trnH-psbA (34 cycles, consistent with Garcia-Robledo *et al*. 2013) (See Supplementary Material S3.2C for full PCR cycle details). PCR success was tested by electrophoresis on a 1% agarose gel with ethidium bromide and viewed under UV light on a Molecular Imager Gel Doc XR plus with Image Lab™ Software (v6.0). A clear band of expected base-pair length indicated amplification. As the Illumina Miseq platform has limited read lengths, shortened primers were necessary for rbcL. We used a mini-barcode derived from the standard rbcL plant DNA barcode region,

using the accepted reverse primer from the standard barcode, and a forward primer developed by Garcia-Robledo et al. (2013) and used by Erickson et al. (2017), which were reported to have increased success rates when sequencing gut contents due to difficulties in sequencing long fragments of degraded DNA. The annealing temperature for the mini-barcode PCR cycle was 50 ºC (39 cycles) (see Supplementary Material S3.2C for full cycle details). This primer pair produces a 379 bp product (for primer sequences, see Supplementary S3.3). The standard trnH-psbA barcode sequence was short enough for use on the Illumina Miseq. In preparation for Illumina Miseq sequencing, all PCR primers were 'Miseq-adapted' with 5' overhangs complementary to Nextera index primers to allow sample identification with individual molecular tags, as used by de Vere et al. (2017) (Supplementary Table S3.4). The same tags were used for the same position and sample in the 96 well plate, across the two markers. Whereas in preparation for Sanger sequencing, PCR primers were end labelled with an M13 tag to allow for big dye attachment. Due to the degraded nature of gut content DNA, in order to reduce PCR failures, two PCRs were carried out for each sample for both markers and the products were pooled prior to the first purification step of the protocol. All products from all samples were kept after this stage, whether showing as successful on the gel or not. Different protocols were followed in preparation for Sanger and Illumina Miseq sequencing.

Sanger protocol and sequence processing

Following methods outlined in Shapcott et al. (2015) and Howard et al. (2016), we used ExoSAP-IT (USB) PCR product clean-up to hydrolyze excess primers and nucleotides and conserve PCR product. Forward and reverse sequencing reactions were performed using a 12 µl sequencing mix consisting of 2.15 µl of 5x sequencing buffer, 0.5 µl of BigDye, 0.02 µl of 100µM M13 primer (forward or reverse), 4 µl of the purified PCR product and 5.33 µl of molecular grade H_20. After cycle sequencing, the product was purified through a Sephadex column. Subsequently, the product was subjected to standard Sanger sequencing in an ABI

3500 Genetic Analyser. Raw sequences were trimmed, checked and manually edited to ensure quality, and forward and reverse reads paired to form contigs of double-stranded sequences using Geneious R8 (v8.1.8). Only sequences over 200 bp in length and with a quality score ('Phred Score') of 20, determined using PRINSEQ v0.20.4 (http://prinseq.sourceforge.net/) or higher (referring to the probability of an incorrect base call as less than 1 in 100) were kept to assign taxonomy using the same method as the Illumina Miseq sequences (see below). Phred scores are a per-base estimate of base caller accuracy. These are calibrated on a scale corresponding to: Quality = $10\log_{10}(Pe)$ where *Pe* is the probability of error.

Illumina Miseq (metabarcoding) protocol and sequence processing

We developed a hybrid protocol based on the Illumina 16s Metagenomic Sequence Library Preparation protocol (Illumina Inc. 2017) and other published methods (de Vere et al. 2017; Hawkins et al. 2015; Kartzinel et al. 2015; Keller et al. 2014). After our initial PCR procedures, the product for each marker was purified using Agencourt AMPure beads (Beckman Coulter) which separate contaminants from the PCR amplicons. We used 16 µl of product with a 1:0.8 ratio of product to AMPure beads. This is a lower than suggested in the Illumina 16S protocol but was previously tested elsewhere and proven to be successful (de Vere et al. 2017). Following this, a second round of amplification was carried out to anneal sample-specific Illumina Nextera XT v2 indices (Supplementary S3.4) so that samples could be separated during bioinformatic processing. We used these same index tags for the same samples on each of the marker plates so that we could sequence 96 samples simultaneously across the two markers on a single run of the Illumina Miseq. The index PCR stage used a 25 µl reaction comprising: 12.5 µl of 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 5 µl of Molecular Grade H_20, 2.5 µl of Nextera XT i7 index primer, 2.5 µl of Nextera XT i5 index primer and 5 µl of purified PCR product. A 1% agarose gel was run to check that

products were within the expected size range. Following this, a second AMPure bead clean-up of the indexed product was carried out. A Quantus Florometer (Promega) was used to measure the DNA concentration of each individual sample using fluorometric quantification with dsDNA binding dye. Concentration in nM was determined by calculating as: concentration (ng/µl)/660(g/mol). Samples with concentrations below 0.5 nM were removed, and individual samples were normalised manually to save costs, then pooled for each marker plate. The two amplicon libraries were normalised and pooled together. Based on methods in the Illumina 16s Metagenomic Sequence Library Preparation protocol (Illumina Inc, 2017), the final sample library was diluted to 4 pM and combined with 5% PhiX library of the same loading concentration and then finally denatured at 96 ^{o}C before being loaded into the Miseq cartridge. Sequencing was carried out using a Miseq Reagent Kit v3 with read lengths of up to 2 x 300 bp. The Miseq Report Generator FASTQ Workflow performed the demultiplexing step to separate data from pooled samples based on the index sequences (Illumina Inc. 2017) so the output obtained was index-matched raw sequence reads only. USEARCH v10.0.240 was used for processing samples. All sequences were trimmed and quality filtered. Sequences with a quality (Phred) score of <20 (which is the equivalent to the probability of an incorrect base call 1 in 100 times (Illumina Inc. 2017)) or with a read length less than 200 bp were discarded using fastq_filter, -fastq_minlen and -fastq_maxns commands. Forward and reverse reads were joined to form contigs using fastq-join command.

Taxonomic identification of sequences

For both Sanger and Illumina samples, sequences that passed the quality control filters were aligned against our marker-specific reference libraries at 98% or above similarity using usearch_global in USEARCH. Additionally, in an attempt to broaden the number of samples with taxonomic assignments, samples without a hit were aligned against a full database for each marker at 98%. We converted the output for each marker into a single matrix showing

the number of reads for each sequence with rows as plant species and columns as samples. We only considered matches with a minimum of 10 reads assigned to reduce results arising from amplification or sequencing errors (CBOL 2009; Hawkins et al. 2015). Additionally, we only considered species that were recorded as being found within the sampling locality by Queensland Regional Ecosystem maps (Neldner et al. 2017) and *ATLAS of Living Australia* (http://www.ala.org.au/). Following Soininen et al. (2017), when we identified a species that was not present in the study area, we adjusted the taxonomic assignment to a less specific level. Genus-level assignments were made if the genera had previously been recorded in the sampling area, but the species had not, and family-level assignments were made if only the family had been recorded in the sampling area, but the genera had not. Throughout the results section, when we refer to OTUs, these are sequences that have been assigned a taxonomic identity to either species, genus or family.

Data analysis

Using our sequence outputs (a matrix of OTUs for each plant taxonomic assignment and Orthoptera samples) for each marker and method, analyses and figure generation were carried out in 'R' v 3.4.1 (R Development Core Team 2014). All analyses that involved comparisons of methods were carried out on a set of 19 Orthopteran samples (that were successfully sequenced using both the Illumina and Sanger methods (Supplementary Material S3.5) unless otherwise stated in the text. We compared sequence length and quality for the Sanger dataset across the two markers using Mann-Whitney U tests, as our data were non-parametric. We created binary food webs using the package 'Bipartite' (Dormann et al. 2009) and compared the number of OTUs (as a measure of dietary richness) across sequencing method (Illumina and Sanger) and marker type (rbcL and trnH-psbA) using Mann-Whiney U tests. Where boxplots were used to present data, the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper whisker extends to the largest value

and the lower whisker extends to the lowest value (both of which are no further than 1.5 * interquartile range (IQR) from the hinge. Data falling beyond this range are "outlying" points that are plotted individually (McGill, R., Tukey, J. W. and Larsen, W. A. 1978). Finally, to determine how much the two markers complement each other, we described the proportion of taxonomic overlap observed across samples. Proportions were measured by taking the number of OTUs in a sample that were sequenced by both markers e.g. for Sample X, if there were 8 OTUs from rbcL and 1 OTU from trnH-psbA. If the single OTU from trnH-psbA matched to the same family as one of the rbcL OTUs, then this was counted as a 100% match at a family level.

3.4 Results

Sequencing yield and sample success rates

The two sequencing methods yielded very different results in many respects. One of the greatest differences was in the initial output of sequences. With Sanger sequencing, 27 sequences were obtained from 22 samples: 15 using trnH-psbA and 12 using rbcL (Table 1). Sequences were obtained from 65 of the same 96 Orthoptera using the Illumina Miseq procedure which produced a total of 1,792,102 readable plant sequences after trimming and quality filtering: 857,967 from 36 samples using trnH-psbA and 894,135 from 65 samples using rbcL (Table 3.1). For the 22 successful Sanger samples, sequences were obtained from 19 of these using the Illumina Miseq method.

Table 3.1: Sample success described as the number of samples that at least one readable DNA sequence was retrieved from after passing quality and length filtering. Percentages are reported in brackets from a sample size of 96.

	TRNH-PSBA		RBCL	
	SANGER	**ILLUMINA**	**SANGER**	**ILLUMINA**
Sample success	15 (15.6%)	36 (37.5%)	12 (12.5%)	65 (67.7%)

Sequence quality and length

Comparisons of quality score ('Phred score') could not be made directly between Sanger and Illumina samples as it would mean comparing scores from a single read from Sanger to that of a large set of repeated reads from Illumina. From a visual comparison of the 19 samples that were successfully sequenced by both methods, the scores from the Illumina dataset showed much less variability than those from Sanger (Figure 3.1 left). It was possible to separate and compare sequences from the two different markers for the Sanger dataset and there was no difference in quality between from the rbcL and trnH-psbA results (Mann-Whitney U, W = 60.5, p = 0.24). While there was no variation in our Illumina Miseq sequence lengths due to the cluster-generation process which limits each sequence to 300 base-pairs, our Sanger sequences did show variation in length (Figure 3.1 right), depending on the quality of the DNA and the marker used. Our Sanger sequences were on average 532 ± 9.04 (1SD) base-pairs in length for the rbcL marker and 459 ± 34.68 (1SD) base-pairs in length for the trnH-psbA marker. However, there was no significance difference in sequence length between the two markers (Mann-Whitney U, W = 60.5, p = 0.24).

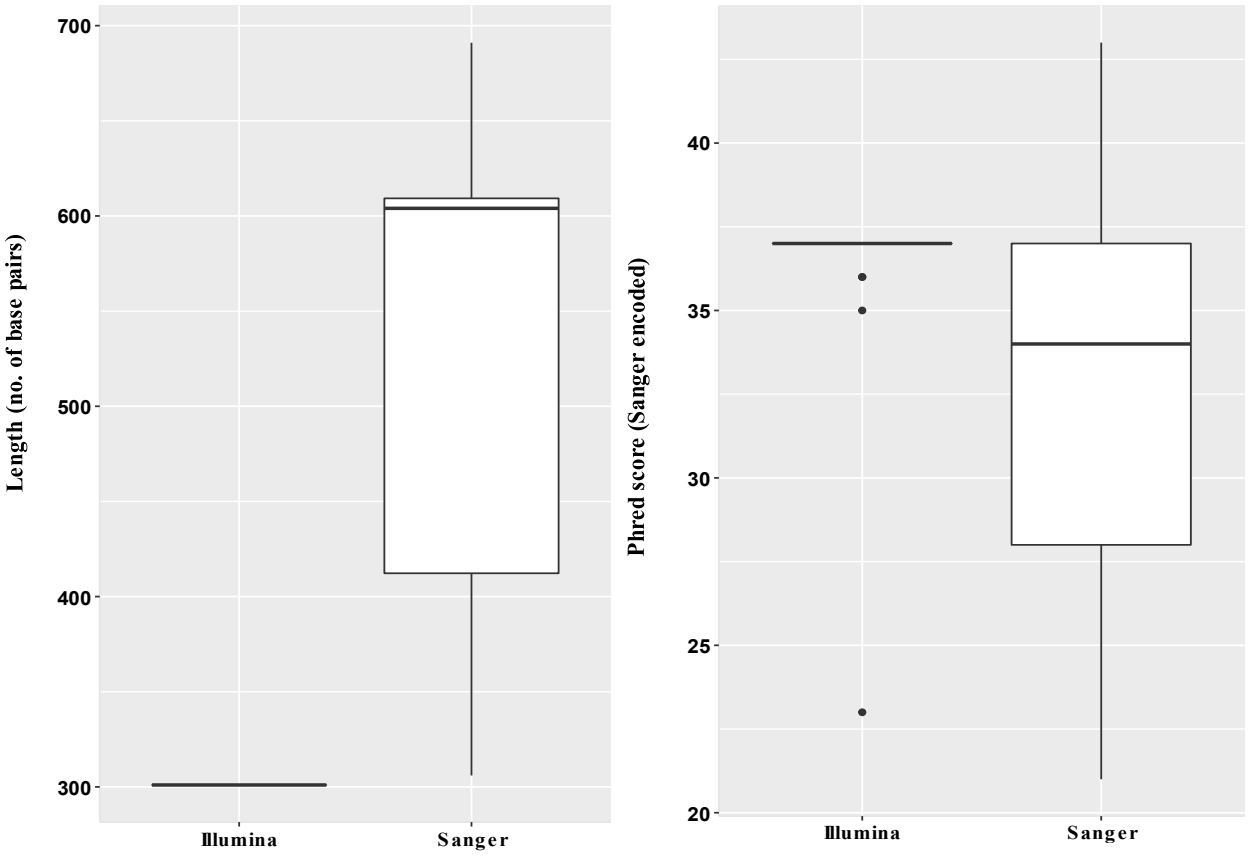


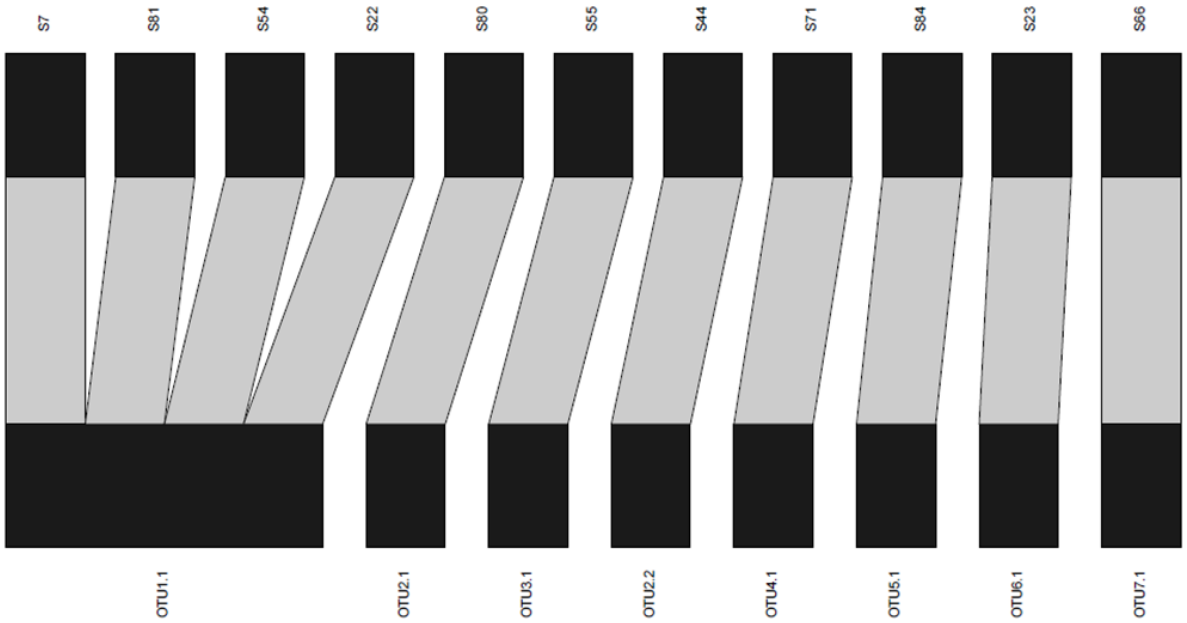
Figure 3.1: Boxplots to show the differences in sequence lengths for Illumina and Sanger samples (left) and the differences in phred scores for Illumina and Sanger samples (right). Boxes show the third and first quartile range of the data and data outliers (explained in 'Data analysis' section).

Interaction analysis

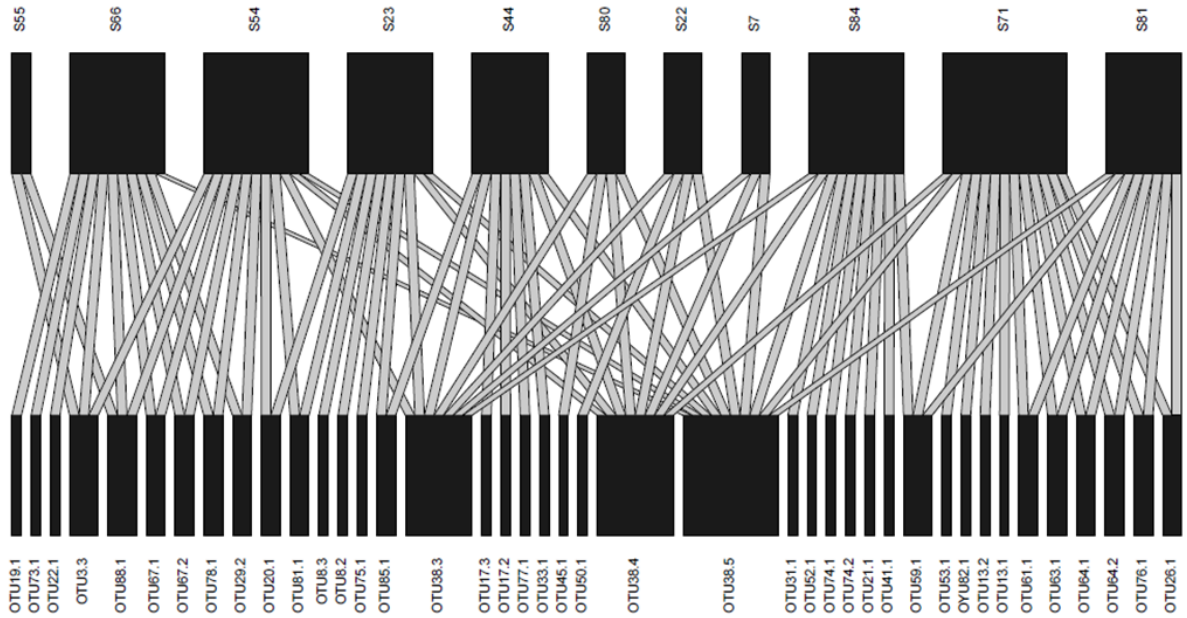
To compare differences in diet composition, determined by both the sequencing method and marker, we present samples that were sequenced using both methods for each marker (this was 11 samples for rbcL and 9 samples for trnH-psbA) as binary food webs. Binary food webs constructed using interaction matrices from Sanger sequencing results show very simple networks and clearly identifying only one food-plant per individual Orthoptera. On the other hand, the larger Illumina Miseq dataset resulted in a much more complex (and informative) interaction network (Figure 3.2 A – D). The Illumina dataset is so much larger and more complex because multiple plant species could be detected from each individual gut

content. A greater number of OTUs were identified by using the rbcL marker for both sequencing methods.

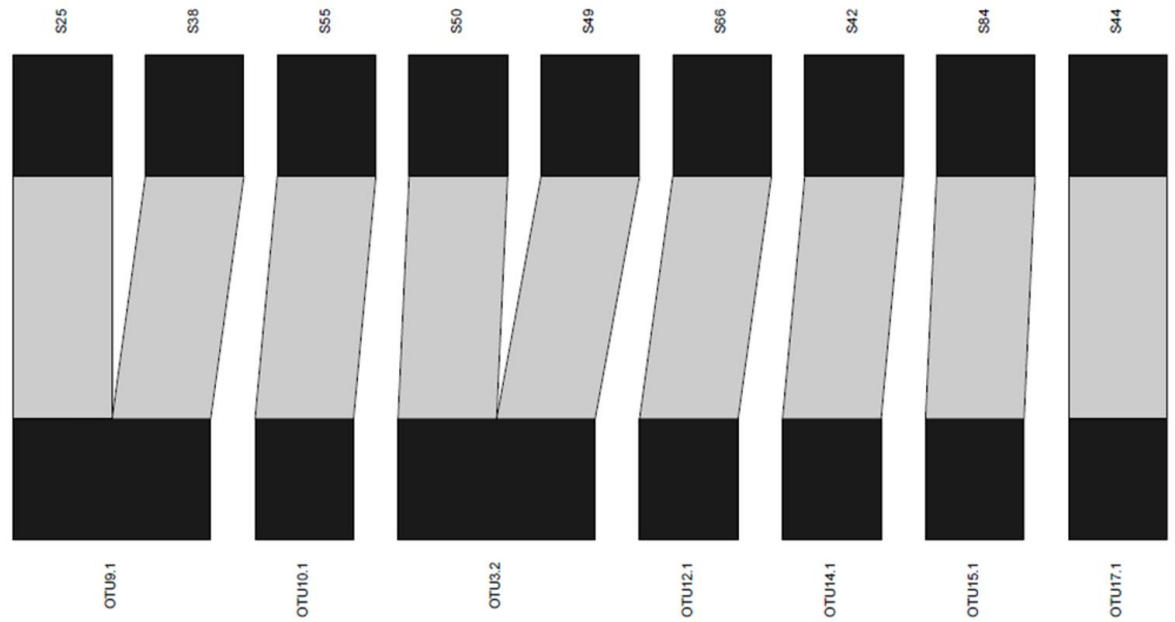
A: Sanger method, rbcL marker



B: Illumina method, rbcL marker



C: Sanger method, trnH-psbA marker



D: Illumina method, trnH-psbA marker

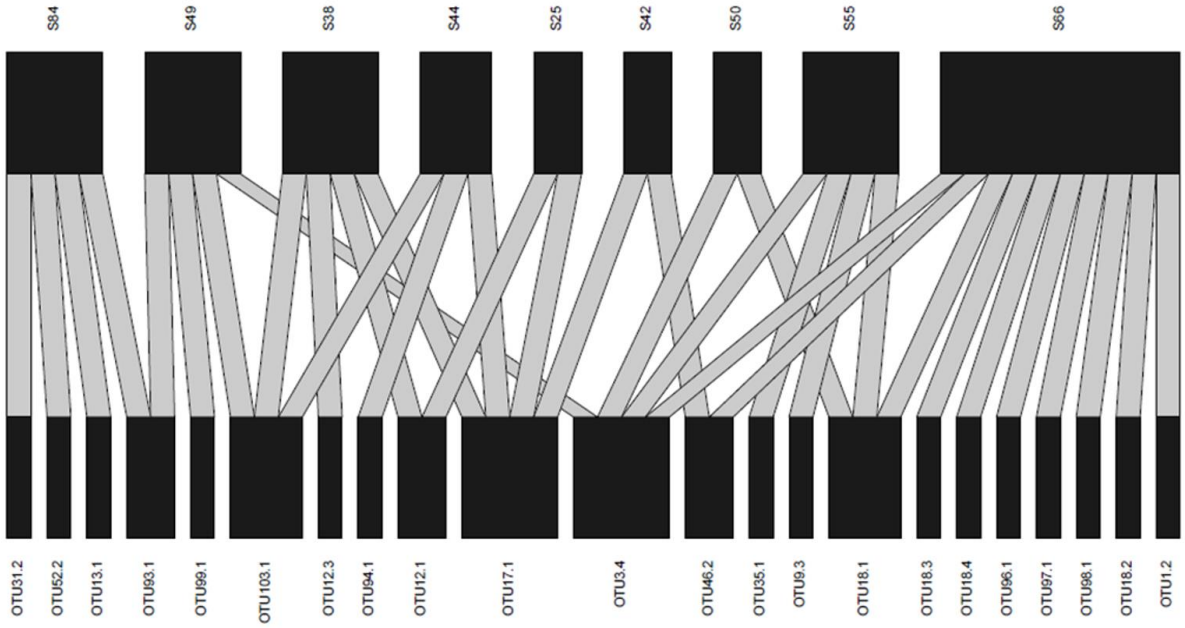


Figure 3.2: Binary food webs network between individual orthopterans (top boxes) and food plants as OTUs (lower boxes) for rbcL (A and B) and trnH-psbA (C and D) using Sanger sequencing (A and C) and Illumina sequencing (B and D).

The number of OTUs identified by each method were significantly different for both the methods used (Sanger and Illumina: Mann-Whitney U, W = 285, p = <0.01). Using Sanger sequencing, a maximum of two OTUs were retrieved per sample (one from rbcL and one from trnH-psbA), whereas using Illumina we retrieved up to 16 different OTUs in a single sample (Figure 3.3 A). When comparing the number of OTUs that were assigned taxonomy for each marker, we retrieved a greater number using the rbcL marker than trnH-psbA (Mann-Whitney U, W = 82, p = 0.01) (Figure 3.3 B).

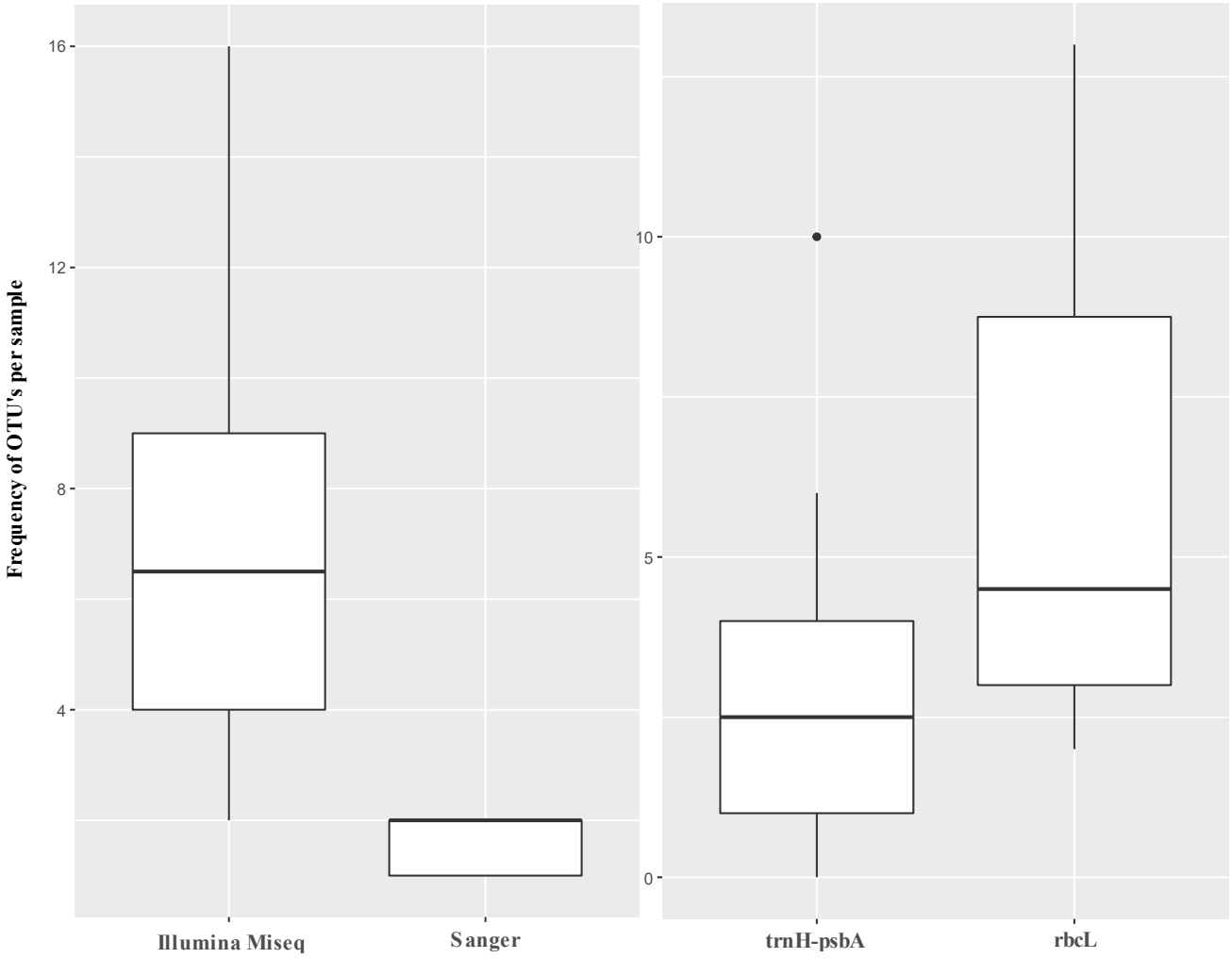


Figure 3.3: The frequency of OTUs obtained per sample using the two sequencing methods (left) and the two markers (right).

Of the samples we have assessed so far, we selected the ones where OTUs were retrieved from both markers and measured the proportion of taxonomic overlap. We found that when both markers were successful in a sample, 67% of the time there was some overlap with the plant taxonomic group (either at a species, genus or family level). Only 11% of these matched to the same species (Figure 3.4).

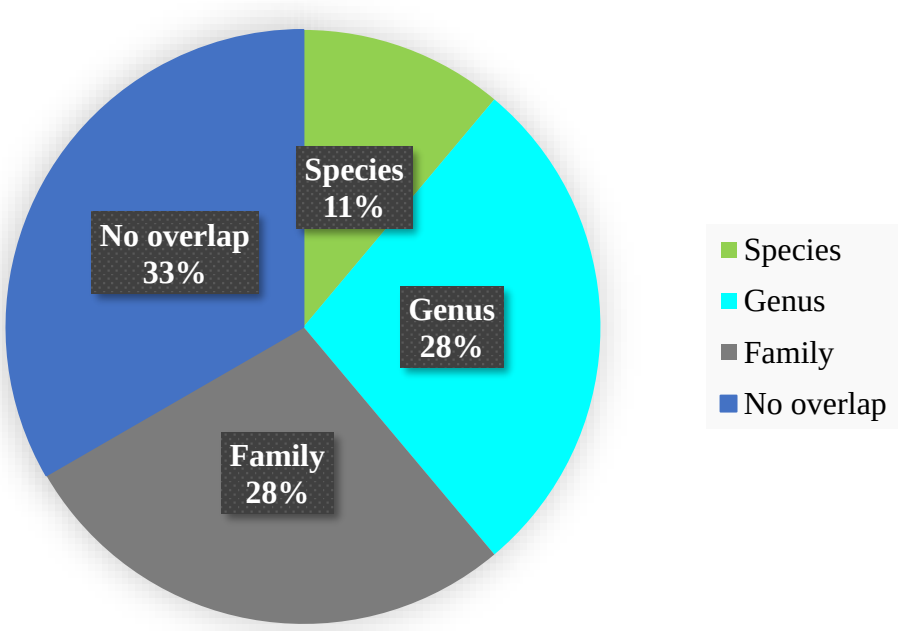


Figure 3.4: Pie chart showing proportions of overlapping OTUs retrieved using the two markers at different taxonomic levels.

3.5 Discussion

The use of DNA barcoding to identify diets has the potential to revolutionise food web ecology where direct observation of interactions is challenging. Both Sanger sequencing and Illumina Miseq sequencing can be successful methods for determining the diets of insect herbivores (Kajtoch 2014). For field-collected samples of herbivores with unknown levels of specialisation, the Illumina Miseq method can provide much more intricate detail than the Sanger method. Each method, however, has benefits and drawbacks. There is variation

between these two sequencing methods with respect to sequencing success rates (if one is unsure of what is in the sample), sequence length and quality and, most importantly, the dietary composition output.

Overall, we found Sanger sequencing to be less effective for determining the diets for the majority of the Orthoptera we examined due, in part, to the much lower success rate for sequencing. The reason for this is probably because the Sanger method requires relatively high concentrations of high quality DNA to be successful (Polz & Cavanaugh 1998). Further, if samples have DNA from multiple plant species within them, Sanger sequencing will be of little utility (Kajtoch 2014). The Sanger sequencing system works by capillary electrophoresis and can only produce one forward and one reverse read per sample and it is highly likely that the low sequence rate we obtained was due to the majority of samples containing mixed plant sequences. This was subsequently confirmed by the Illumina Miseq dataset. In addition, for the Sanger analysis, the use of the standard length rbcL marker (680 bp) could have prevented the amplification of degraded DNA. Next-generation sequencing platforms such as the Illumina Miseq operate only with shorter DNA fragments but have the benefit of being able to sequence millions of fragments of DNA in parallel (Behjati & Tarpey 2013). The standard trnH-psbA gene is just within the length limit but shortened marker sequences are necessary for the use of the rbcL gene. The Illumina Miseq method can sequence very low concentrations of DNA (Shokralla et al. 2015) and therefore is useful for obtaining food web interaction information from degraded DNA.

Sequence quality and length are two key factors when aligning reads against a reference library: greater quality and longer length will increase the likeliness of higher taxonomic resolution in the matching process. This is, however, a trade-off as higher sequencing depth can be achieved with shorter reads such as those from Illumina platforms (Paula et al. 2016; Pompanon et al. 2012). We had a much greater sequencing success rate from the Illumina

Miseq, and this method gave a clearer picture of diet breadth and composition. However, if you have a sample which is not-mixed (e.g. only contains one plant or animal species), higher confidence to species-level resolution could be achieved when using longer Sanger sequences of >600 bp.

It is well documented that a two-locus system will produce better taxonomic resolution (Garcia-Robledo et al. 2013; Hollingsworth, PM, Forrest, et al. 2009; Hollingsworth, PM, Graham & Little 2011; Jurado-Rivera et al. 2009; Kajtoch 2014; Kress, J et al. 2015). This is often reported as a method to confirm species within samples if both markers result in the same taxonomic alignment (Kress, WJ & Erickson 2007). In our case we found that our levels of overlap across the trnH-psbA and rbcL markers occurred at a relatively low rate. This could be because different genes tend to pick up different plant families (Hollingsworth, PM, Forrest, et al. 2009), or that one of our markers is not aligning to the correct species. As we only used sequences that had passed strict quality control measures, we suggest that using different markers can be useful for increasing the network completeness, rather than only being used to confirm species identities. Overall, the rbcL marker resulted in a wider taxonomic breadth of plant species for both methods. This is because we had a more comprehensive reference library for this marker, for both our personalised reference library for the area, and our downloaded GenBank libraries. Caution should be taken with species-level identifications with the rbcL marker as some have suggested it should be discounted as a species-level discriminator (Salazar et al. 2003). Despite this, others have found it to be useful, particularly as it can be easily amplified in most land plants and is regarded as a benchmark locus in that it can provide reliable taxonomic placement of a taxon into a plant genus or family (Kress and Erickson 2007). With rbcL we often had multiple hits to different species from the same plant genus. Although it is not unusual that an orthopteran would feed on multiple plants from the same genus, we suggest that extra care is taken when using rbcL

(e.g. by ensuring plants have been previously recorded within the sampling regions and by only using sequences of high quality). Although we found that using the two different sequencing methods would likely increase reliability of results (for example, as Sanger could be used to confirm species using longer DNA sequences), we suggest the best method for understanding generalist insect herbivore diets is by maximising the number of markers used instead, because a broader group of plants is then encompassed.

The use of a reference database that contains only the plant species in a particular sample environment is highly advantageous in assigning samples taxonomically (Erickson et al. 2017; Garcia-Robledo et al. 2013) and reduces the risks of false positives. However, this does means that the taxonomic breadth of detection of plant species is directly related to the comprehensiveness of that database (Paula et al. 2016). Although we used a customised reference database of high quality sequences that encompassed all the plants recorded in the region (Neldner et al. 2017; Shapcott et al. 2015), it may, nevertheless, not have included all species. We used a full GenBank reference library with samples where no taxonomic result was produced with our reference library, and only reported the taxonomy if the hit was over 98% similarity and was found within our sampling region. We also only used USEARCH, as other methods of alignment such as BLAST (Basic Local Alignment Search Tool) (Altschul 1990) can align hits based on short sections of the query sequence and, accordingly, should be used with caution (Keller et al. 2014). Given that only 20% of land plants worldwide have been barcoded (Wilkinson et al. 2017), confirming precise diets using DNA barcoding will remain a challenge in herbivory studies for some time to come.

In conclusion, we suggest that for insect herbivores, DNA-based plant identification has the potential to resolve some long-standing and challenging questions surrounding diet identification and the functioning of ecological communities (Navarro et al. 2010). We found that, when working with degraded DNA, of unknown and potentially mixed species, a

metabarcoding approach using an Illumina Miseq provided more comprehensive and useful information than Sanger sequencing. There remain issues with accuracy when using DNA barcodes to identify an exact species of host plant. For single species barcoding for identification, the Sanger method can be highly reliable and accurate, out-performing the more recent metabarcoding methods, as it can produce longer read lengths. However, for mixed sample sequencing or for simultaneous sequencing of multiple genes, using an Illumina Miseq system is an efficient method. We recommend this approach for analysis of the diets of generalist (or unknown) herbivore diets. Both approaches to DNA barcoding have many applications and their value will continue to increase as the taxonomic coverage in reference databases increases. We suggest that these methods are the future for understanding of trophic interactions.

3.6 Acknowledgements

This study was funded through an Australian Research Council Discovery Grant DP160102078. JLH was supported, in addition, by a Griffith University postgraduate research scholarship and a research grant from the Equities Trustees Charitable Foundation. Laboratory work was undertaken in the University of the Sunshine Coast's Genecology molecular research lab and AS was funded by German Australian research exchange program. We extend our thanks to Lois Kinneen and Erica Odell for their assistance in the field, to Wiebke Sickel and Rachele Wilson for their assistance in the laboratory and to David Erickson and Alexander Keller for their helpful feedback on this manuscript. All research methods and sampling were approved under the auspices of Scientific Purposes permit WITK17907216 issued from the Queensland Government Department of Environment and Resource Management. We thank these organisations for their vital inputs.

3.7 S3 Supplementary material

Table S3.1: Table of Orthoptera families and habitats that they were collected from and number of readable plant sequences that were obtained using each method (Sanger and NGS) and marker (rbcL and trnH-psbA). Habitat abbreviations stand for: *Eucalyptus* dominated woodland (EuWo), Tropical rainforest (For), Tropical rainforest edge (ForEd), *Eucalyptus* forest (EuFor).

Orthopteran Family	**Habitat**	**Number of individuals**	**Sanger:** rbcL	**Sanger:** trnH-psbA	**NGS:** rbcL	**NGS:** trnH-psbA
Gryllidae	EuWo	25	3	3	23	6
Gryllidae	For	6	0	0	3	1
Gryllidae	ForEd	3	0	0	3	2
Gryllidae	EuFor	2	0	0	0	1
Gryllacrididae	EuWo	2	0	1	2	1
Gryllacrididae	ForEd	1	1	0	1	0
Tettigoniidae	EuWo	5	0	0	2	1
Tettigoniidae	ForEd	1	1	1	1	0
Tettigoniidae	EuWo	3	1	2	3	3
Tettigoniidae	EuFor	2	1	0	1	0
Acrididae	EuWo	5	0	1	4	5
Acrididae	EuFor	1	0	0	1	1
Acrididae	EuWo	32	4	5	28	12
Tetrigidae	For	3	0	1	1	0
Tetrigidae	ForEd	3	0	0	3	1
Pyrgomorphidae	EuWo	2	1	2	2	1
Total:		96	12	16	78	35

Protocol Material S3.2: Solutions list (A) and detailed laboratory protocol for DNA extraction methods (methods used by Shapcott et al. (2015) and consistent with Ivanova, Fazekas and Hebert (2008) and Kress et al. (2009).

A. Solutions list

Insect Lysis Buffer

To make 200 ml:

- GuSCN 700 mM – 16.5 g
- EDTA 30 mM pH 8.0 – 12 ml
- Tris-HCl 30mM pH 8.0 - 5 ml
- Triton-x-100 0.5% - 1 ml
- Tween-20 5% - 10 ml
- Na2SO3 54 mM – 0.13g
- PVP 4000 1% - 0.5 ml
- Molecular grade water – make up to 200ml

Binding Buffer

To make 500 ml:

- GuSCN 6 M – 354.6 g
- EDTA 20 mM pH 8.0 – 20 ml
- Tris-HCl 10 mM pH 6.4 – 50 ml
- Triton-x-100 4% - 20 ml
- Molecular grade water – make up to 500ml

Plant Binding Buffer

To make 96 ml:

- Binding buffer – 80 ml
- ddH20 – 16 ml

Protein Wash Buffer

To make 100 ml:

- Binding buffer – 50 ml
- EtOH 96% - 50 ml

B. DNA extraction protocol

Place whole specimens were placed into each tube of a 96 well block along with 5-8 (2.3 mm) disposable zirconia/silica beads and 0.1 mm of zirconia/silica powder (to assist with the grinding process). Place lids onto tubes and then freeze the whole block using liquid nitrogen for 2 minutes. Immediately after freezing, disrupt the samples using an automated TissueLyser (25 Hz for 1 minute). Repeat this 2 – 3 times, or until tissue is reduced to powder form. Centrifuge the plate for 3000 rpm for 2 minutes and then add 300 ul of Insect Lysis Buffer to each well. Tape hard plastic cover onto the well plate and mix once by gentle inversion then centrifuge at 3000 rpm for 1 minute. Add cap lids to the plate and then place the plate into an incubator at 56°C for a minimum of 6 hours (or overnight) and turn on gentle shaker. Once incubated, centrifuge at 2500 rpm for 4 minutes to remove the condensation from the cap lids. Add 200 ul of Plant Binding Buffer to each well of a new Eppendorf plate and transfer 100 ul of the lysate to the new plate. Incubate at room temperature for 5 minutes. Mix the lysate solution by pipetting 5 – 10 times and then transfer all lysate into a PALL GF Filter plate, which sits on top of a square well catch plate. Seal this plate and then centrifuge at 3000 rpm for 5 minutes to bind the DNA to the GF membrane. Add 180 ul of Protein Wash Buffer to each well and then seal and centrifuge at 3000 rpm for 2 minutes. Dispose of catch plate waste then replace catch plate and add 750 ul of Wash

Buffer to each well. Seal with a new cover and centrifuge at 3000 rpm for 5 minutes. Discard of the catch plate. Remove the seal from the GF filter plate and incubate at 56°C (along with a bottle of 0.1 Tris pH 8) for 30 minutes to evaporate any residual ethanol. Place the plate on top of a labelled PCR collection plate and add 200 ul of pre-warmed 0.1 Tris pH 8 to each well and incubate at room temperature for 1 minute. Seal and centrifuge for 5 minutes at 3000 rpm to collect the eluate. Place 140 ul of DNA into new tubes and store as stock at -20°C. Keep the remaining 40 ul as working stock in a PCR plate.

C. PCR cycle conditions

rbcL (normal length for Sanger sequencing)

1. 95°C for 3 mins
2. 92°C for 10 secs
3. 55°C for 10 secs
4. 72°C for 40 secs
5. Repeat steps 2 – 4 39 X
6. 72°C for 5 mins
7. Hold at 12°C

trnH-psbA (normal length for Sanger and Illumina sequencing)

1. 95°C for 3 mins
2. 92°C for 10 secs
3. 55°C for 10 secs
4. 72°C for 1 min
5. Repeat steps 2 – 4 34 X
6. 72°C for 5 mins
7. Hold at 12°C

rbcL (mini-barcode for Illumina sequencing)

1. 95°C for 3 mins
2. 92°C for 10 secs
3. 50°C for 20 secs
4. 72°C for 40 secs
5. Repeat steps 2 – 4 39 X
6. 72°C for 5 mins
7. Hold at 12°C

Table S3.3: Table of primer sequences used for amplification. PsbA_3f/trnH_05 and rbcLa_for/rbcLa_rev were used for PCR's in preparation for Sanger sequencing and psbA3_f/trnH_05 (miseq adapted) and shortened rbcL_for/rbcL_rev (miseq adapted) were used for PCR's in preparation for Illumina Miseq sequencing.

Primer name	Sequence
psbA3_f (forward) trnH_05 (reverse)	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC
rbcL*a*_for (forward rbcL*a*_rev (reverse)	ATGTCACCACAAACAGAGACTAAAGC GTAAAATCAAGTCCACCYCG
psbA3_f (forward **Miseq adapted**) trnH_05 (reverse **Miseq adapted**)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTTATGCATGAACGTAATGCTC **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACG**CGCGCATGGTGGATTCACAATCC
rbcL_for (forward **Miseq adapted**) rbcL*a*_rev (reverse **Miseq adapted**)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CTTACCAGYCTTGATCGTTACAAAGG **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACG**GTAAAATCAAGTCCACCRCG

Table S3.4: Table of Nextera XT adapter sequences used for tagging samples in preparation for Illumina Miseq sequencing.

Bases in adapter	i7 index name	Bases for sample sheet
TCGCCTTA	N701	TAAGGCGA
CTAGTACG	N702	CGTACTAG
TTCTGCCT	N703	AGGCAGAA
GCTCAGGA	N704	TCCTGAGC
AGGAGTCC	N705	GGACTCCT
CATGCCTA	N706	TAGGCATG
GTAGAGAG	N707	CTCTCTAC
CCTCTCTG	N708	CAGAGAGG
AGCGTAGC	N709	GCTACGCT
CAGCCTCG	N710	CGAGGCTG
TGCCTCTT	N711	AAGAGGCA
TCCTCTAC	N712	GTAGAGGA
CTCTCTAT	S502	CTCTCTAT
TATCCTCT	S503	TATCCTCT
AGAGTAGA	S504	AGAGTAGA
GTAAGGAG	S505	GTAAGGAG
ACTGCATA	S506	ACTGCATA
AAGGAGTA	S507	AAGGAGTA
CTAAGCCT	S508	CTAAGCCT
CCTAGAGT	S516	CCTAGAGT

Table S3.5: Table of dietary results for individual orthopterans that were successfully sequenced using both markers. Codes in the table stand for the method and marker that a sequence was obtained by: R-San = rbcL Sanger, R-ILL = rbcL Illumina, P-SAN = trnH-psbA Sanger, P-ILL = trnH-psbA Illumina. Grey squares indicate where sequences were obtained by both rbcL and trnH-psbA.

		ACRIDIDAE							GRYLLIDAE						TETTIGONIIDAE				GRYLLACRIDIDAE	
Taxonomic level	**Taxonomic ID**	**S54**	**S55**	**S25**	**S22**	**S38**	**S23**	**S15**	**S81**	**S80**	**S50**	**S14**	**S7**	**S49**	**S44**	**S71**	**S84**	**S42**	**S66**	**S64**
Species	*Achnatherum petriei*																		R-ILL	
Species	*Amaranthus hybridus*																P-ILL			
Species	*Alphitonia excelsa*							SAN-P												
Species	*Argyrodendron actinophyllum*																	P-SAN		
Species	*Austrostipa nitida*										R-ILL			R-ILL	R-ILL	R-ILL		R-ILL		
Species	*Baloghia marmorata*		P-ILL																	
Species	*Bouchardatia neurococca*													R-ILL						
Species	*Brachychiton bidwillii*															R-ILL				
Species	*Caldcluvia paniculosa*															R-ILL				
Species	*Carex horsfieldii*	R-ILL			R-ILL		R-ILL								R-ILL	R-ILL	R-ILL		R-ILL	
Species	*Cyperus rupicola*								R-ILL											
Species	*Digitaria ciliaris*																	P-ILL	P-ILL	
Species	*Diospyros geminata*						R-SAN													
Species	*Erigeron sumatrensis*																P-ILL			
Species	*Eucryphia jinksii*															R-ILL				
Species	*Euroschinus falcatus*														R-ILL					
Species	*Flindersia schottiana*										R-ILL			R-ILL						
Species	*Glochidion ferdinandi*																P-SAN			
Species	*Glycine stenophita*			P-SAN		P-SAN														
Species	*Hydrocotyle pedicellosa*			P-ILL											P-ILL/P-SAN					
Species	*Karrabina benthamiana*															R-ILL				
Species	*Melodinus acutiflorus*																P-ILL			
Species	*Microlaena stipoides*	R-ILL																	R-ILL	
Species	*Olearia canescens*								R-ILL								R-ILL			
Species	*Oplismenus aemulus*		P-ILL								P-ILL	SAN-P							P-ILL	
Species	*Oplismenus compositus*	R-ILL																		
Species	*Oplismenus undulatifolius*																		P-ILL	
Species	*Oxalis corniculata*			P-ILL		R-ILL/P-ILL													P-SAN	
Species	*Palmeria racemosa*																		R-SAN	
Species	*Panicum bisulcatum*																		P-ILL	
Species	*Panicum pygmaeum*	R-SAN			R-SAN				R-SAN				R-SAN							
Species	*Phyllanthus tenellus*																R-ILL			
Species	*Plectranthus nitidus*																			P-SAN
Species	*Poa costiniana*	R-ILL	R-ILL						R-ILL	R-ILL	R-ILL			R-ILL						
Species	*Poa sieberiana*																		R-ILL	
Species	*Pomaderris notata*															R-ILL/R-SAN				
Species	*Pseudoweinmannia lachnocarpa*																			R-ILL/P-SAN
Species	*Rhagodia parabolica*													P-ILL						
Species	*Schinus terebinthifolia*														P-ILL					
Species	*Sicyos australis*									R-SAN					R-SAN					
Species	*Solanum furfuraceum*	R-ILL												R-ILL						
Species	*Solanum opacum*	R-ILL						R-ILL						R-ILL						
Species	*Urtica incisa*								R-ILL							R-ILL/P-SAN				
Species	*Vulpia bromoides*																		R-ILL	
Genus	*Actephila sp.*														R-ILL					
Genus	*Arundinella*																		P-ILL	
Genus	*Austrostipa sp.*	R-ILL							R-ILL					R-ILL						
Genus	*Carex sp.*									R-ILL			R-ILL	R-ILL					R-ILL	
Genus	*Eugenia sp.*													R-ILL						
Genus	*Glycine sp.*		P-ILL	R-ILL/P-SAN		R-ILL/P-SAN				P-ILL										
Genus	*Hydrocotyle sp.*			R-ILL/P-ILL											R-ILL/P-ILL/P-SAN					
Genus	*Medicosma sp.*										R-ILL			R-ILL						
Genus	*Oxalis sp.*			R-ILL/P-ILL		R-ILL/P-ILL														
Genus	*Plectranthus sp.*																			R-ILL/P-SAN
Genus	*Poa sp.*	R-ILL	R-ILL/P-ILL/R-SAN				R-ILL				P-ILL/P-SAN			P-ILL/P-SAN					R-ILL/P-ILL	
Genus	*Sauropus sp.*																R-SAN			
Genus	*Solanum sp.*																R-ILL		R-ILL	
Genus	*Syzygium sp.*													R-ILL						
Genus	*Wahlenbergia sp.*																	R-ILL		
Family	Acanthaceae						R-ILL						R-ILL							
Family	Asteraceae														R-ILL					
Family	Cunoniaceae								R-ILL/P-ILL							R-ILL/P-ILL/R-SAN				
Family	Fabaceae											R-ILL								
Family	Poaceae	R-ILL/P-ILL/R-SAN	R-ILL/P-ILL/R-SAN/P-SAN				R-ILL	R-ILL	R-ILL/R-SAN		R-ILL/P-ILL/P-SAN	R-ILL/P-SAN		P-ILL/P-SAN	R-ILL/R-SAN	R-ILL	R-ILL	R-ILL/P-ILL	R-ILL/P-ILL	
Family	Rosaceae													P-ILL						
Family	Trebouxiophyceae									R-ILL										
Family	Lamiaceae						R-ILL													
Family	Lauraceae																	P-ILL		

Chapter 4

Chapter 4 is a co-authored paper which has been **prepared** for publication. The bibliographic details of the co-authored paper, including all authors, are:

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My contribution to the paper involved all field work to collect specimens, all laboratory work to sequence the samples and all data processing and data analysis. Associate Professor Alison Shapcott supervised the project, trained me in the lab both in preparation and during sequencing the samples. Dr. Sarah Maunsell assisted with field work, lab work and general supervision of the project. Professor Roger Kitching and Professor Nigel Stork supervised this project and were responsible for direction and guidance in regard to the design of the project and scope and structure of the manuscript.

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Chapter 4: What's eating what? A diet assessment of Orthoptera using rbcL and trnH-psbA

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4.1 Abstract

Insect herbivory is of prime importance for ecosystem functioning, but the interactions between insects and plants can be difficult to measure. Orthoptera (grasshoppers and crickets) are an abundant insect Order that are largely herbivorous, yet little is known about their diets in natural environments. Here, we use a metabarcoding approach to compare the use of two gene markers (rbcL and trnH-psbA) for the purpose of identifying Orthoptera diets. We investigate each marker in terms of the diversity and structure of plant species observed across the same samples recovered from Orthoptera. Additionally, we carry out the first dietary assessment of two coexisting Orthoptera genera: Merrinella (family: Gryllidae) and Methiolopsis (family: Acrididae), from an open eucalypt woodland habitat in southeast Queensland. We found that the rbcL marker performed better than trnH-psbA. The number of samples where a readable sequence was retrieved was greater with rbcL (65 samples, compared to 36) and a greater diversity within the diets was identified when compared to

trnH-psbA. We also found that *Merrinella* sp. and *Methiolopsis* sp. are both broad generalists occupying the same habitat type but their dietary overlap (measured by Pianka's overlap index at a plant family level) was low at 0.096, suggesting that there is little competition for resources between these two species. As our food webs are not complete using these two markers, we suggest using a three-marker method for future insect herbivore studies, especially when host plants are previously unknown. Gaining further information about orthopteran interactions and feeding ecology is important for the greater understanding of herbivory, species competition and niche-overlap in communities and ecosystems and DNA metabarcoding is an excellent tool to aid these studies.

4.2 Introduction

Plants and herbivores represent the major component of eukaryote biodiversity (Futuyma & Agrawal 2009) within the so-called 'green' food web (Zou et al. 2016). They form the basis of virtually all terrestrial food webs. Despite this, the trophic interactions of many species are poorly understood or identified with only limited confidence. Insect herbivores are of prime importance for ecosystem functioning and stability: herbivory influences biodiversity and productivity (Ewers et al. 2015; Worm & Duffy 2003) and the insects themselves are one of the major conduits of energy flow to higher trophic levels (Futuyma & Agrawal 2009). Variation in insect herbivore diets mediates the coexistence of competitors and can influence the stability of ecosystem networks (Buchi & Vuilleumier 2014). The top-down effects of predators on primary production can be controlled by the level of herbivore specialisation (Singer et al. 2014). Identifying and quantifying these complex interactions is challenging (Matheson et al. 2008) but is fundamental to our understanding of food webs and ecosystem structure and function (De la Cadena et al. 2017; Juen & Traugott 2006). Further, the relationship between insect species and their plant hosts, although frequently used as a basis

for estimates of global species richness of insects and all eukaryotes (Erwin 1982; Stork 2018; Stork et al. 2015), is only beginning to be understood (Forister et al. 2015).

The feeding preferences of arthropods are difficult to study (Matheson et al. 2008). However, in the late 1990s, it was first demonstrated that DNA from prey items from within the gut of invertebrate predators could be detected using the polymerase chain reaction (PCR) (Zaidi et al. 1999). This was followed by studies utilising this method to investigate the feeding ecology of a variety of predator species (e.g. Symondson 2002; Juen and Traugatt 2006). This period saw the widespread use of cytochrome C oxidase (CO1) 'barcoding' within insect taxonomy (Hebert et al. 2003) and predator diet analysis (Traugott et al. 2013). In contrast, however, the use of the CO1 gene for plant identification was deemed ineffective for species-level discrimination as the mitochondrial genes in plants have a lower rate of sequence change than that of animals (Kress et al. 2005; Kress and Erickson 2007). Finding a plant equivalent to the CO1 gene has proved difficult (Hollingsworth, PM, Graham & Little 2011).

The chloroplast trnL intron has been suggested as an appropriate target region (Taberlet et al. 2007) and, subsequently eight different plant species were successfully identified from the gut contents of insect herbivores with the use of the chloroplast rbcL gene (Matheson et al. 2008). Since then, three regions of the chloroplast genome (rbcL, matK and trnH-psbA), and the nuclear ITS region have been widely used for plant identification as they show high levels of interspecific divergence (Kress et al. 2005; Hollingsworth et al. 2011). The ITS2 region alone has been highly discriminatory for medicinal plant (Chen et al. 2010) and pollen species (e.g. Sickel et al. 2015) identification, and trnH-psbA was found to be the most viable candidate for a single-locus barcode when testing 43 different plant families that included angiosperms, gymnosperms, ferns, mosses and liverworts (Kress, WJ & Erickson 2007). There remains, however, no consensus on a single locus that can discriminate to the species

level with the same success as the CO1 gene in animals (Herbert et al. 2003; Kress and Erickson 2007). Therefore, it has been suggested that the use of multiple genes (e.g. Kress and Erickson 2007; Kress et al. 2009) is the most successful way to confirm taxonomic assignments. Complementing a rapidly evolving locus such as the trnH-psbA spacer with a more conservative locus (such as the coding locus rbcL) may minimise these errors. RbcL can discriminate at the family or genus level and trnH-psbA can then be used for species identification (Galimberti et al. 2016; Kress, WJ & Erickson 2007), although with an accurate reference library, rbcL has been used to successfully identify to the species level (de Vere et al. 2017). The choice and number of DNA gene markers used for amplification has varied in herbivore diet studies. For example, rbcL and ITS were used to investigate the diets of rolled-leaf beetles (Chrysomelidae) (Garcia-Robledo et al. 2013); trnH-psbA and rbcL were used to determine the diets of weevils (Curculionidae) (Kajtoch 2014); trnL-P6 and ITS were used to evaluate niche partitioning in large African herbivores (Kartzinel et al. 2015). For metabarcoding, standard barcode markers are often too long for the requirements of the sequencing platform and require modification for their use (Erickson et al. 2017). Using shortened barcode markers can increase the recovery of degraded DNA (Garcia-Robledo et al. 2013) and although the use of the mini-rbcL barcode is 30% shorter than the full length rbcL marker, it retains 90% of the identification power and is highly effective in species discrimination (Erickson et al. 2017).

Orthoptera (grasshoppers and crickets) have a wide range of feeding habits, with some species being broad generalists and others having very specialised diets (McClenaghan et al. 2015). As an abundant insect Order that occupy many habitat types, particularly grasslands and forests, it is important to gain an understanding of their feeding behaviours, given that insect herbivores influence ecosystem functioning (McClenaghan et al. 2015). High herbivore diversity can be promoted if insect-plant interactions are specialised, as finely partitioned

resources can promote species coexistence (Lewinsohn & Roslin 2008). On the contrary, generalist herbivores are thought to mediate indirect interactions, such as apparent competition (Holt 1977). This is an indirect interaction where the presence of one species lowers the density of another, even when there is no direct competition, due to increased predation pressures (Joern 1979). It is thought that this may structure assemblages when resource competition is not pervasive (Abrams et al. 1995). Amongst generalist Orthoptera occupying the same habitats, studies have found dietary niche overlap to be low, indicating that competition may be reduced by different species utilising different resources (McClenaghan et al. 2015). However, there are few studies on this Order to support this. Due to the power that DNA barcoding offers in revealing the strength of trophic interactions (Erickson et al. 2017), we use metabarcoding for the identification of plants from the gut contents of Orthoptera, sampled from a range of sub-tropical habitats in southeast Queensland, Australia. Using a customised plant reference database for the area, we compare the performance of the chloroplast coding rbcL and the non-coding trnH-psbA region. These markers were chosen as these had been used successfully for barcoding plants within the region (Shapcott et al. 2015) and for successful barcoding of mammalian herbivore diets (Galimberti et al. 2016). We address to what extent the two markers complement each other by detecting the same plant species within the diets of Orthoptera and carry out a detailed diet assessment for the first time for two abundant species of Orthoptera (*Merrinella* sp. (family: Gryllidae) and *Methiolopsis* sp. (family Acrididae)) from a sub-tropical eucalypt woodland habitat.

4.3 Methods

Sample collection

Orthoptera were collected from a eucalypt open woodland and sub-tropical rainforest in Lamington National Park (28° 13’ S 153° 08’E), and a eucalypt forest in Toohey Forest (27°

32’ S 153° 03 E), within southeast Queensland, Australia (Table 4.1). In each case sweep-netting was used as a collecting method. The different habitat types were chosen so as to collect a broad range of species that would be feeding on a wide range of plants, to test the performance of the markers. Forty-six individuals were collected from the Suborder Caelifera and sorted into the families Acrididae, Tetrigidae and Pyrgomorphidae, and fifty individuals were collected from the Suborder Ensifera and sorted into the families Gryllidae, Gryllacrididae and Tettigoniidae (Supplementary Table S3.1 from Chapter 3). Immediately after collection, each specimen was placed into 98% ethanol and stored at −20ºC within 4 hours of collection.

Table 4.1: Descriptions of habitat types where sampling was carried out (Nelder et al. 2017).

Habitat Type	Description
Rainforest	Closed forests characterised by dense foliage and high plant diversity of predominantly non-sclerophyllous species
Eucalypt woodland	Form transitional zone between higher rainfall and forested margins. Open woodland with understoreys of grasses to shrubs.
Eucalypt forest	Characterised by tall eucalypts and their relatives (*Corymbia, Angophora, Syncarpia and Lophostemon*). Canopy cover of 50-80%. Shrub layer hard-leaved and fire tolerant.

Plant reference library

As described in Chapter 3, a customised reference database of plant barcodes was created for each of the plant gene markers. This was compiled using a comprehensive DNA barcode library of southeast Queensland rainforest plants, created by Shapcott et al. (2015) and Howard et al. (2016). This reference database was supplemented with additional plant species based on comprehensive local lists and regional ecosystem maps for the area Neldner et al. (2017) which were downloaded from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). For the specific sample locations, the rbcL reference library contained 3606 species and the trnH-psbA reference library contained 1416 species. A separate database was created using all sequences available from the NCBI website for each marker to use for samples that did not successfully match to any sequences in the smaller customised reference library. These contained approximately 100,000 species for each marker.

Sample preparation and DNA extraction

Before DNA extraction, all Orthoptera specimens were rinsed with 98% ethanol to remove any external plant DNA. Whole genomic DNA was extracted from the entire bodies of all 96 specimens following methods used by Shapcott et al. (2015) and consistent with Ivanova, Fazekas and Hebert (2008) and Kress, J et al. (2009). For solutions list and detailed laboratory protocol see Supplementary Material S3.2. Extracts were tested for the presence of DNA using gel electrophoresis (1%) and viewed on agarose gel under UV light, and then stored at −20°C.

PCR amplification

Limited read lengths of the Illumina Miseq platform meant that shortened primers were necessary for rbcL; therefore, we used mini-barcodes derived from the standard rbcL plant

DNA barcode, which produce a 379bp product. We used a forward primer, CTTACCAGYCTTGATCGTTACAAAGG designed by Garcia-Robledo et al. (2013) and used by Erickson et al. (2017), with the standard rbcL reverse primer, GTAAAATCAAGTCCACCRCG. The standard trnH-psbA barcode sequence was short enough for use on the Illumina Miseq. The forward primer sequence used for trnH-psbA was GTTATGCATGAACGTAATGCTC and the reverse was CGCGCATGGTGGATTCACAATCC. PCR amplification tests were performed following established methods used by Shapcott et al. (2015) in standard 96-well plate formats (for further detail on PCR cycle methods, see Chapter 3 'PCR amplification'). A standard PCR cycle was followed for each primer with the annealing temperature at 50^{o}C for rbcL (40 cycles) and trnH-psbA (34 cycles). PCR success was tested by electrophoresis on a 1% agarose gel with ethidium bromide and viewed under UV light on a Molecular Imager Gel Doc XR Plus with Image Lab™ Software (v6.0). A clear band of expected base-pair length indicated amplification. In preparation for Illumina Miseq sequencing, all PCR primers were 'Miseq-adapted' with 5' overhangs complementary to Nextera index primers to allow sample identification with individual molecular tags, as used by de Vere et al. (2017) and (Erickson et al. 2017) (Supplementary Table S3.4 from Chapter 3). Due to the degraded nature of gut content DNA, in order to reduce PCR failures, two PCRs were carried out for each sample for both markers and the products were pooled for sequencing. All product from all samples were kept from this stage, whether showing as successful on the gel or not. As the Illumina Miseq is highly sensitive to low concentrations of DNA (Shokralla et al. 2015), we sequenced all samples, even those that did not show up on agarose gels. For full details of our sequencing protocol, see Chapter 3 'Illumina Miseq (metabarcoding) protocol and sequence processing' section.

Data processing

USEARCH v10.0.240 (Edgar 2010) was used for processing samples. Sequences were screened for quality by filtering out any with a quality (Phred) score of <20 (referring to the probability of an incorrect base call as less than 1 in 100) or a length of <200bp using fastq_filter, -fastq_minlen and -fastq_maxns commands. Forward and reverse reads were joined to form contigs using the fastq-join command. Remaining sequences were matched against our marker-specific reference libraries at 99 and 100% similarity using the -usearch_global command. Additionally, in an attempt to broaden the number of samples with taxonomic assignments, sequences without a hit were kept and these were matched against our full databases for each marker at 99 and 100%. We converted the output for each marker and each match percentage into a single matrix showing the number of reads for each sequence with rows as plant species and columns as samples. We only considered matches with a minimum of 10 reads assigned to reduce results arising from amplification or sequencing errors (CBOL Plant Working Group, 2009; Hawkins et al. 2015). Additionally, we only considered species assignments that were recorded as being found within the sampling locality by Queensland Regional Ecosystem maps and *ATLAS of living Australia* (http://www.ala.org.au/). Following Soininen et al. (2017), when we identified a species that was not present in the study area, we adjusted the taxonomic assignment to a higher taxonomic level. Genus-level assignments were made if the genera had previously been recorded in the sampling area, but the species had not, and family-level assignments were made if only the family had been recorded in the sampling area, but the genus had not.

Data analysis

Using our sequence outputs (matrices of operational taxonomic units (OTUs) for each plant taxonomic assignment and Orthoptera samples) for each marker, analyses were carried out in

'R' v 3.4.1 (R Development Core Team 2014). Plots were created using the 'ggplots2' package (Wickham 2009), unless otherwise stated. Comparative analyses were carried out on all samples where OTUs were obtained by both markers (38 samples). We compared diversity across samples by calculating the Shannon-Wiener Index (H) using the 'VEGAN' package (Oksanen et al. 2008) and tested for differences across the two markers using a two-sample t-test. To test for differences in taxonomic level (e.g. species, genus or family) that could be assigned to a sample by each marker, we used Chi Square Tests of Independence. Before comparing the dietary composition of the two morphospecies of Orthoptera (*Merrinella* sp. and *Methiolopsis* sp.), species accumulation curves were plotted using a sample-based rarefaction method. To do this, we used the 'specaccum' function in the 'Vegan' package in 'R' using the 'exact' method (Oksanen 2018), which shows the mean dietary richness. We compared the dietary composition of *Merrinella* and *Methiolopsis* as determined by the two different markers by calculating Jaccard's index and Jaccard's nestedness partition (using presence/absence sequence data). Dietary composition differences for these two morphospecies, across markers, were tested using Analysis of Variance (ANOVA). Additionally, for *Merrinella* sp. and *Methiolopsis* sp., detailed food webs were constructed using the 'Bipartite' package (Dormann et al. 2009). For these two species, we used the numbers of OTUs obtained for each plant family to calculate dietary similarity across the two species using Pianka's index (Pianka 1973):

$$O_{jk} = \Sigma p_{ij}\, p_{ik} / (\Sigma p_{ij}^2 \Sigma p_{ik}^2)^{1/2}$$

where pi is the frequency of occurrence of prey item i in the diet of species j and k. Pianka's index (O) varies between 0 (total separation) and 1 (total overlap).

4.4 Results

Overall, we identified 111 plant species, from 29 families from the gut contents of 69 individual orthopterans. When comparing results produced by rbcL and trnH-psbA, we found a greater number of plants were identified, and a greater number of sequences were assigned taxonomy using the rbcL marker (Table 4.2). Alpha diversity per sample was significantly greater using rbcL, when compared to trnH-psbA (Figure 4.1: $t = 6.846$, $df = 65.466$, $p = < 0.01$). When comparing sequences that had been assigned taxonomy, there was no difference in the proportions of sequences that were assigned to a plant species, genus or family across the two markers (Figure 4.2: X^2: 0.768, $df = 2$, $p = 0.681$).

Table 4.2: Summary statistics of DNA output data. *number of samples where taxonomy was assigned to our personalised database, **number of samples where taxonomy was assigned using our database and the full database for that marker.

	rbcL	**trnH-psbA**
Total number of reads passing filter	1.78M	1.92M
Number of sequences assigned taxonomy	309K (17.4%)	143K (7.4%)
Number of samples where taxonomy was assigned (N=>10) at >99% similarity	60*/65**	19*/36**
Total number of samples sequenced by at least one marker (n=96)	69 (71.9%)	

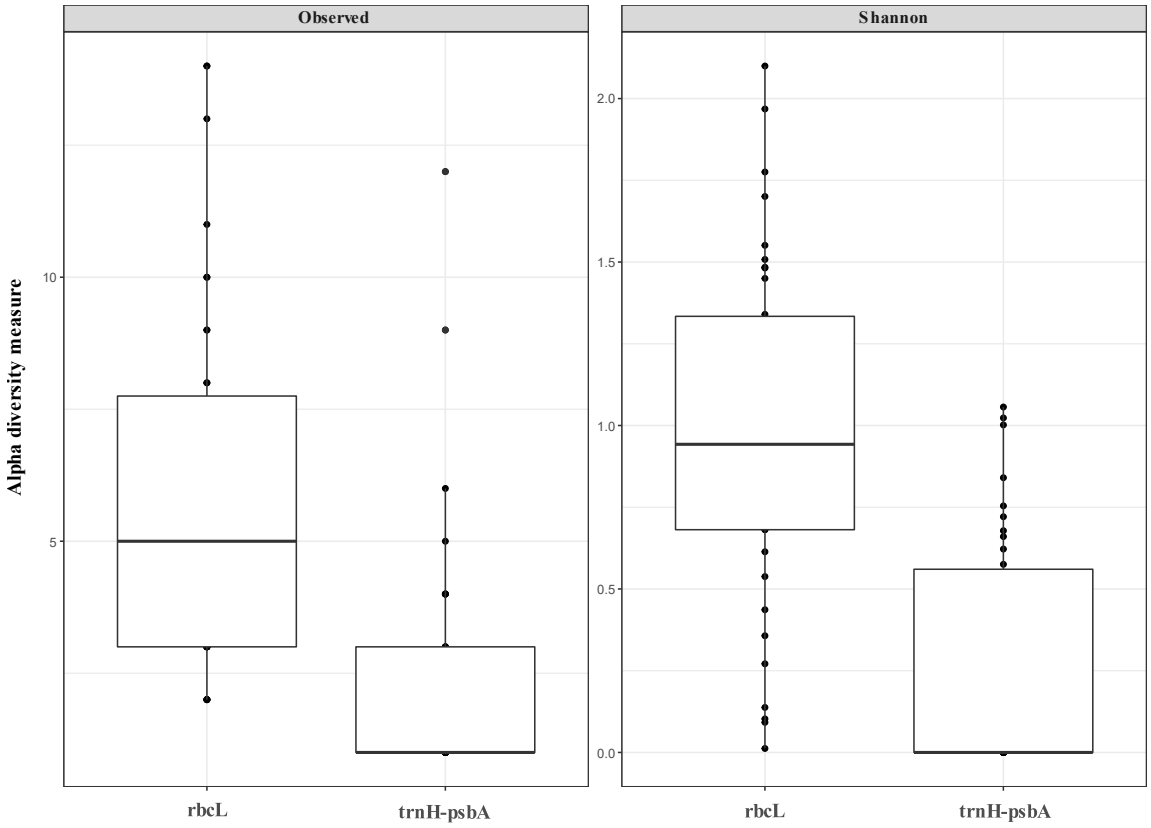


Figure 4.1: Observed dietary diversity (left) and Shannon-Wiener Index (right) per individual Orthoptera, for each marker.

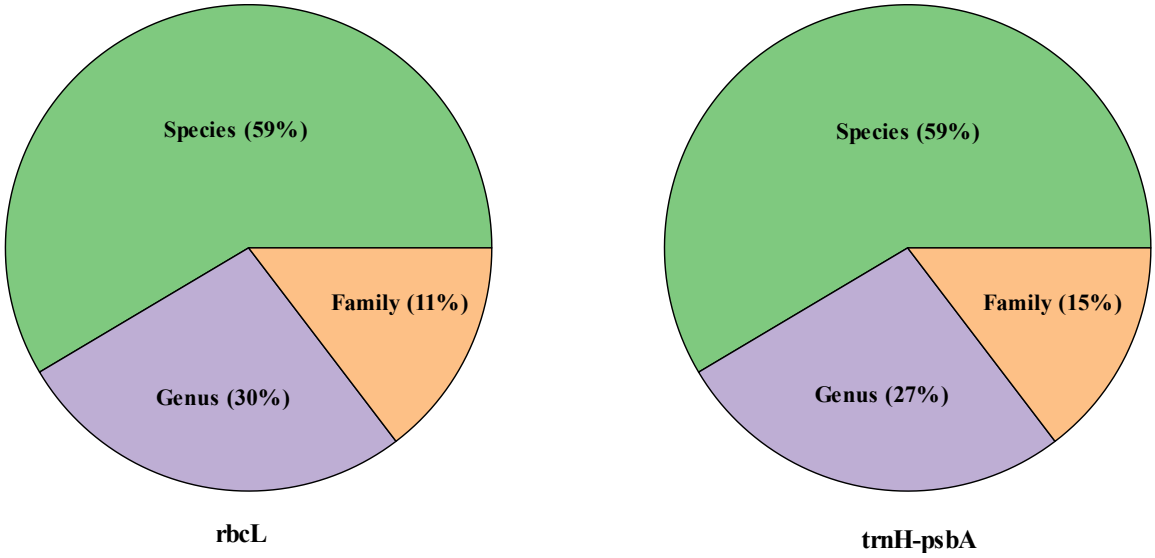


Figure 4.2: Proportions of plants (n = 111) from gut contents (n = 69) identified to species, genera and family- level using for each marker (rbcL: left, trnH-psbA: right).

Within the diets of the range of orthopterans that yielded identifiable plant sequences, we found various different plant types including grasses, herbs, trees, climbers/vines, trees/shrubs and green algae. For definitions of these categories see Supplementary Material S4.1. To investigate which plant groups could be identified by each marker, we compared the proportions (based on number of unique OTUs within each group, rather than abundance of sequences) that were found in each sample, and these were found to vary. Overall, trees/shrubs and grasses were the most common dietary plant type across the samples (Figure 4.3). rbcL identified a higher proportion of trees/shrubs and was the only marker to identify any sedges and algae (although the latter only occurred in one sample). TrnH-psbA identified a higher proportion of grasses and herbs. The proportion of climbers/vines identified were very similar across the two markers (Figure 4.3). Overall, plant types identified by each marker were not significantly different ($X^2 = 7.99$, $df = 4$, $p = 0.09$).

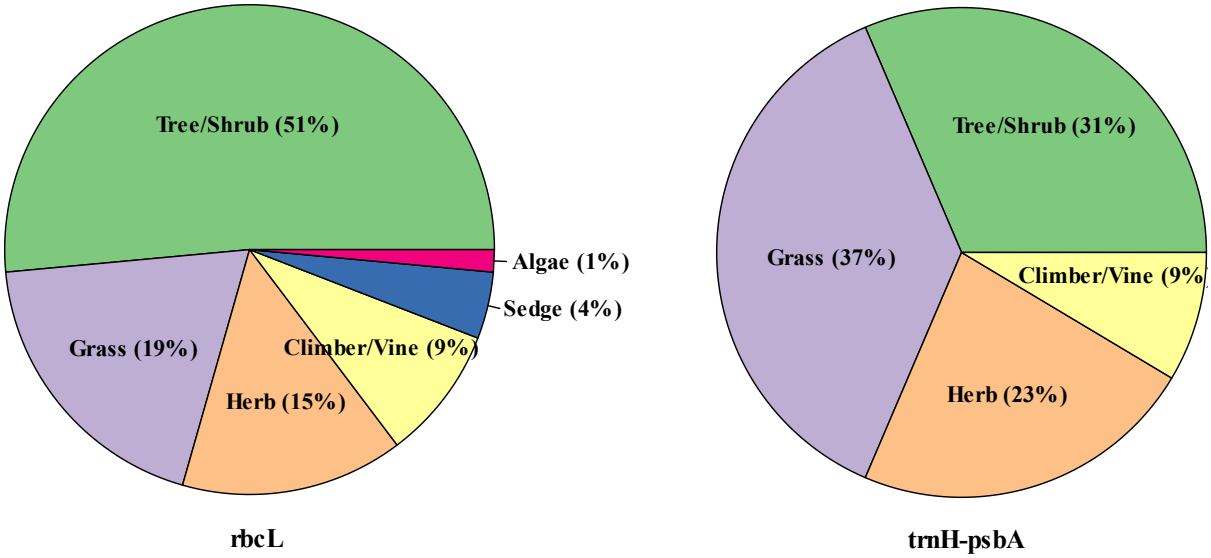


Figure 4.3: Proportions of different plant types (n = 111) identified in samples (n = 69) using the two gene markers (rbcL: left, trnH-psbA: right).

Next, we investigated, in detail, the diets of the two most common species of orthopteran in our dataset. This included 11 individuals from the genus *Methiolopsis* from the Acrididae (grasshopper) family and seven individuals from the genus *Merrinella*, from the Gryllidae (cricket) family. Although from different families, these two genera occupy the same habitat type – a sub-tropical eucalypt woodland in Lamington National Park. First, we assessed whether we had captured the full dietary richness of these species using accumulation curves (Figure 4.4). The curves suggest that both of these species have very broad diets and that we would need a greater sample size to find out the true diet breadth. When comparing the measure of dietary nestedness across the two markers, we found it to be significantly different for *Merrinella* sp. (ANOVA: $F_{1,12} = 6.18$, $p = 0.029$) (Figure 4.5), but not for *Methiolopsis* sp. (ANOVA: $F_{1,20} = 0.58$, $p = 0.45$) (Figure 4.5).

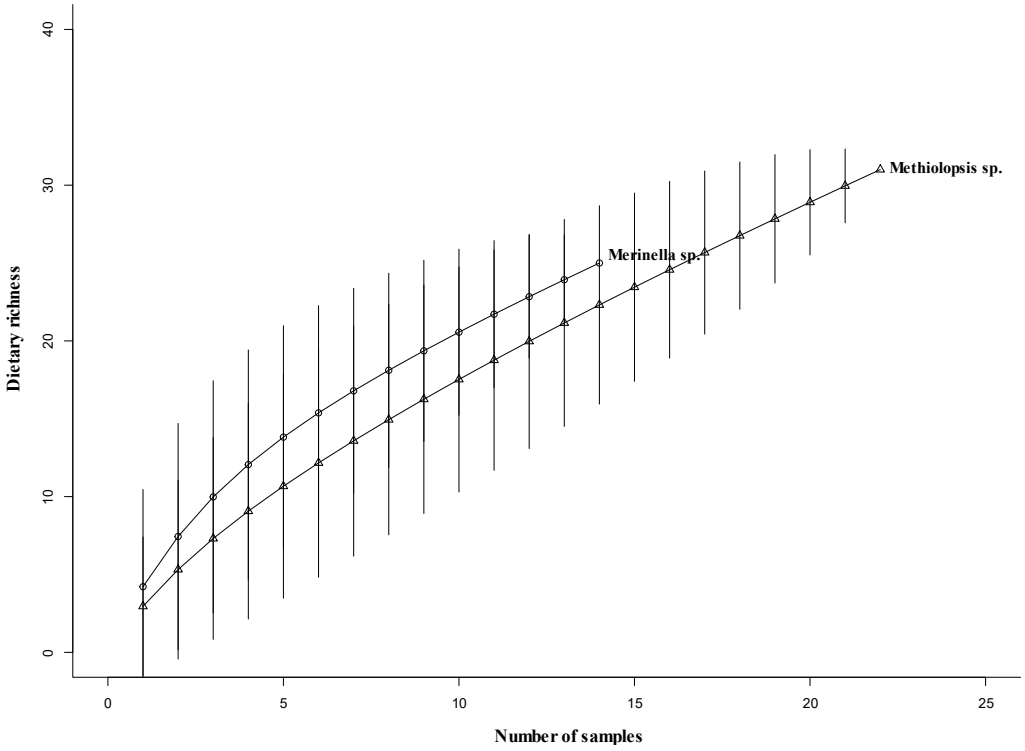


Figure 4. 4: Species accumulation curves based on dietary richness. Dietary richness increases with number of samples that were sequenced for two species of Orthoptera (*Merrinella* sp. *and Methiolopsis* sp.)

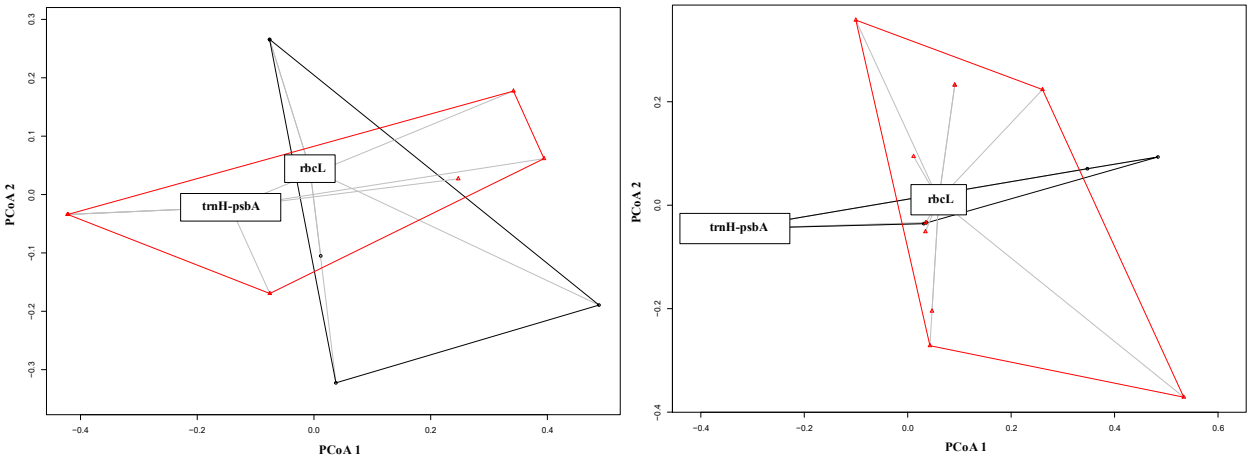
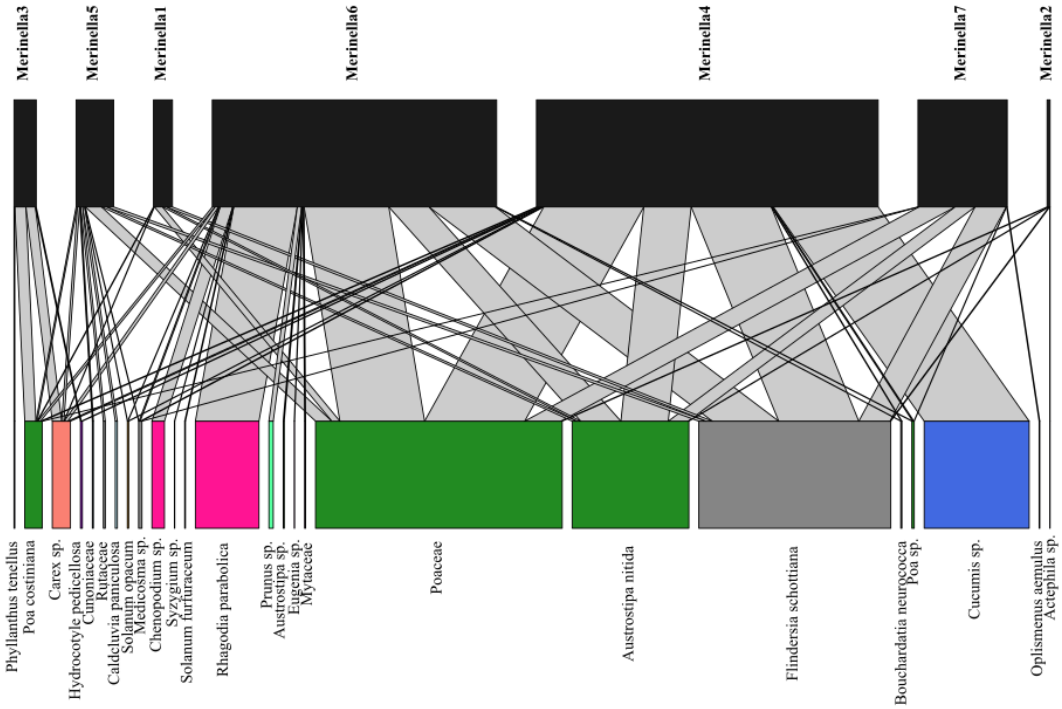


Figure 4.5: PCoA shows differences in dietary nestedness across the two markers for *Merrinella* sp. (left) and *Methiolopsis* sp. (right).

By combining the two markers and creating food webs for *Merrinella* sp. and *Methiolopsis* sp. we identified some of the most important food types for both species. Grasses were the most common food type in the diets (as indicated by the green boxes in Figure 4.6A and B) but many other plant families were also abundant within the diets of both species. Sequences of Rutaceae were abundant in the diets of three *Merrinella* individuals (Figure 4.6A, grey box), and sequences of Euphorbiaceae were abundant in the diets of three *Methiolopsis* individuals (Figure 4.6B, navy blue box). From seven individuals of *Merrinella* sp. we identified 11 different plant families and from 11 individuals of *Methiolopsis* sp. we identified 14 different plant families (for plant list see Supplementary Material S4.2). Overall, dietary similarity between the two species was low (Pianka's index: 0.096).

A)



B)

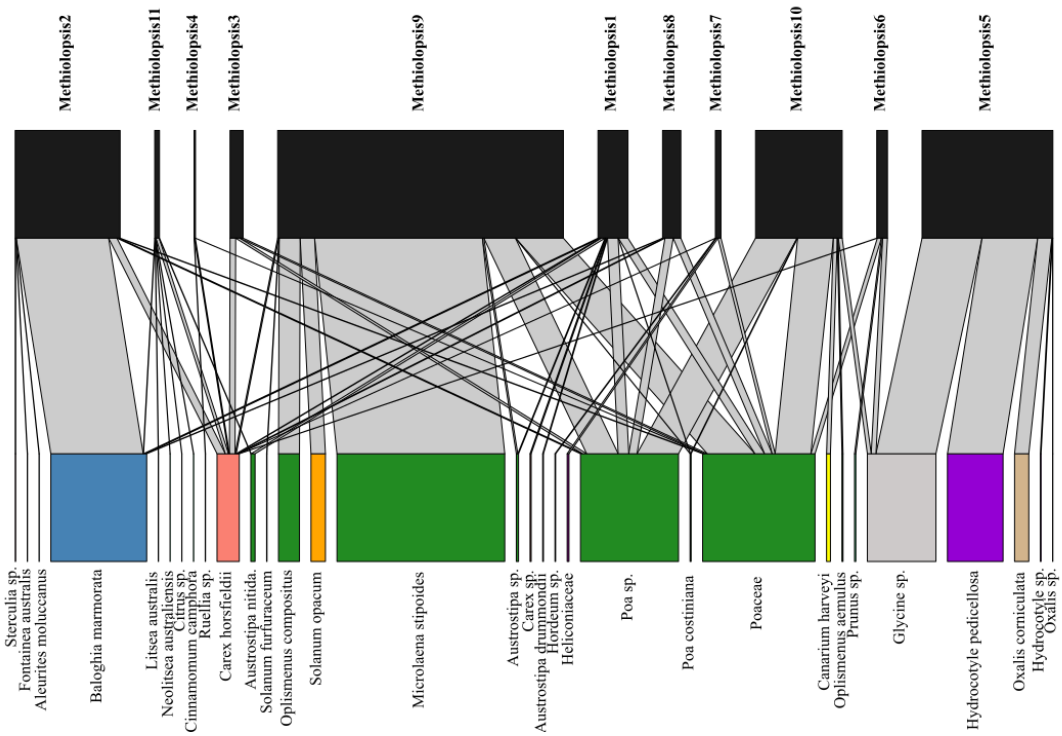


Figure 4.6: Food webs created using proportional sequence data for *Merrinella* sp. (A) and *Methiolopsis* sp. (B). Different colours represent different plant families.

4.5 Discussion

Metabarcoding is an excellent tool for increasing knowledge of insect-host interactions in the natural environment (Mollot et al. 2014), which are otherwise difficult to measure. Our study is one amongst a handful of others (beetles: Jurado-Rivera et al. 2009; Garcia-Robledo et al. 2013; Kishimoto-Yamada et al. 2013; Kajtoch 2014, grasshoppers: McClenaghan et al. 2015, and bees: Hawkins et al. 2015; de Vere et al. 2017) that have used DNA barcoding techniques to reveal the host plants of insect herbivores. Only a few studies have utilised metabarcoding (de Vere et al. 2017; Hawkins et al. 2015; Kajtoch 2014; McClenaghan et al. 2015) rather than the traditional Sanger sequencing method. We founnd that, rbcL and trnH-psbA can reveal different dietary composition for the same set of species, which could be linked to

which plants are available in reference libraries, or because different gene markers can detect different plants (Hollingsworth, ML, Andra Clark, et al. 2009). For both these reasons, when studying generalist species, the use of two or more gene markers is necessary to create the most complete food web possible.

Studies have had varying results in terms of plant species resolution from using different gene markers across various habitat types and plant groups. The trnH-psbA region alone has been highly effective in many studies, showing between 90% and 100% species resolution (Kress and Erickson 2005, Kress et al. 2009, Fazekas et al. 2008). However, the trnH-psbA marker (along with the nuclear ITS2) also has low levels of universality, meaning that in mixed samples some species are likely to be undetected (Coissac, Riaz & Puillandre 2012). Therefore, for our food web created with results from trnH-psbA, we expected species identification errors to be low, but that not all species within each sample were identified. Our results were consistent with this as we had greater species diversity per sample using the rbcL marker. RbcL is known as a core barcode and has been shown to have the highest universality of proposed markers (CBOL 2009). Some experimental studies using the rbcL region, however, have found it to demonstrate insufficient sequence variation to distinguish among closely related species (e.g. Kress and Erickson 2007; Kress et al. 2009). Therefore, we can be less confident with species-level identifications using rbcL when they did not match with plants identified using trnH-psbA. We agree with Kress et al. (2009) that a three-locus barcode using rbcL, trnH-psbA and MatK (or ITS2, which has proved successful in other studies e.g. Sickel et al. 2015) would be most appropriate for maximising confidence in species identities and constructing more complete food webs.

A key factor to increase species identities using DNA metabarcoding is the comprehensiveness and quality of the reference database that the sequences are compared to (de Vere et al. 2017). The flora of southeast Queensland is well documented (Neldner et al.

2017; Shapcott et al. 2015) and therefore assisted us with the correct species identities of host plants. However, as comprehensive reference libraries only tend to exist for specific geographic ranges (e.g. Shapcott et al. 2015) or taxonomic groups (e.g. Garcia-Robledo et al. (2013)), many taxa are yet to be barcoded and this will be the case for some time to come.

In addition to underscoring the importance of the use of multiple markers to increase the completeness of a food web, our results present the first partial food webs for the diets of two orthopteran genera (*Merrinella* sp. and *Methiolopsis* sp.). We demonstrate, that by using metabarcoding, host plants can be identified from the diets of insect herbivores from their natural habitats. This method has the potential to determine how generalist herbivore diets differ across species, as well as any changes spatially and temporally. With this information, better conclusions can be made about how herbivore diets impact community structure and assemblages. Insect herbivores are often grouped based on their degree of dietary specialisation, but, in the past, this has been based on fairly arbitrary observations (Ali & Agrawai 2012). We found that both *Merrinella* sp. and *Methiolopsis* sp. showed strong evidence of dietary mixing – a mechanism used by generalist insect herbivores to balance nutrients in their diets (Franzke et al. 2010). However, even though these two genera occupied the same niche space, a eucalypt woodland habitat, their dietary overlap was low. In Orthoptera, it has been found that coexisting species are divergent in relevant niche space, which facilitates the coexistence of generalist herbivore communities (Behmer & Joern 2008; Chase 1996). We believe this to be the case with the two genera studied here. If resources are limiting, however, competition can arise (Behmer & Joern 2008; Chase 1996). Phylogenetics effects can also be pronounced, with past studies finding that different sub-families of Orthoptera feed on different proportions of plant types (e.g. Joern, A (1979) found that individuals from the subfamily Gomphocerinae fed primarily on grasses, whereas individuals from Melanoplinae fed mostly on forbs). Therefore, the differences seen in the diets of

Merrinnella sp. and *Melanopsis* sp. could simply be different food preferences based on different families. A larger study could provide more conclusive results. As ecological niches are central to explaining the structure of communities, further research of these genera could address niche overlap and competition across resources. Further investigations could also use CO1 to test whether the diets of *Merrinella* sp. are truly herbivorous, as some Gryllidae species are reported to have omnivorous diets (e.g. Kaufman and Klug (1991)).

Metabarcoding is transforming food web ecology with interactions proving to be more complex than previously realised (Roslin & Majaneva 2016). In conclusion, we demonstrate that metabarcoding is a powerful tool for the analysis of insect herbivore interactions, but we recommend that three gene markers are necessary for reconstructing accurate and complete species links, especially in the case of generalist herbivores. We found that two abundant species of Orthoptera in a sub-tropical eucalypt woodland habitat were both generalists, but that further investigations are needed to understand their full feeding behaviours. We suggest that with a large sample size, a comprehensive reference library of host plants and three plant gene markers, detailed dietary information can be obtained for insect herbivores.

4.6 Acknowledgements

This study was funded through an Australian Research Council Discovery Grant DP160102078. JLH was supported, in addition, by a Griffith University postgraduate research scholarship and a research grant from the Equities Trustees Charitable Foundation. Laboratory work was undertaken in the University of the Sunshine Coast's Genecology molecular research lab and AS was funded by German Australian research exchange program. We extend our thanks to Lois Kinneen and Erica Odell for their assistance in the field. All research methods and sampling were approved under the auspices of Scientific

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4.7 S4 Supplementary material

Table S4.1: Descriptions of plant types, taken from PlantNET [1], Putz et al. 1989[2] and Acreman 1994[3]

Plant type	Description
Tree	A woody plant usually with a single distinct trunk and generally more than 5 m high[1]
Shrub	A much-branched woody plant less than 8 m high and usually with many stems[1]
Vine/Climber	A plant that cannot remain free-standing to any appreciable height[2]
Herb	A plant that does not produce a woody stem, although it may be woody at the base[1]
Grass	A plant belonging to the family Poaceae[1]
Sedge	A plant belonging to the family Cyperaceae[1]
Algae	Thallophyte plant lacking roots, stems, and leaves[3]

Table S4.2: List of plant families detected within the diets of *Merrinella* sp., and *Methothiopsis* sp. detected using DNA metabarcoding with rbcL and trnH-psbA barcodes.

Plant families detected in *Merrinella* sp. diets	**Plant families detected in *Methiolopsis* sp. diets**
Araliaceae	Acanthaceae
Chenopodiaceae	Araliaceae
Cucurbitaceae	Burseraceae
Cunoniaceae	Cyperaceae
Cyperaceae	Euphorbiaceae
Mytaceae	Fabaceae
Phyllanthaceae	Heliconiaceae
Poaceae	Lauraceae
Rosaceae	Malvaceae
Rutaceae	Oxalidaceae
Solanaceae	Poaceae
	Rosaceae
	Rutaceae
	Solanaceae

Chapter 5

Chapter 5 is a co-authored paper which has been **prepared** for publication. The bibliographic details of the co-authored paper, including all authors, are:

Hardwick, J. L.[1], Maunsell, S. C.[1,2], Stork, N. E.[1], Yusah, K. M.[3], & Kitching, R. L.[1]

(prepared manuscript) **Assemblages of Orthoptera and forest conversion in Borneo.**

My contribution to the paper involved all field work to collect specimens, with assistance from Dr. Sarah Maunsell in the field. Dr. Kalsum Yusah assisted with obtaining research permits in Sabah, Malaysia. Professor Roger Kitching, Professor Nigel Stork and Dr. Sarah Maunsell supervised this project and were responsible for direction and guidance regarding the design of the project and scope and structure of the manuscript.

(Signed) _____ (Date) 08.05.2018

Jane Louise Hardwick

(Countersigned) _____ (Date) 08.05.2018

Co-author of paper: Sarah Maunsell

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Chapter 5: Assemblages of Orthoptera and forest conversion in Borneo

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5.1 Abstract

Deforestation is occurring at a rapid rate in Malaysia, mainly for the landscape-scale planting of oil palm crops. Due to the rapid rate of change, the impacts of logging and conversion of forest to plantation on many arthropod taxa are yet to be understood but it is of prime importance to monitor the changes taking place. The insect Order Orthoptera is pivotal in food webs, with many species representing primary consumers. However, no studies to date have measured the impact of logging and habitat change to oil palm on this taxon. We investigated the species abundance, richness and community composition of Orthoptera across a habitat gradient in Sabah, Borneo. Orthoptera were twice-sampled at 48 points across primary forest, logged forest, and intensive oil palm plantations. Specimens were identified to family and sorted into morphospecies for analysis. Overall, species abundance and richness were lowest in two of the logged forest sites (Logged2 and Logged3) when compared with two other logged sites, two primary forest sites and two plantation sites. We

found that community composition was significantly altered by habitat change, particularly from primary forest to oil palm where only 43% of species from the primary sites were also found in plantations. Forest quality and leaf area index had effects on orthopteran community composition. Two families in particular were affected negatively by the conversion of forest to plantations, Mogoplistidae and Trigonidiidae, and had a much lower abundance in plantations than in primary forest. We conclude that although plantations have high species richness and abundances of Orthoptera, many forest dwelling species are unable to survive the habitat change and therefore the systems and functions are likely to be altered. To prevent loss of naturally occurring forest families, such as Mogoplistidae and Trigonidiidae, primary forests must be preserved.

5.2 Introduction

Deforestation is increasing at a rapid rate from conversion of forest to agricultural land (Gaveau et al. 2016). In the lowland tropics, entire landscapes are being changed from tropical rainforest, the most species-rich of all terrestrial ecosystems (Turner, IM & Corlett 1996), to uniform oil palm (*Elaeis guineensis*) plantations (Turner, EC & Foster 2009). Understanding these human-altered landscapes has become one of the central goals of conservation biology due to global concerns about loss of species and ecosystem function (Basset, Aberlenc, et al. 2001; Gibson et al. 2011; Sodhi et al. 2004). Together, Malaysia and Indonesia produce more than 80% of the world's palm oil (Koh and Wilcove 2007; Palm Oil Analytics, 2017) and it is these countries that also hold more than 80% of southeast Asia's remaining primary forests, which are critically under threat (Donald 2004). Even small-scale changes to habitats can alter trophic interactions between species and this can have cascading effects at a population level (Metcalfe et al. 2014).

Arthropods represent more than 80% of all described biodiversity (Borror, Triplehorn & Johnson 2004), with the highest species richness found in tropical forests (Lamarre et al. 2016).

As arthropods are important at several different trophic levels, changes to communities are expected to trigger changes in ecosystem function (Sobrinho et al. 2015). Despite an increasing focus on the impacts of forest conversion from primary forest to logged forest to oil palm plantation over the last decade (e.g. Fayle et al. 2010; Koh 2008, Wilcove and Koh 2010) the consequences of this conversion for many invertebrate taxa are far from understood (Turner, EC et al. 2008; Turner & Foster, 2009). Given that the demand for palm oil is expected to almost double by 2050 (Corley 2009), understanding these impacts is vital; if the status of biodiversity within plantations and the impacts on different taxa are known, then careful management may decrease the impacts on ecosystem function (Turner, EC et al. 2008).

Among arthropods, the impacts of the conversion of forest to oil palm plantations has been best documented for ants (Brühl & Eltz 2010; Fayle et al. 2010; Lucey & Hill 2011; Luke et al. 2014; Pfeiffer, Tuck & Lay 2008; Wang, WY & Foster 2015). In Malaysia, species richness of ground ants was severely reduced in plantations regardless of the age, undergrowth cover or proximity to neighbouring forest of the plantation when compared to primary forest (Brühl & Eltz 2010). Elsewhere, species richness decreased by 64% between forest and oil palm plantation and non-native species were found to be abundant in the canopy of plantations (Fayle et al. 2010). In addition, conversion of forest to oil palm has been shown to result in significant changes to community structure and diversity of moths (Alonso-Rodríguez, Finegan & Fiedler 2017; Chey 2006; Chey, Holloway & Speight 1997), butterflies (Koh 2008; Lucey & Hill 2011; Tscharntke et al. 2002), beetles (Chung et al. 2000; Gray et al. 2014), aquatic Heteroptera (Cunha, Montag & Juen 2015), dragonflies (Luke et al. 2017) and bees (Liow, Sodhi & Elmqvist 2001).

Selective logging is the most common approach for producing commercial timber in southeast Asia, and Borneo, in particular, has been intensively logged (Gaveau et al. 2016). This modifies the structure of forests and has large impacts on regeneration dynamics and

community composition of tree species (Okuda et al. 2003). Logging can cause a reduced abundance of key functional arthropod groups within tropical forests, which can lead to a decreased contribution towards essential ecosystem processes (Ewers et al. 2015). For some taxa, the impacts are greater than others; for example, butterfly species richness, abundance and evenness were reduced in logged forest compared to unlogged forests in Indonesia (Hill et al. 1995) but for ground-dwelling ants no difference was found except in the population density of certain species (Vasconcelos, Vilhena & Caliri 2000). Gibson et al. (2011) found Coleoptera were more sensitive to forest disturbance than Hymenoptera and Lepidoptera, and that, overall, arthropod biodiversity was more sensitive to disturbance than plants and mammalian taxa.

Orthoptera in particular, are among the largest insect Orders (Stork 2018) and thus have been grouped as important herbivores within tropical ecosystems (Lamarre et al. 2016; Novotny, V. et al. 2010). Their role in providing links to higher trophic levels through food webs has been recognised (Ingrisch & Kohler 1998; Morelli et al. 2015) and in addition, they have been shown to be indicators of habitat disturbance (Anderson, AN et al. 2001; Samways & Lockwood 1998). Their community composition can be sensitive to anthropogenic change with greater diversity and richness being recorded in undisturbed forest than disturbed habitat in a moist deciduous forest in India (Joshi et al. 1999). In Australia, however, Orthoptera have been found to be resilient to the effects of logging and fire disturbance in eucalypt forests (Abbott et al. 2002). They have also been found to be unaffected by logging in a tropical forest in northern Borneo (Edwards et al. 2012). Although the impacts of land conversion for different forms of agriculture (e.g. grassland land-use (Batáry et al. 2007; Braschler et al. 2009; Fabriciusová, Kaňuch & Krištín 2011)), on Orthoptera have been examined, the impacts on Orthoptera of degraded tropical rain forest or conversion to oil palm plantations are largely unknown.

In tropical forests, there is an extraordinary number of interactions between species. Therefore, understanding how these ecosystems respond to habitat degradation and land-use change is challenging (Fayle et al. 2015). The Stability of Altered Forest Ecosystems (SAFE) project is a landscape scale ecological experiment in Sabah, Malaysian Borneo, with a main aim to address the impacts of logging, fragmentation and conversion to oil palm plantations, on a range of different species (Ewers et al. 2011). This is possible as a large area has been designated for conversion to oil palm plantation by the Malaysian Government, but 800 ha of this cultivatable land is being retained for the purpose of research, providing a unique opportunity to study the effects of landscape-scale habitat change (Ewers et al. 2011). Given that beta-diversity is a key concept for understanding ecosystem processes (Legendre, Borcard & Peres-Neto 2005), here, we provide the first assessment of how orthopteran assemblages respond to tropical rainforest degradation and to the conversion of forest to oil palm plantations. We assess species and family richness, abundance and community composition across the SAFE landscape in Sabah, Malaysia.

5.4 Methods

All sampling was conducted in Sabah, Malaysian Borneo, between May and August 2015, and was based at the Stability of Altered Forest Ecosystems (SAFE) project sites (for our logged and plantation sites) and at Maliau Basin Conservation Area (4°49′N, 116°54′E) (for our primary forest sites). The SAFE project area encompasses 72 km sq. of lowland rainforest within the Kalabakan Forest Reserve (4°43’N, 117°35’E), which previously has been logged twice, and oil palm plantations are located approximately 7 km west of this area (Figure 5.1). The Maliau Basin Conservation Area encompasses almost 60 km sq. of lowland rainforest and was declared a World Heritage site in 2003. Although some of the dipterocarp forest within the Maliau area was selectively logged in the early–mid 1990s, it has never been commercially logged. The forest quality here is very high (Ewers et al. 2011) and the area

contains some of the last floristically and faunally intact forest in southeast Asia (Brodie & Giordano 2011), making this location appropriate for our primary forest sampling sites (Figure 5.1).

Orthoptera specimens on the surface of leaf litter and to ~2 m above ground were captured using sweep-netting along transects within four logged forest sites with varying disturbance levels and two oil palm plantations within the SAFE project research area (Figure 5.1). Orthoptera were also collected at two primary forest sites within the Maliau Conservation Area (Figure 5.1). All sampling plots used were established as part of the SAFE project and were orientated to minimise pseudo-replication and other factors that have potential to confound the effects of land-use change, such as latitude, slope and elevation (Ewers et al. 2011). At each site, six plots were twice-sampled using four 25 m transects per plot totalling 288 transects across 48 plots (24 sampling plots across four logged forest sites, 12 across two oil palm plantation sites and 12 across two primary forest sites). A greater number of survey points were sampled in logged forest as we expected this habitat type to be more heterogeneous (Luke et al. 2014) and we wanted our plots to span a gradient of disturbance across all habitats. When orthopterans were caught in the net, they were immediately removed and placed into 95% ethanol. Each individual was then categorised as ‘adult’ or ‘nymph’ and sorted to morphospecies and family where possible based on Rentz (1996). The morphospecies approach was used due to incomplete species databases for Orthoptera and as a way to increase practicability by reducing the time required for taxonomic identification of specimens. This is an appropriate method for landscape-scale assessments (Kremen 1992; Kremen et al. 1993). Nymphs were removed from all analysis involving morphospecies due to difficulties with identification.

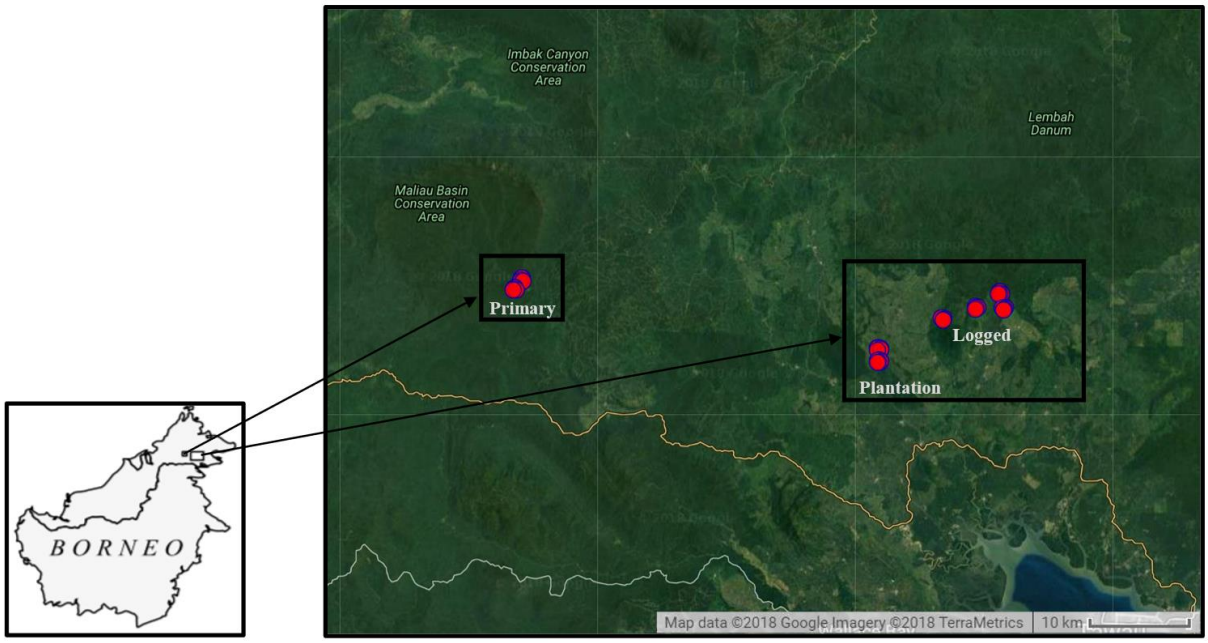

Figure 5.1: Map of sampling sites (two primary forest sites, four logged forest sites and two plantation sites) and plots (12 primary, 24 logged and 12 plantation) within the SAFE project experimental area (right rectangle) and Maliau Basin Conservation Area (left rectangle) in Sabah, Malaysia.

Data analysis

All statistical analyses were conducted using R 3.4.2 (R Development Core Team 2014) and data were grouped by each of the six sampling plots per site, unless otherwise specified. We used all adult specimens for species-level analysis but removed 122 specimens for family-level analysis that could not be identified. We assessed species and family richness and sample coverage with a combined method of extrapolation and rarefaction using both species accumulation curves and sample coverage (Chao & Jost 2012) using the iNEXT package (Hsieh, Ma & Chao 2016). We used 100 bootstrap runs to obtain 95% confidence intervals and extrapolated to 300 individuals. We used Analysis of Variance (ANOVA) to test for differences of both species and family richness across sampling sites and a Tukey HSD test to determine

significant differences between means. These results are visualised using boxplots where the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper whisker extends to the largest value and the lower whisker extends to the lowest value (both of which are no further than 1.5 * interquartile range (IQR) from the hinge. Data falling beyond this range are "outlying" points that are plotted individually (McGill, R., Tukey, J. W. and Larsen, W. A. 1978). We tested for effects of spatial autocorrelation using Mantel tests using the Vegan package (Oksanen et al. 2013). We fitted Generalised Linear Models (GLMs) using the MVABUND package (Wang, Y et al. 2012) to test for differences in community composition across the sites. We ran these models using a negative binomial distribution on count data, using our eight sites as categorical predictor variables to assess the impacts of habitat change on community composition of Orthoptera. We used the ANOVA function to produce an analysis of deviance table which uses likelihood ratio tests (LRT) (to compare the goodness of fit) and resampled p values to look for significant effect of sites on the community data. Dissimilarity of species communities across the habitat type was visualised using principal component analysis (PCoA) plots, created using the 'Betapart' package with the Bray–Curtis metric using species abundance data. Using the numbers of individuals from each family at each site, proportions were calculated and visualised on a stacked bar chart. For each of our 48 sampling plots, environmental variables were obtained from Pfeiffer and Ewers (2018). These included measures of leaf area index (LAI), above ground biomass, forest cover and forest quality. LAI scores ranged from 0 (bare ground) to > 10 (dense forest) and were used to determine the interception of solar energy for photosynthesis. The scores were corrected for clumping of leaves at plot level (25 m x 25 m). Above ground biomass (AGB) was derived from the diameter at breast height (DBH) and height of trees for individuals >= 10 cm DBH using an index developed for mixed-species forest stands in East Kalimantan, Indonesia (Basuki et al. 2009). Forest cover was measured as

fractional canopy cover using hemispherical images. Forest quality scores were scored based on a qualitative scale of 1 – 5 introduced by Ewers et al. (2011), where (1) was very poor with no standing trees, open canopy with ginger, vines or low scrub; (2) was poor, meaning open canopy with small trees over a ginger and vine under growth; (3) was okay, with small trees abundant and canopy at least partly covered; (4) was good, with abundant trees including some large trees and a closed canopy; (5) was very good, with abundant large trees, a closed canopy and no evidence of logging. As these scores were only used for forests and not plantations, we gave the plantation sites a score of zero for these analyses. To identify and measure the associations among these variables and our species composition dataset, we used canonical correlation analysis (CCA). This method is a multivariate analysis of correlation which has been recommended as the most appropriate for partitioning spatial variation of community composition data among environmental components (Legendre, Borcard & Peres-Neto 2005). We applied a log+1 transformation to our species abundance matrix in order to correct for possible statistical errors associated with rare or very common species before selecting predictor variables (as above) that best explained our species matrix using a stepwise model ('ordistep' function). We calculated Variance Inflation Factors (VIF), and removed any that were >10, as this indicated that collinearity was present with another variable. The significance of the relation between predictor variables and community composition was tested at $p < 0.05$, using the 'anova.cca' function on the final model with 999 permutations.

5.4 Results

A total of 1791 orthopterans were collected across the sites. Nymphs were removed, leaving 1190 adults (Table 5.1) which resulted in 79 morphospecies in seven families. One-hundred and twenty-two specimens could not be identified to family and were subsequently removed from family-level analyses. Out of 35 morphospecies collected in primary forest, 22 (63%) were also collected in logged forest and 15 (43%) in oil palm plantations. Of the 42

morphospecies collected in logged forest, 18 (43%) were found in plantations. Sample coverage of species varied across sites (Figure 5.2), but sample completeness was estimated to be greater than 90% for all sites except for one logged forest site (Logged3) at 87%. At a family level, sample coverage was estimated to be 100% across all sites except for two of the logged forest sites (Logged2 at 98% and Logged3 at 99%) (Figure 5.2). Observed species richness varied significantly with sample site (ANOVA: $F_{7,40} = 4.55$, $p = 0.0008$) (Figure 5.3). A Tukey HSD test revealed that significant differences were between two logged sites and both of the primary and plantation sites (Logged2 and Primary2: adjusted $p = 0.01$, Logged 3 and Primary 2: adjusted $p = 0.03$, Logged2 and Plantation1: adjusted $p = 0.01$, Logged2 and Plantation 2: adjusted $p = 0.008$, Logged3 and Plantation1: adjusted $p = 0.03$, Logged3 and Plantation2: $p = 0.02$). Mantel tests showed that there was no spatial effect on the data for species richness ($r = 0.03$, $p = 0.25$) or abundance ($r = -0.02$, $p = 0.56$).

Table 5.1: Orthoptera abundance (total) across all sampling sites.

	Logged1	Logged2	Logged3	Logged4	Primary1	Primary2	Plantation1	Plantatio
Acrididae	0	2	0	3	22	4	14	9
Gryllidae	50	23	14	59	35	78	63	49
Mogoplistidae	14	5	26	78	7	16	3	0
Tetrigidae	24	18	24	28	19	25	80	32
Tettigoniidae	3	2	1	1	0	0	11	16
Tridactylidae	0	0	0	0	0	1	19	62
Trigonidiidae	21	6	4	32	16	46	1	2
Unknown	13	1	2	8	10	14	44	30
Total	125	57	71	209	109	184	235	200

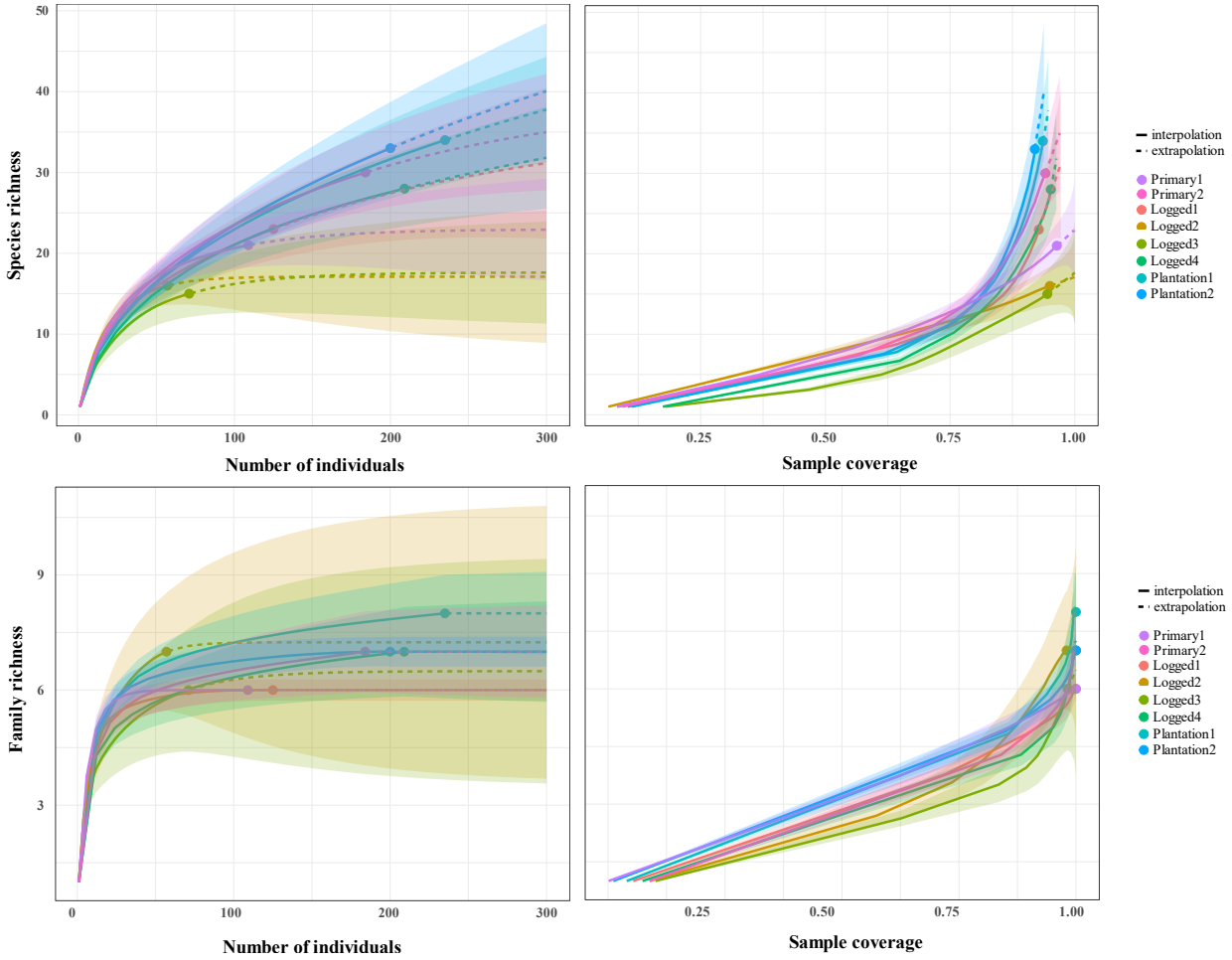

Figure 5.2: Extrapolation-rarefaction curves (Chao and Jost 2012) for species (top left) and family (bottom left), and sample coverage for species (top right) and family (bottom right). Different colours represent different sampling sites.

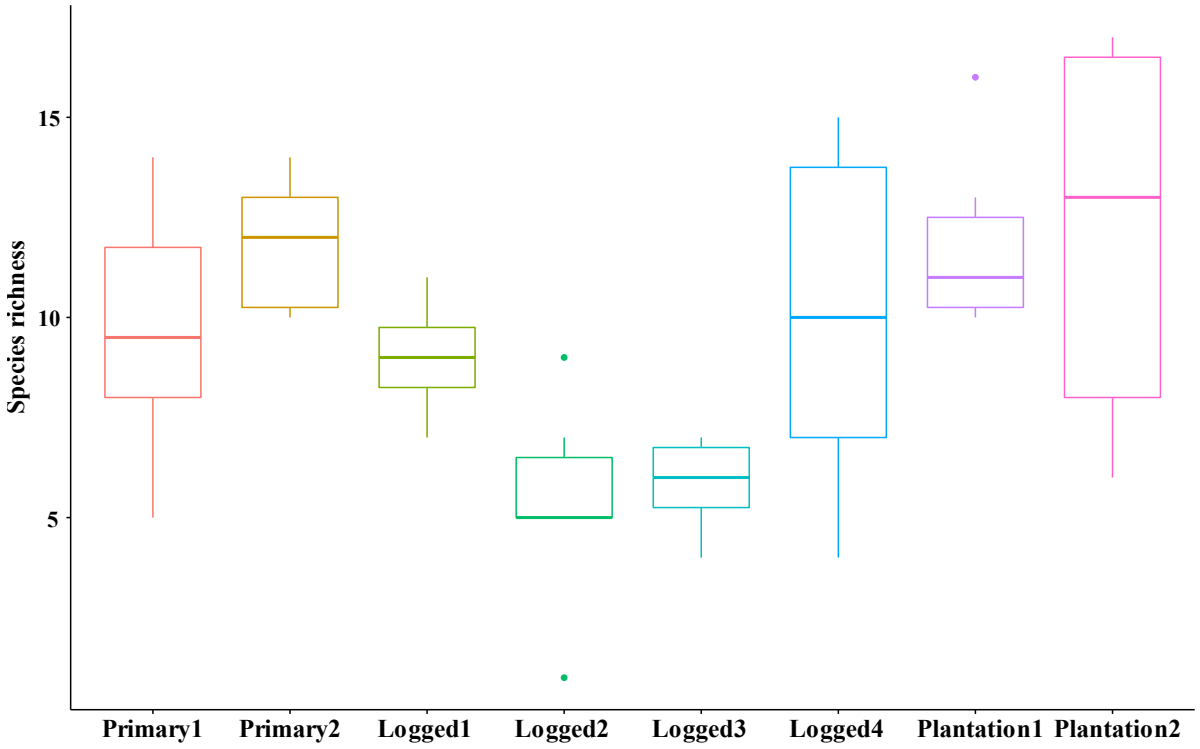

Figure 5.3: Observed species richness across different sites. Boxplots show observed differences in species richness across the sites (showing the third and first quartile range of the data and data outliers - explained in 'Data analysis' section).

The composition of Orthoptera significantly differed across the sites at both a species (ANOVA: LRT = 1103, p = 0.001) (Figure 5.4) and family level (ANOVA: LRT = 239.5, p = 0.001) (Figure 5.4). Univariate tests confirmed that the composition of all families were significantly affected by site (Acrididae: LRT = 32.66, p = 0.001; Tetrigidae: LRT= 18.4, p = 0.028; Tettigoniidae: LRT = 18.82, p = 0.02; Trigonidiidae: LRT = 43.68; p = 0.001 Tridactylidae: LRT = 53.25, p = 0.001; Mogoplistidae: LRT = 42.43, p = 0.001 and Gryllidae LRT = 30.24, p = 0.003). Gryllidae were the most abundant family overall, followed by Tetrigidae (Table 5.1). These two families were well-represented across primary forest (Gryllidae mean n = 36.5, Tetrigidae mean n = 23.5), logged forest (Gryllidae mean n = 56.5, Tetrigidae mean n = 22) and oil palm plantation sites (Gryllidae mean n = 56,

Tetrigidae mean n = 56) (Figure 5.5). Tettigoniidae were the least abundant family overall (Table 5.1) and were only mostly only found in plantations (mean n = 13.5). Very few Tettigoniidae were recorded in logged sites (mean n = 1.75) (Figure 5.5). Acrididae were also found to be in low abundance (Table 5.1) but were found in all habitat types (primary forest mean n = 13, logged forest mean n = 1.25, and plantations mean n = 11.5). They were, however, absent from two logged sites (Logged1 and Logged3) (Figure 5.5). Mogoplistidae were found to be a dominant family across the logged sites (mean n = 30.75) but their abundance decreased in primary forest (mean n = 11.5) and even more dramatically in plantation sites (mean n = 1.5) (Figure 5.5). Trigonidiidae also had very low abundance in the plantations (mean n = 1.5) but were the second most abundant family, after Gryllidae, in the primary sites (mean n = 31) and were also found across the logged sites (mean n =1 5.75) (Figure 5.5). Tridactylidae were abundant in the plantations (mean n = 40.5) and were absent in logged sites. Only one individual was collected from primary site (mean n = 0.5) (Table 5.1 and Figure 5.5).

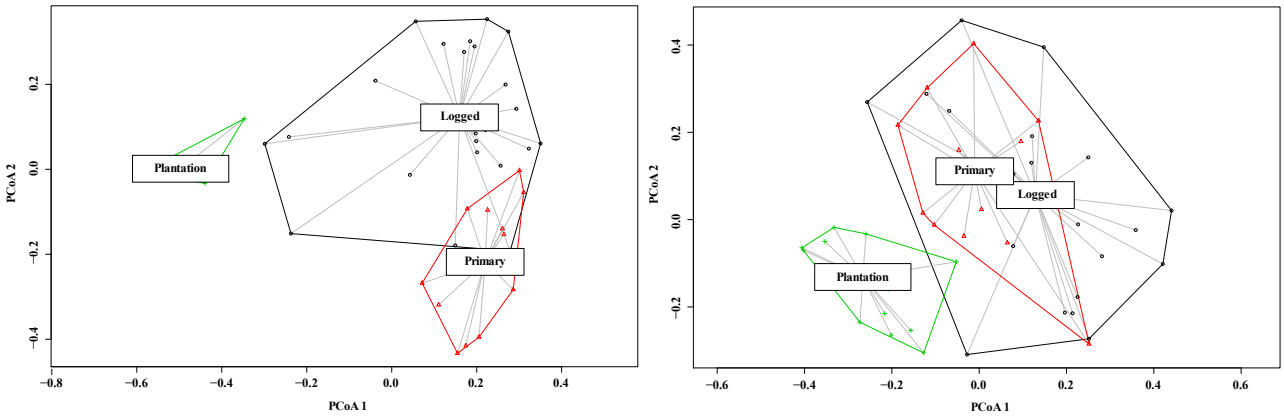

Figure 5.4: Bray-Curtis PCoA ordination of species (left) and family (right) composition across habitat types.

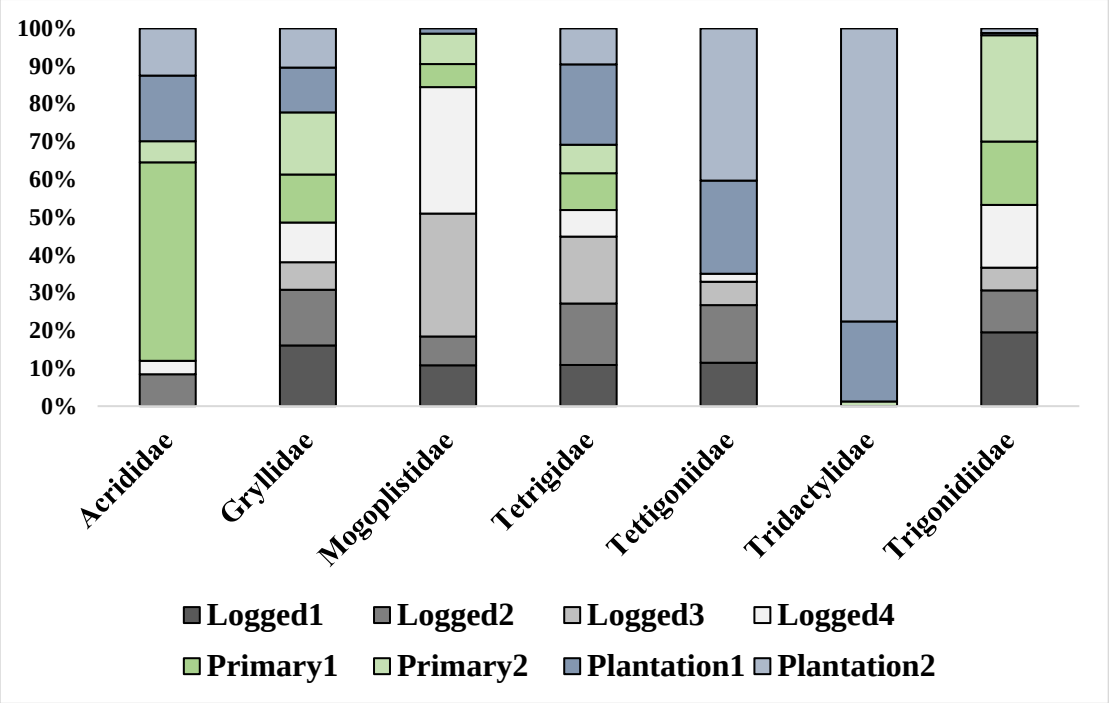

Figure 5.5: Stacked bar charts show the proportions of seven Orthoptera families across different sites.

Using CCA analysis we tested the effects of forest quality, leaf area index (LAI), above ground biomass (AGB) and canopy cover on the community composition of Orthoptera at both a species and family level. We found forest quality and LAI had significant effects on species composition (forest quality: ANOVA: $F_{1,44}$ = 6.64, p = 0.001, LAI: ANOVA: $F_{1,44}$ = 2.23 , P: 0.011) (Figure 5.6) and family composition (forest quality ANOVA:$F_{1,44}$ = 13.76, p = 0.001, LAI: ANOVA:$F_{1,44}$ = 3.64, p = 0.025) (Figure 5.6). When identifying the effects on different families, both forest quality and LAI had the strongest negative effects on Tridactylidae and Tettigoniidae (Figure 5.6).

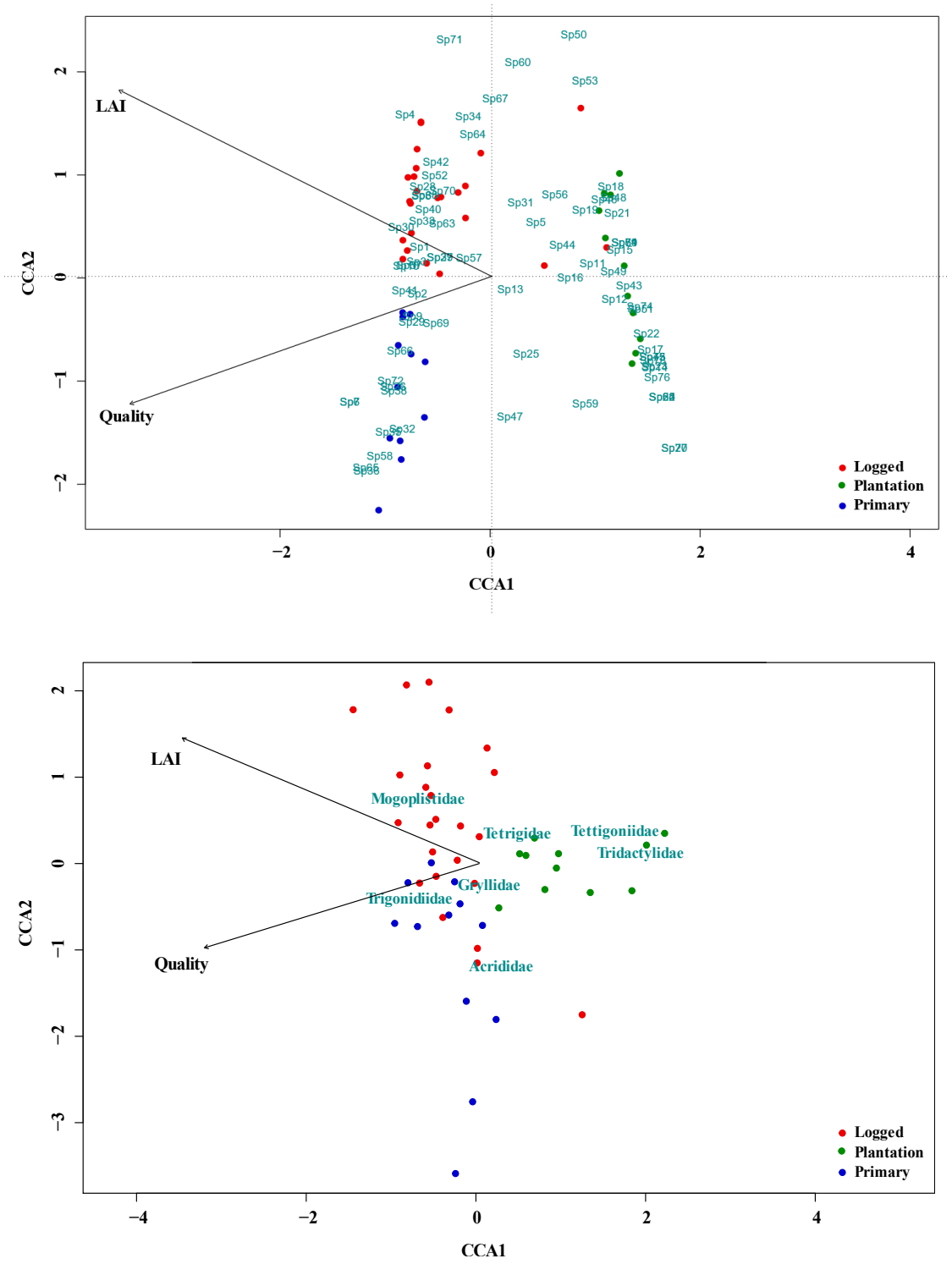

Figure 5.6: CCA analysis showing the effect of leaf area index (LAI) and forest quality on Orthoptera species (top figure) and families (bottom figure).

5.5 Discussion

As different taxonomic groups are affected differentially in terms of changes to community structures and ecological functions, there is an urgent need to gain a better understanding of the impacts of logging and oil palm agriculture on biodiversity (Koh 2008; Turner, EC et al. 2008). This study is the first to examine the impacts of these two forms of forest degradation on Orthoptera, and the findings show that although species richness was high in plantations, only 43% were overlapping with forest-dwelling species and community composition varied markedly with habitat type and taxon. Among the few previous studies of arthropods communities and land-use change in the tropics, similar species compositional changes have been recorded (e.g. Lamarre et al. 2016; Fayle et al. 2010; Luke et al. 2014).

Logging is one of the more widespread forms of forest disturbance (Basset, Charles, et al. 2001), and in our study area, logging intensities are some of the highest on Earth (Edwards et al. 2012). Orthopterans are known to have rapid response to habitat disturbances (Parmenter, Macmahon & Gilbert 1991) such as fire responses (Clayton 2002) and grazed grasslands (Fartmann et al. 2012) but little is known about the impacts of tropical forest clearance. We found that species richness was lowest in our logged sites compared to all other sites and that there were significant declines in species richness from both primary forest sites compared to two of the logged forest sites. Other studies have recorded varying impacts on different arthropod species. For example, species richness declines were found in termites in Malaysian Borneo (Luke et al. 2014) and butterflies in Indonesia (Hill et al. 1995) but species richness inclines in ants in Malaysian Borneo have been recorded (Luke et al. 2014), and a broader study on insect herbivores in Guyana also found overall increases, especially in Psyllidae (Basset, Charles, et al. 2001). Either way, our study, along with others, has found that there is a response by arthropod species to logging. We also found that two of the logged sites had significantly lower species richness than both of the plantation sites but that there

was no difference between the primary forest and plantation sites. Although surprising, less than half (43%) of species overlapped in both habitats. Both species and family composition of Orthoptera were altered across these sites. The environment of an oil palm plantation is very different to that of the forest: for example, there is a decrease in leaf litter complexity and the microclimate is hotter and drier than that of forest areas (Hardwick et al. 2015; Turner, EC & Foster 2006). Although concerning, this outcome is not surprising. A greater number of overlapping orthopteran species were recorded across primary and logged forests where habitats, and presumably plant species, are similar, than with the shift to plantations. Others have found arthropod species (e.g. ants) losses of 81% from primary forest to plantations and dramatic reductions in species richness (Fayle et al. 2010).

When species identifications are problematic, examinations of the responses at higher taxonomic levels, such as the familial level, can be made (Nakamura et al. 2009). We found certain families of Orthoptera, such as Mogoplistidae and Trigonidiidae, were almost completely absent from plantations. Very little is known about these two families and further habitat conversion in the tropics could put them at risk of local extinctions, highlighting why studies such as this one are vital. Gryllidae and Tetrigidae were abundant across primary forest, logged forest and plantations which suggests that these families are more resilient to habitat change than the others. Although there are few studies on Gyllidae and habitat change, Orthoptera in general have been previously found to be unaffected by logging and Gryllidae in particular have been found to be the most abundant family (Strehlow et al. 2004). Another study found that Gryllidae populations inhabiting leaf litter made a rapid recovery after forest fires (Bess et al. 2002). Being highly mobile enables Orthoptera to respond quickly to spatial and temporal environmental changes, allowing them to search for suitable microhabitats (Groning, Krause & Hochkirch 2007; Kindvall et al. 1998). Tetrigidae have also been recorded occupying many different habitat types in other studies including

primary and secondary habitats (Groning, Krause & Hochkirch 2007) but also gravel, sand pits and edges of rivers (Kočárek et al. 2011) which suggests they may be able to find microhabitats within a wide range of ecosystems. Tridactylidae were one family that we only collected from within plantation sites (except for one individual found in the primary forest). Tridactylidae have previously been recorded as 'algae feeders', alongside Tetrigidae (Andersen et al. 2000). We noted that areas where Tridactylidae were collected were mossy patches in plantations which could explain their high abundances at these sites. Tettigoniids and Acrididae have been found to be especially sensitive to disturbances (Anderson, AN et al. 2001). We sampled very few Tettigoniidae and these are also known to be abundant in canopies (Nickel 2009) which may explain why we collected very few from ground-level sampling. We found Acrididae abundance to be particularly low in logged sites, compared to primary forest and plantation sites. Other studies have also found that most Acrididae species were in high abundances in grasslands after deciduous forest clearance and in lower abundances in clear-cut areas (sliacka & Kristin 2012).

Forest quality and LAI were found to influence the species and family composition of Orthoptera. Measures of forest quality have been found to affect arthropod taxa in other studies (e.g. Hill et al. 1995; Turner and Foster 2009: Luke et al. 2014) but few have used LAI as a predictor variable. LAI has been found to be strongly related to microclimate (Hardwick et al. 2015), as where canopies are dense, and when LAI is high, 95% of the visible light can be prevented from reaching the forest floor and therefore it is cooler beneath the canopy (Hardwick et al. 2015). Anthropogenic disturbances lead to significant changes in LAI, which in turn, alters microclimate and leads to knock-on effects on ecosystem functioning (Hardwick et al. 2015). Microclimate has been recorded as a key factor in the occurrence of most Orthoptera species (Ingrisch & Kohler 1998) and although there are many other variables that could drive orthopteran species composition, we suggest that LAI (and

associated microclimate) is an important factor to consider in these types of studies on arthropod communities. Logged forests are on average 2.5 C warmer than primary forests, and oil palm 6.5 C warmer than primary forests (Hardwick et al. 2015) and many taxa are not resilient to these temperature increases.

As with most natural ecosystem studies, there are some limitations to this work. Some of our results presented in this study are based on morphospecies, as documentation of orthopteran species in Malaysia and Southeast Asia remain incomplete (Tan, Ming Kai, Yeo & Hwang 2017b). Although the concept of morphospecies is useful for assessing changes in species assemblages, there are some limitations with using this method involving errors in human interpretation to define a species. Sexual dimorphism can lead to overestimation of species numbers (Derraik et al. 2002) which could have occurred in our dataset. Immature forms can also lead to confusion and this was avoided in our study by removing them from the main dataset. Finally, variation in morphology among species can be vast, leading to overestimations of species, or in contrast, two species can be morphologically very similar but actually genetically distinct (Derraik et al. 2002). Additionally, although Mantel tests were undertaken to assess the possibility of the effect of distance between our sampling sites and no evidence of spatial effect was determined, some have found Mantel tests to have lower statistical power than its raw-data counterparts (Legendre, Fortin and Borcard, 2015) and therefore, there could have still been some effects of regional biogeographic differences driving the species compositional data.

To conclude, we found that forest degradation in the form of logging and land conversion to oil palm plantations had affected Orthoptera assemblages. Despite plantations holding high species richness and abundances of Orthoptera, the community composition was altered, and many primary forest dwelling species are unable to survive within plantations. This kind of shift can alter ecosystem functioning as changes in insect communities can impact

ecosystems in terms of productivity, energy flow and nutrient cycling (Hillstrom & Lindroth 2008). As the tremendous amount of arthropod biodiversity in tropical forests has only just begun to be described and understood (Stork 2018), it is essential to continue surveying in the hope that any future detrimental effects on populations and ecosystems can be reduced. Orthoptera in particular, are proving to be a fundamental component of tropical ecosystems and to prevent loss of naturally occurring forest families, such as Mogoplistidae and Trigonidiidae, primary forests must be preserved. Further work must continue within these changing environments so that appropriate management recommendations can be made.

5.6 Acknowledgements

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Chapter 6

Chapter 6 is a co-authored paper which has been **prepared** for publication. The bibliographic details of the co-authored paper, including all authors, are:

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My contribution to the paper involved all field work to collect specimens, with assistance from Dr. Sarah Maunsell in the field. Dr. Kalsum Yusah assisted with obtaining research permits in Sabah, Malaysia. Associate Professor Alison Shapcott supervised this project, trained me in the lab in preparation and during sequencing the samples and provided guidance and comments on drafts. Professor Roger Kitching and Professor Nigel Stork supervised this project and were responsible for direction and guidance in regard to the design of the project and scope and structure of the manuscript.

(Signed) _____ (Date) 08.05.2018

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Chapter 6: An insight into the diets of Tetrigidae (Orthoptera) across an altered landscape in Sabah, Malaysia, using DNA metabarcoding

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6.1 Abstract

Tetrigidae are a basal group of Orthoptera, abundant within tropical habitats, and are recognised for their unusual diets which mainly consist of detritus and lower plants. Here, we examine whether habitat degradation affects Tetrigidae species composition and diet. Individuals were collected across 60 sites in primary forest, logged forest, salvage logged forest and oil palm (*Elaeis guineensis*) plantations across the Stability of Altered Forest Ecosystem (SAFE) project sites in Sabah, Malaysia. Experimental feeding trials confirmed preferred food items and 96 individuals from six morphospecies were selected for gut content analysis using DNA metabarcoding. Differences were found in tetrigid community structure across the different habitat types. Feeding trials revealed that tetrigids only fed on mosses,

leaf litter and soil detritus and identified variation in food preferences among the same species collected in different habitat types. Metabarcoding indicated that dietary composition varied across both species and habitat types. Forest-dwelling Tetrigidae had ≥80% angiosperm species (assumed to be from leaf litter and/or soil detritus) and ≤20% mosses in their diets. In contrast, the diets of individuals from oil palm had greater proportions of mosses (approximately 80%), with the remaining 20% comprising angiosperm species. These results are consistent with other studies and confirm that leaf litter/detritus dominates the diets of forest Tetrigidae. A shift in dietary composition, however, occurs in plantations, where mosses are the predominant food source. This is the first dietary metabarcoding study for this family and suggests Tetrigidae are likely to be important decomposers in tropical forests

6.2 Introduction

Invertebrates dominate terrestrial ecosystems and play major roles in driving all fundamental ecological processes including herbivory, predation, pollination, decomposition and dispersal (Didham 1997). The insect Order Orthoptera includes grasshoppers (Suborder Caelifera) and crickets/katydids (Suborder Ensifera), which are among the most common terrestrial macro-invertebrates on Earth. Species and assemblages vary greatly in biology and abundance (Samways & Lockwood 1998), occupying most terrestrial habitats, from tropical rainforests to grasslands and mangroves, and microhabitats from the leaf litter to the forest canopy (Rentz 1996). Being so widespread, they are a significant part of the food chain, both as an important prey item for many species (e.g. civets (Fung et al. 2018), birds (Jones et al. 2018), frogs (Teles et al. 2018)), and because they form strong interactions with plants influencing vegetation structure and decomposition (Badenhausser et al. 2015). Despite Orthoptera being an abundant and influential taxon within the ecosystems they occupy, very little is known about species that occur in tropical rainforests. Previous studies have tended to focus on

species of economic importance to agriculture, such as locusts that swarm and cause financial losses to farmers (Barbosa, Letourneau, and Agrawai 2012; Samways and Lockwood 1998; Schell and Lockwood 1997; Usmani, Nayeem, and Akhtar 2012).

Agricultural expansion is one of the major drivers of tropical biodiversity loss worldwide (Green et al. 2005). Within the tropics, habitats are being lost as natural environments are rapidly converted to land suitable for agriculture and timber production. In Malaysian Borneo, for example, logged forests and oil palm plantations now dominate the landscape (Bryan et al. 2013). Plantations of oil palm (*Elaeis guineensis*), native to Africa, have been responsible for an average of 270,000 ha of forest conversion annually from 2000 – 2011 in major palm oil exporting countries (Henders, Persson & Kastner 2015). Of the 43 countries where oil palm is currently grown, Malaysia and Indonesia account for 80% of global oil palm fruit production (FAO 2014). With the demand for oil palm projected to increase substantially in the future (Corley 2009; Vijay et al. 2016), understanding the impacts of forest clearance for oil palm plantations on biodiversity is vital. Studies to increase understanding of these impacts on arthropods have been carried out for certain taxa, but nothing to date has been published on the large and pervasive Order, Orthoptera. Although logged forests may still retain many of the species found in primary forests (e.g. Edwards et al. 2012), many taxa and the spatial patterns they display are strongly affected by disturbance (Ewers et al. 2015; Kitching et al. 2013) and a global assessment has found that primary forests are irreplaceable for sustaining tropical biodiversity (Gibson et al. 2011).

Within Orthoptera, the family Tetrigidae (commonly known as groundhoppers or pygmy grasshoppers) are widely distributed across the world, particularly within tropical habitats. They are a basal group of Caelifera that have been described as exhibiting a conservative feeding strategy associated with the consumption of detritus and lower plants (Kuřavová et al. 2016). The intake of food types including mosses, algae and fungal spores has been found

previously to be affected by habitat associations. Although proportions of food types have been found to differ across species, no association at the level of subfamilies of groundhoppers has been found (Kuřavová et al. 2016; Paranjape 1985). Habitat and diet, however, have been shown to be associated, with those in grassland patches consuming more mosses when compared with those in forests (Kuřavová, et al. 2016). The dietary preferences of a range of Tetrigidae species from both temperate (Europe) and tropical (Borneo) zones, estimated by microscopy by measuring the proportions of food types in the digestive tract, were almost identical and consisted of approximately 80% detritus, 15% moss fragments and 5% other food items (Kuřavová et al. 2016).

DNA barcoding is a method of taxon identification using a standardised region of a gene (Hebert 2003). In recent years, this field has seen many advances and it has become increasingly popular in the field of ecology and conservation. One advance is the use of next-generation sequencing, which allows a single mixed sample to be 'metabarcoded', revealing multiple species of plants or animals within a diet or environmental sample (Ji et al. 2013; Taberlet et al. 2012). This has become a popular tool in ecology, with emerging studies using the technology to measure trophic interactions from a range of taxa (e.g. identifying pollen sources from bees (De Vere et al. 2017), interactions between rolled-leaf beetles and their host plants (Garcia-Robledo et al. 2013) and host ranges of chrysomelid beetles (Kishimoto-Yamada et al. 2013), often when these interactions would otherwise be difficult to measure. Although there are risks of biases affecting PCR-based inference of trophic links, such as accidental ingestion of non-food material (e.g. ingestion from secondary predation or probing) or a contamination of the sample with DNA from other sources, experiments have found that these risks can be reduced by taking careful precautions in the laboratory (De la Cadena et al. 2017). Despite this, molecular methods may be one of the most accurate approaches available to understanding food web ecology (Valentini, Pompanon & Taberlet

2009). Recently, mammalian herbivore diets have been explored using DNA metabarcoding (e.g. large African herbivores (Kartzinel et al. 2015), deer (Erickson et al. 2017) and lemmings (Soininen et al. 2017)) but few studies of insect herbivore networks are available.

In this study, the diets of Tetrigidae were assessed using metabarcoding across four different habitat types in Malaysian Borneo. The four habitat types used were primary forest, twice-logged secondary forest (forests that have been selectively logged twice over an approximately 30 year period), salvage logged forest (forests clear-felled for woody material prior to plantation establishment) and oil palm plantations. Previous studies have found that detritus can dominate Tetrigidae diets (Kuřavová et al. 2016).). As it would be impossible to determine whether plants detected based on the DNA output are from detritus or fresh plant material, we use a series of laboratory-based feeding trials, on separate specimens, prior to DNA analysis to determine the main food sources.

As an oil palm plantation offers a different environment to that of a rainforest, for example, it has lower plant diversity (Fitzherbert et al. 2008) and a warmer/drier micro-climate (Hardwick et al. 2015), the following predictions are made:

1. distributions of species of Tetrigidae will vary according to habitat type, with the greatest difference being between primary forest and plantations,

2. diet composition will differ across both species and habitat types, and

3) diet breadth of Tetrigidae species will be narrower in plantations when compared with forest species

Additionally, as it has been previously suggested that Tetrigidae feed primarily on lower plants, we assess the relative proportions of each plant type (monocot, dicot, gymnosperm, angiosperm) within the diets of target species using metabarcoding.

6.3 Methods

In collaboration with the Stability of Altered Forest Ecosystems (SAFE) project in Malaysian Borneo (as described in detail in Chapter 5), sampling was conducted between the months of May and August 2015. Orthoptera were sampled using a sweep-net in salvage logged (labelled as 'Salvage' in figures) and twice-logged forest (labelled as 'Logged' in figures) and oil palm plantations (labelled as 'Plantations' in figures) in the SAFE project experimental area in the Kalabakan Forest Reseve (4°43'N, 117°35'E). Sampling was also conducted in primary forest (labelled as 'Primary' in plots) at Maliau Basin Conservation Area (4°49′N, 116°54′E). Once caught in the net, specimens were immediately placed into 90% ethanol and then into a freezer at the field station. Specimens were exported to Australia (under permit number JKM/MBS.1000-2/3 JLD.2 (29)) and DNA laboratory work was carried out at the University of the Sunshine Coast. Tetrigidae were separated from samples and only adult specimens were used for analysis due to the difficulties in separating taxonomic groups of nymphs. As colour polymorphism and wing dimorphism are common in Tetrigidae species (Tan et al. 2017), specimens were grouped by JH into morphospecies based on distinguishable morphological features and subfamily identity keys by Rentz (1996), Tumbrinck (2014) and by Ming Kai Tan's recommendations (an orthopteran taxonomist at the National University of Singapore).

Feeding trials

A second field season included a 10-week period between June and August 2016 during which we carried out feeding trials using the three of the most commonly encountered morphospecies of Tetrigidae (Tet1, Tet2 and Tet5). These three chosen species were among six that were also used in DNA diet analysis (Tet1 – Tet6). The goal was to identify food types in their diets and food preferences as a positive control independent of the DNA

analysis. Of the tetrigid species used in feeding trials, two species (Tet1 and Tet5) were collected from more than one habitat type. Tet1 was collected from plantations and logged forests and Tet5 was collected from logged and primary forests. The third species (Tet2) was only found within plantations.

A series of choice and no-choice experiments were carried out for each species. The 'no-choice experiments' were used initially to determine what tetrigids would and would not feed on, and choice experiments were used afterwards to determine food preferences. For all experiments, between 50 and 60 individuals from each species were used. If an individual did not survive until the end of the experiment, the feeding data for that individual was not used in analysis. Approximately twenty specimens from one species were collected at a time and housed in separate plastic containers with mesh lids. To fast the tetrigids and clear the gut content without causing dehydration, water was provided but no food was given for the first 18 hours. For all experiments, tetrigids were offered a food type or a combination of food types for 48 hours. Within this time, observations were taken on any feeding behaviour every 15 minutes over a six-hour period each day. Food items offered for the 'no-choice' experiments included a variety of fresh plant materials (herbs, grasses, trees and mosses) including the leaf laminae and the stems, soil detritus and leaf litter. Leaf litter was only collected from forest sites (as it was absent in plantations) and therefore was dominated by dipterocarp species. In the first two weeks of 'no-choice' trials, it was apparent that none of the three species would feed on any fresh plant material, except for mosses, and therefore all grasses, trees and herbs were subsequently removed from the experiment. In the choice experiments, each tetrigid was offered the three food items predetermined by the no-choice experiments; leaf litter, soil detritus and moss. As the 'no-choice' experiments were used to determine potential food sources and not feeding preferences, only data from the 'choice' experiments were analysed in this study.

We selected ninety-six specimens from six morphospecies (Tet1 – Tet6) for diet analysis. Each specimen was cleaned with 90% ethanol and DNA was extracted from the whole insects following methods that were consistent with Shapcott et al. (2015), Ivanova et al. (2008) and Kress et al. (2009). Plant DNA from within the gut contents was targeted using the barcode markers trnH-psbA, and ITS*2* gene markers. These gene regions were chosen as previous studies had found them successful in amplifying plant groups such as mosses (trnH-psbA: Liu et al. 2010) – which have been found previously to be important components of Tetrigidae diets (Kuřavová et al. 2016). PCR was carried out twice for each marker for each individual to increase success and this product was pooled before sequencing. Sequencing was carried out on an Illumina Miseq using a 2x300 reagent kit following an adapted Illumina 16 s methodology (Illumina Inc. 2017) (for detailed methods, see Chapter 3). Sequences were processed using a customised python script using USEARCH commands within a linux shell (as described in Chapters 3 and 4). Sequences were joined and filtered for quality by discarding those with less than 200bp and with phred scores less than 20. To assign taxonomic names to these remaining sequences, we used a reference library of plants created for each marker and downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov/) and aligned our sequences against each database at a 98% minimum alignment threshold using USEARCH (Edgar 2010). Because we did not have a specific reference library for the region, we only considered plant identities to family level. As previous studies have shown it is advisable to discard extremely rare sequences from metabarcoding analysis, we followed McClenaghan et al. (2015) and omitted any operational taxonomic units (OTUs) with less than 10 sequences from our analyses.

Data analysis

All analyses were carried out using R (V.3.4.3) (R Development Core Team, 20174). For assessing community composition of Tetrigidae among the sampling sites, the ‘metaMDS’

package within the ‘Vegan’ package (Oksanen et al. 2009) was used to calculate Bray–Curtis dissimilarity. We used the default settings to regress the data and reduce stress and then we examined a stress plot to check for scatter around the regression between interpoint distances in the final configuration against their original dissimilarities. We then visualized these on non-metric multidimensional scaling (NMDS) plots. We also calculated Jaccard’s index to assess the amount of overlap in species among sites. Observed species richness and abundance were tested among the sites using analysis of variance (ANOVA) tests.

For feeding trials, stacked bar charts were created to show the proportions that each species were recorded feeding on the different food types. Differences in food preference were tested across species and habitat types using Pearson’s Chi Square tests.

For DNA metabarcoding analyses, sequence data was combined from the two gene markers. Accumulation curves were plotted, using the ‘BiodiversityR’ package (Kindt & Coe 2005), for each tetrigid species at each site to evaluate whether we had enough individuals to make inferences about diet breadth. The “exact” method was used which finds the expected (mean) species richness to calculate the species accumulation curves (Kindt & Coe 2005). To test if there were any differences in diet composition across species and sites, we first computed a Bray–Curtis index using sequence abundance data (square root transformed) and used a multivariate analysis of variance (MANOVA) with the ‘PERMANOVA’ package in R (Anderson, MJ 2001). Food webs were created using the ‘plotweb’ function in the ‘Bipartite’ package in R with connection lines proportional to interaction strength (Dormann et al. 2009). We also created stacked bar charts to show differences in plant types (angiosperm, pteridophyte and bryophyte) in tetrigid diets among habitat types and tested differences in these proportions using Chi Square tests.

Finally, we assessed whether there were any differences in diet breadth across the sites (at plant family level) by calculating Levins' Index (Levins 1968) for each individual and comparing across groups using ANOVA. This is a measure of dietary breath estimated by measuring the uniformity of distribution of individuals among the resources. It is calculated using the following formula:

$$\hat{B} = \frac{1}{\sum p_j^2}$$

Where:

$\hat{B}$ = Levins' measure of niche breadth

p_j = Proportion of individuals found in or using resource or fraction of items in the diet that are of food category j (estimated by N_j / Y) ($\sum p_j = 1.0$)

N_j = Number of individuals found in or using resource state j

$Y = \sum N_j$ = Total number of individuals sampled

6.4 Results

Species composition

Using the individuals collected for diet analysis, we found that community composition was different across the sites; for example, we found that Species Tet2 and Tet3 were only found in plantations (Figure 6.1). Jaccard's index showed that the logged and primary sites were 60% similar in terms of composition of Tetrigidae species, whereas plantation sites were only 33% similar to logged sites and only 20% similar to primary forest sites. There was no

difference in Tetrigidae abundance (ANOVA: $F_{3,37} = 0.16$, $p = 0.092$) or species richness among the sites (ANOVA: $F_{3,37} = 1.91$, $p = 0.14$) (Figure 6.2).

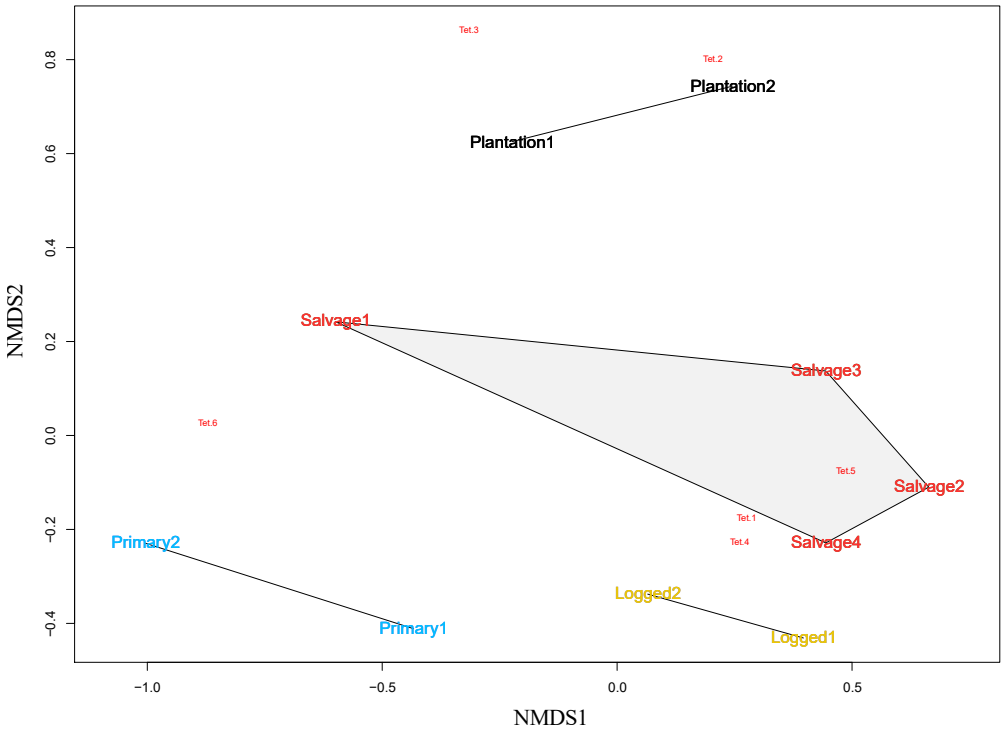

Figure 6.1: Nonmetric multidimensional scaling plot to show Tetrigidae species (Tet1 – 6 in red) composition across habitat types (Plantation sites 1 and 2 in black, Salvage sites 1, 2, 3 and 4 in red, Primary sites 1 and 2 in blue and Logged sites 1 and 2 in yellow).

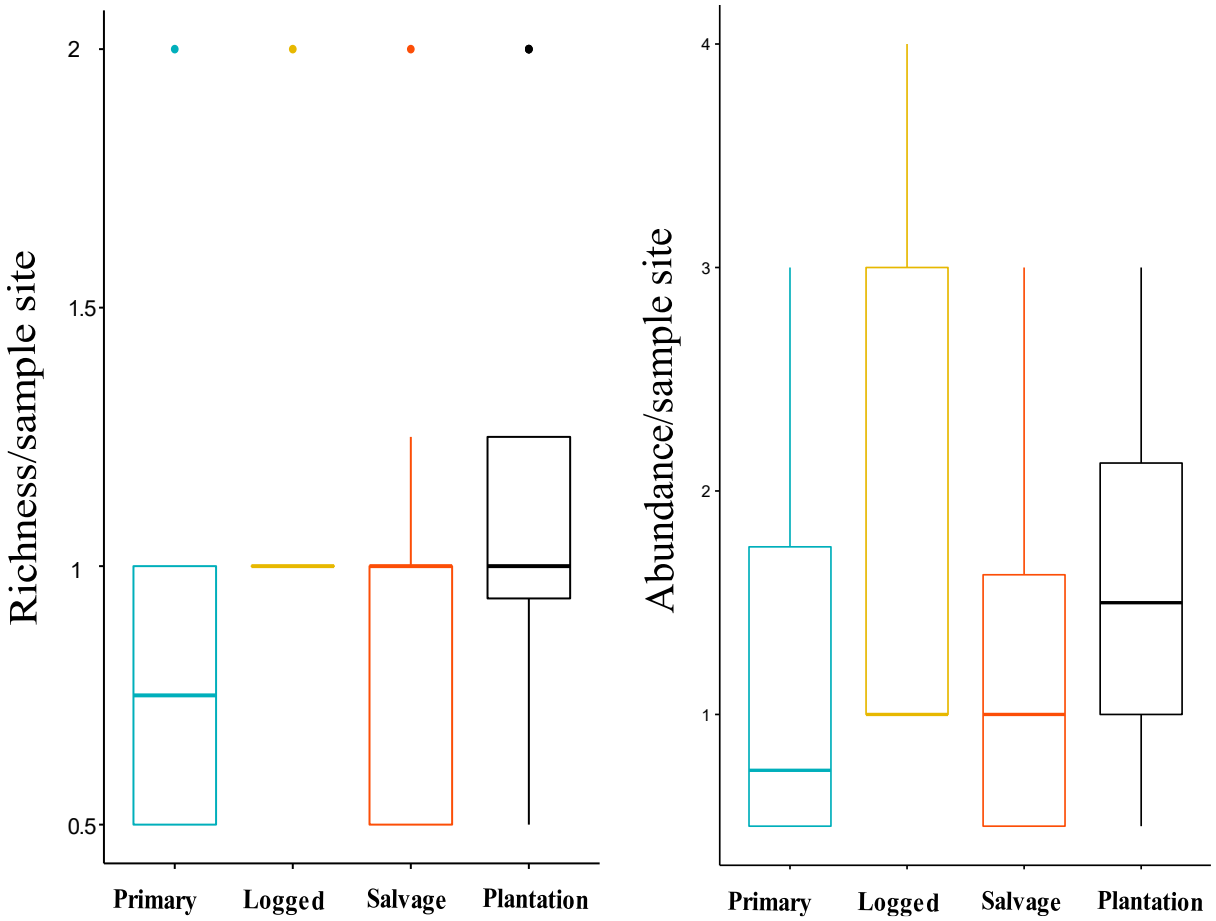

Figure 6.2: Tetrigidae species richness (left) and abundance (right) across sampling sites (12 in primary forest, 12 in logged, 24 in salvage logged and 12 in plantations).

Feeding trials

Feeding trial data confirmed that none of the three Tetrigidae species (Tet1, Tet2 and Tet5) fed on any fresh plant material offered except for mosses. However, all species fed on moss, leaf litter and soil detritus. Food preferences were found to differ among the same species when collected from different sites, but not among different species collected from the same sites. When offered the same three food choices (moss, leaf litter and soil detritus), food preferences of Tet1 differed among those collected from logged sites and those collected

from plantations (χ^2 (2) = 17.67, p = < 0.01). Tet1 individuals from plantations were observed feeding more frequently on mosses and less on leaf litter or soil detritus when compared to Tet1 individuals from the logged sites (Figure 6.3). Similarly, the food preferences of Tet5 individuals collected from primary forest were found to differ from Tet5 individuals from logged forest (χ^2 (2) = 27.77, p < 0.01). Tet5 individuals from the primary forest were observed feeding more frequently on mosses and less frequently on leaf litter or soil detritus when compared to Tet5 individuals from the logged forest. No difference was found between the feeding preferences of Tet1 (collected from plantations) and Tet2 (collected from plantations) (χ^2 (2) = 0.27, p = 0.87) or between Tet1 (collected from logged forest) and Tet5 (collected from logged forest) (χ^2 (2) = 1.07, p = 0.59) (Figure 6.3).

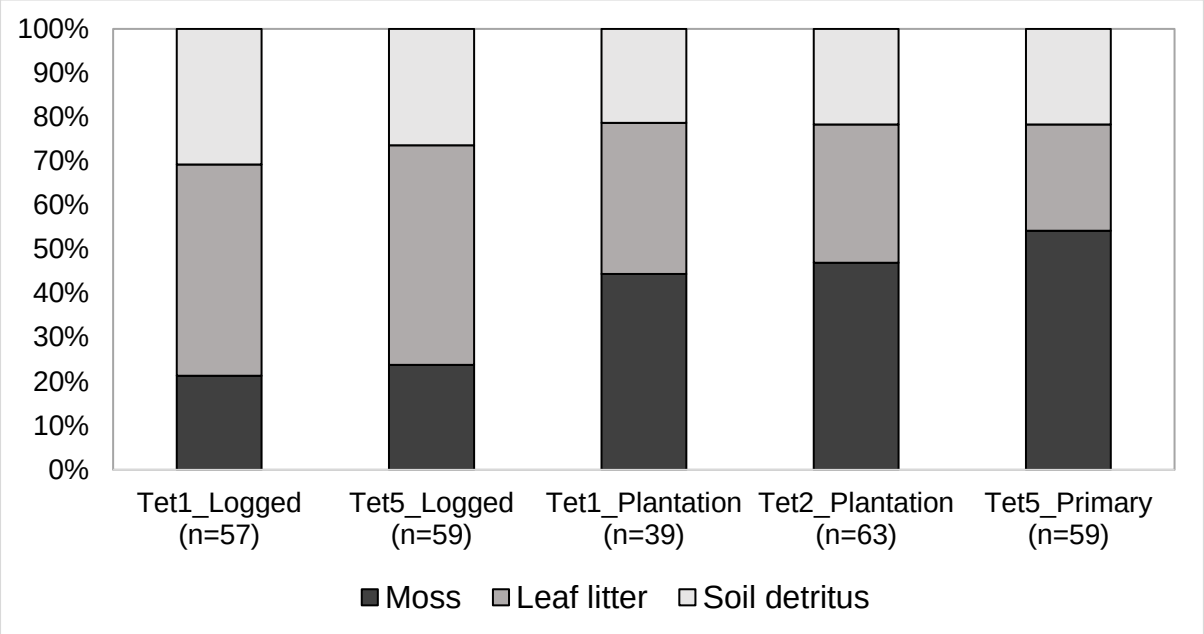

Figure 6.3: Stacked bar charts show proportion of feeding observations recorded for Tetrigidae species (T=Tet) (grouped by different habitat types) during choice experiments.

DNA metabarcoding results

In total, 613,552 sequences matched at 98% or above to our reference libraries after filtering. From this, we identified 27 plant families from the diets of 33 individuals (34% of samples). The trnH-psbA and ITS2 markers yielded a very similar number of sequences (306,774 sequences and 306,788). Accumulation curves of plant families found within individual guts indicated that a greater number of individual grasshoppers would be required fully to understand the diet breadth of these six Tetrigidae species across the habitat gradient (Figure 6.4). The only species (grouped by sites) that plateaued in plant family accumulation were one of the primary forest species (Tet6) and one from the salvage logged forest (Tet5).

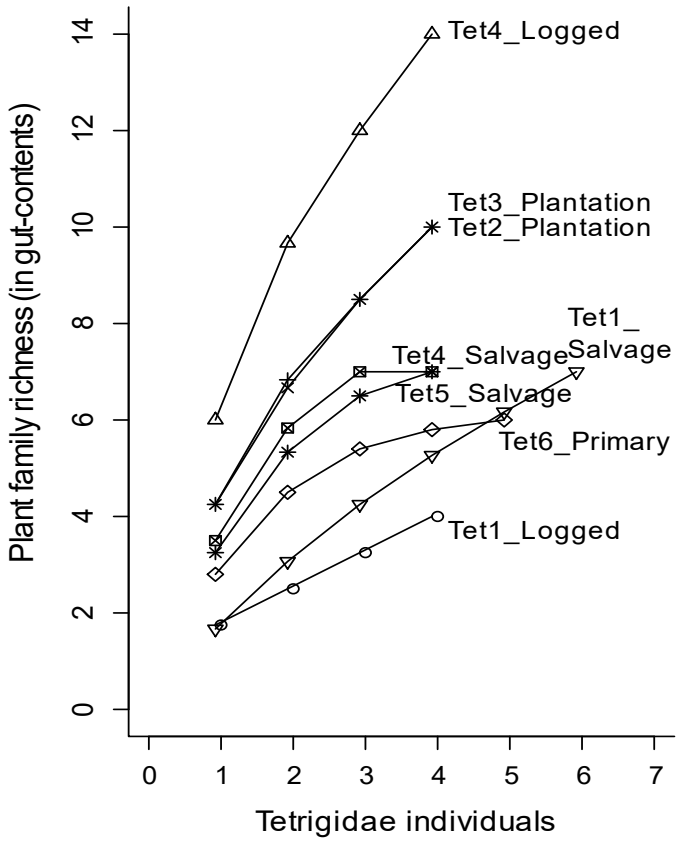

Figure 6.4: Sampling curves to assess dietary richness (at the family level) across different Tetrigidae species (T=Tet) per site.

Differences in diet composition across species and habitat

The most commonly detected plant families across all tetrigid species were two families of mosses (Grimmiaceae and Fissidentaceae), which were detected in 50% of species sampled, although these did not always dominate samples. No individual plant family was detected across all tetrigid species. Using abundance data with DNA sequences and the Bray–Curtis index, differences in dissimilarity were present in diet composition across species ($F_{5,37}$ = 2.45, R^2 = 0.31, p = 0.001), site ($F_{3,29}$ = 2.03, R^2 = 0.17, p = 0.006) and species when pooled by site ($F_{7,25}$ = 1.97, R^2 = 0.35, p = 0.001). When pooling species into habitat types (Primary,

Salvage, Logged and Plantation) and grouping plants into broader groups (Angiosperms, Pteriophyte and Bryophytes) there were large differences in proportions of sequences for each food type in diets among the sites (χ^2 (3) = 274.53, $p < 0.001$). Angiosperms dominated (between 60–100%) the diets of species in forest sites (both primary and logged sites), and mosses dominate (80%) the diets of Tetrigidae within oil palm plantations (Figure 6.5). There were no differences in diet breadth (Levins' Index) among sites ($F_{3, 31} = 0.484$, $p = 0.69$) or species ($F_{5, 29} = 0.87$, $p = 0.51$). However, all species of Tetrigidae sampled had at least three different plant families within their diets and one species (Tet4) had 14 different plant families in the diets of four individuals (Figure 6.6).

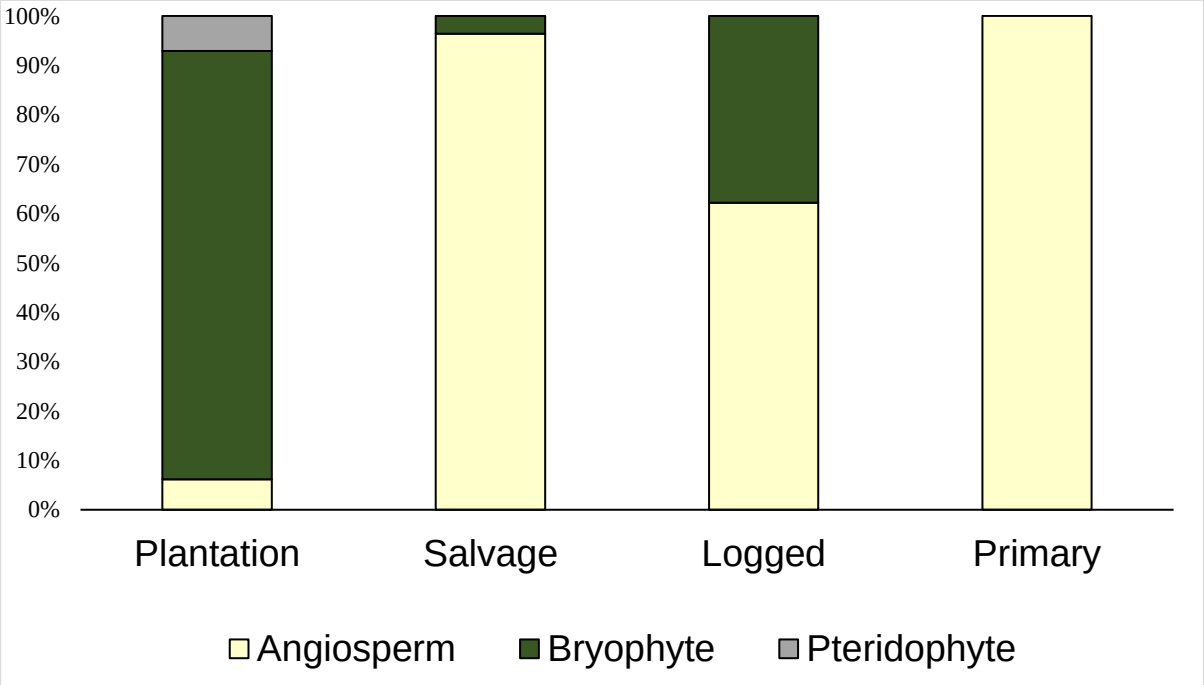

Figure 6.5: Proportions of plant types in diets, calculated by number of DNA sequence reads for each plant type (Angiosperm, Bryophyte and Pteridophyte) in the diets of Tetrigidae in plantations (n=8), salvage logged forest (n=12), logged forest (n=8) and primary forest (n=4).

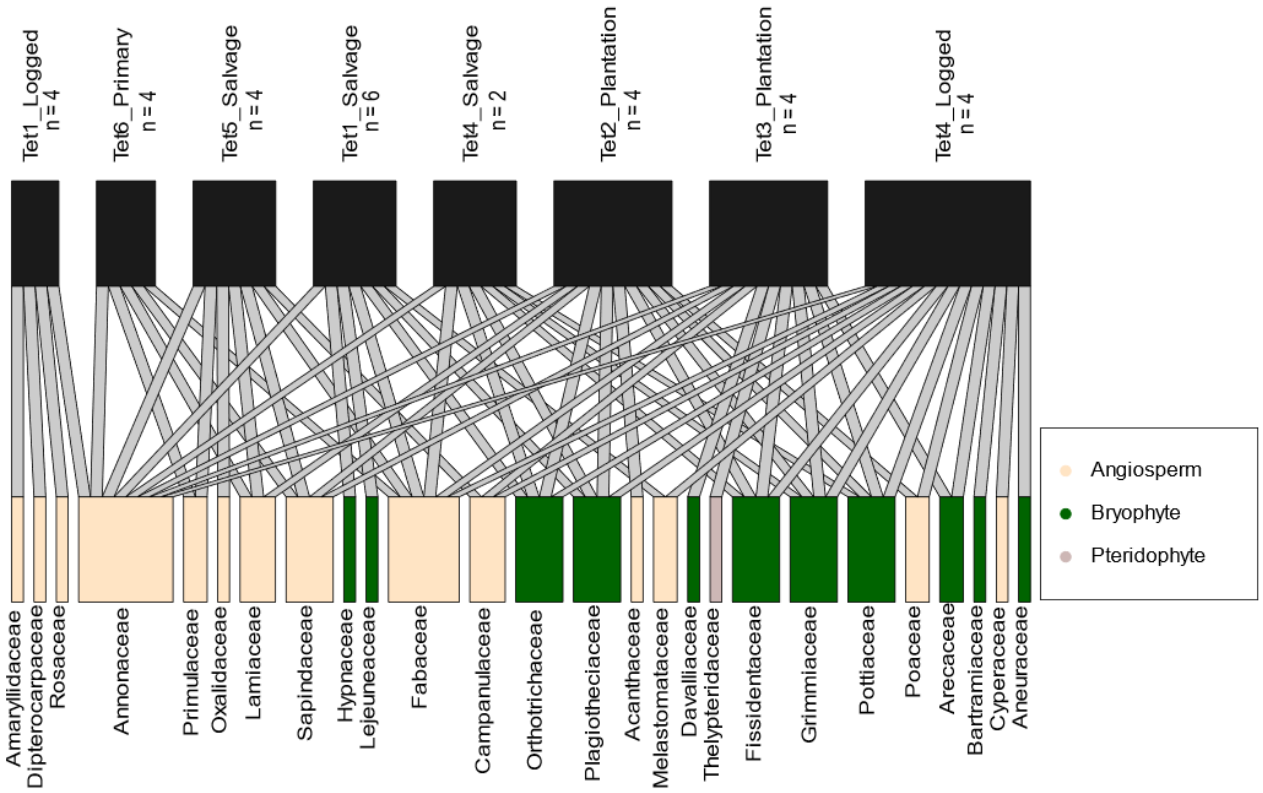

Figure 6. 6: Binary food web showing Tetrigidae species (grouped by site) and their food-plants. Width of boxes are proportional to the sum of interactions involving the species and width of connecting lines are proportional to interaction strength. Different colours represent different plant families.

6.5 Discussion

Global habitat degradation and conversion of natural habitats to agriculture has led to marked changes in species composition (Tilman et al. 2001). With habitat loss occurring at a rapid rate worldwide it is vital to gain a better understanding of how changed environments affect species interactions (Turner, EC et al. 2008; Tylianakis, Tscharntke & Lewis 2007). Orthoptera, although abundant in tropical habitats, have been seldom studied. Our research assesses Tetrigidae, a family of grasshoppers that inhabit many habitats but especially tropical rainforests, to determine if habitat change (from primary tropical rainforest to logged

and salvage logged forest to oil palm plantations) alters their community composition and feeding behaviours. Although the diets of Tetrigidae have been reported from examinations of alimentary tracts, this is the first study to use DNA metabarcoding for gut content analysis of this family, and one of the first for the Order. We found that both community composition and diet composition differed among habitat types, with the greatest difference being seen in the plantation community. Although we found no difference in Tetrigidae species richness or diversity among habitat types, changes in species composition can lead to altered food web structure (Tylianakis, Tscharntke & Lewis 2007) and therefore it is important to monitor when these changes occur.

Our feeding trials confirmed that Tetrigidae consume mosses, soil detritus and leaf litter. No instances of feeding on fresh plant material other than mosses were recorded. Although feeding trials do not necessarily reflect natural preferences, our results are consistent with findings from other studies on Tetrigidae diets (Kočárek et al. 2011; Kuřavová & Kočárek 2015; Kuřavová et al. 2016). Differences in dietary preferences were recorded within species across different habitat types, but not across species within the same habitat types. This indicates that habitat type (and subsequently, resource availability) are drivers of tetrigid feeding preferences, more so than the effect of different species. Similarly, habitat associations have been previously found to be a stronger driver of feeding patterns than phylogenetic relationships (Kuřavová & Kočárek 2017).

Plant DNA detected from gut contents successfully indicated 14 different angiosperm families, and 11 different bryophyte families (all mosses) within the diets of six Tetrigidae species. Differences in plant family composition in the gut contents were recorded both among different species and habitats types using the DNA metabarcoding output. The angiosperm species detected were assumed to be from leaf litter or soil detritus, given the outcome of the feeding trials. In the forested habitats (primary, logged and salvage forest)

tetrigid diets were at least 60% angiosperm materials, compared to less than 10% in the plantation-dwelling tetrigids. Previously Tetrigidae have been reported to be 'bryo-detritivores' with soil detritus and other decaying organic matter as a main component of their diets (Kuřavová & Kočárek 2015) but their importance as leaf litter decomposers is seldom mentioned. Leaf litter decomposition is a fundamental process in terrestrial ecosystems for the recycling of carbon and nutrients (García-Palacios et al. 2016) and our study indicates that Tetrigidae in forested habitats play an important role in this process.

We found that species of Tetrigidae inhabiting plantations had much greater proportions of mosses in their diets (~80%) compared with forest-dwelling species (~20%). Mosses are an unusual food choice for insects (Kuřavová & Kočárek 2017) as calorific returns and digestibility are low, and mosses have a large amount of terpenoids and flavonoids which act as antifeedants (Kuřavová & Kočárek 2015). Tetrigidae are known to favour damp environments (Tan, M. K., Yeo & Hwang 2017a) and therefore it is likely that tetrigids in drier locations may feed selectively on desiccation-tolerant mosses due to their high water content. In oil palm plantations in Sabah, Malaysia, microclimate has been measured to be an average of 6°C warmer than the forest (Hardwick et al. 2015). In our study, it is probable that the plantation species have such high reliability on mosses because they are a highly abundant food source and have a high water content. This last feature may be important in the hotter, drier microclimate of plantations. This also supports evidence that some tetrigids are dependent on microhabitats (Tan, M. K., Yeo & Hwang 2017a) and warrants the need for further study.

The use of DNA metabarcoding in food web ecology has enabled researchers to measure trophic links without *a priori* knowledge of the consumed species (Pompanon et al. 2012). This is extremely useful in situations where study taxa are difficult to assess in nature. There remain, however, limitations. In this study, for example, it is unknown whether the

angiosperm plant families within the diets of Tetrigidae were from fresh plant materials, leaf litter or soil detritus. In addition, we had a high failure rate across samples at PCR or sequencing stages. This is likely because plant DNA from the gut content is highly degraded and leaf litter/soil detritus even more so, and accordingly difficult to replicate. There can also be limitations with PCR inhibitors which can cause sequencing errors (Khanuja et al. 1999).

Our study gives further insight into the diets of Tetrigidae and indicates that habitat degradation alters community structure and dietary composition of species in this family. We demonstrate that DNA metabarcoding can be useful for understanding dietary composition, even for bryo-detritivorous species. Our results also suggest that Tetrigidae play an important role in forested habitats as leaf litter decomposers. We recommend that further studies investigate the importance of microhabitats for tetrigids in altered habitats and address their role as leaf litter decomposers in forests. Food web ecology is a complex field, but with rapidly changing landscapes for timber and agricultural purposes (such as the extensive and expanding oil palm industry) it is essential to gain a greater understanding of species interactions. With knowledge on the robustness and fragility of biological communities, key species can be identified which can be the foundation of conservation management (Cagnolo 2018). Some species are not as resilient as others (e.g. certain species of forest-dwelling Tetrigidae were not found in plantations), suggesting that, as habitats change, they may be unable to survive in the drier habitat with a warmer microclimate without much of their normal dietary requirements. As plants are removed from logged forests and other areas, there are shifts in insect communities which will in turn have knock-on effects for entire ecosystems.

6.6 Acknowledgments

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Chapter 7: General discussion and conclusions

7.1 Introduction

As interactions between insects and their host plants dominate terrestrial ecosystems (Jander & Howe 2008), it is surprising that there is a disproportionate research effort into more charismatic groups such as birds and large mammals (Stork 2007, 2018). E.O Wilson called insects "the little things that run the world" (Wilson 1987). It is estimated that 80 – 95% of insect species are yet to be described, and even for the 850,000+ species that have been named, very little is known of their trophic ecology (Stork 2007). There is much greater insect biodiversity within tropical regions relative to temperate regions, and this latitudinal gradient in species diversity is strongly driven by the parallel increase in plant diversity (Novotny, V et al. 2006). Many plant species have evolved chemical defences against herbivores, and in turn, herbivores have developed strategies to overcome plant defences (Lowman & Rinker 2004; Mello & Silva-Filho 2002). This stepwise co-adaptation, known as 'coevolution' (Ehrlich & Raven 1964), undoubtedly plays a central role in driving patterns of biodiversity.

Within Orthoptera there are 25,000 described species which are broadly diverse in terms of behaviour and ecology (Bidau 2014). Although most orthopterans are herbivorous, the Order also has some omnivorous species and some families such as the Tetrigidae feed primarily on mosses and dead organic materials (Kuřavová et al. 2016; this thesis, Chapter 6). In the few studies that have examined Orthoptera in tropical habitats, however, they have been categorised as 'major herbivores', and many species have been found to have broad dietary (host) ranges, often across multiple plant families (Novotny, V. et al. 2002). By measuring the host specificity of insect herbivores, the richness of tropical herbivore assemblages has been estimated by calculating the average number of herbivorous species per host plant

species and multiplying this by the known number of plant genera from a particular region, in this case, in New Guinea, (Novotny, V. et al. 2002).

Given that knowledge of insect-plant interactions remains limited (Mello & Silva-Filho 2002), further food web studies utilising methods such as those described in this thesis will help build a better understanding of evolution, ecology and biodiversity.

Over the preceding chapters, I have pursued the four specific aims stated in Chapter 1, discussed in turn below. The overarching aim of this thesis has been to add knowledge on the ecology of Orthoptera and to document the impacts of land-use change in the tropics on their species composition and diets. In addition, a central goal has been to demonstrate the utility of DNA barcoding as an aid in food web ecology. By sampling Orthoptera across different habitat types (including sub-tropical open eucalypt woodland, tropical forests, logged tropical forests and oil palm plantations), this research has:

- provided detailed molecular methodology that can be used in future food web studies
- demonstrated that certain species of Orthoptera have very broad diets and this is likely how they coexist with others in high abundance
- found that Orthoptera communities are impacted by tropical forest degradation and that certain families are more responsive than others to land-use change
- shown that the Tetrigidae are found across a variety of primary and degraded habitats, but those occupying agricultural oil palm plantations have very different diets when compared with forest-dwelling species.

The broader implications of these findings, how DNA barcoding can aid the field of food web ecology and the importance of monitoring insect community changes, are discussed here.

7. 2 Revisiting aims

The research in this thesis has tested the utility of DNA barcoding and metabarcoding for insect herbivore food web analysis (Aim 1, Chapter 3).

Significant advances in molecular techniques offer exciting opportunities for ecologists to gain a better understanding of ecosystem functioning (Evans et al. 2016). In Chapter 3, we compared the use of traditional DNA sequencing (Sanger sequencing) with modern next generation sequencing (or metabarcoding) using an Illumina Miseq on the same set of Orthoptera gut contents. The findings from the study show that, if measuring insect-plant interactions when host plants and diet breadth are largely unknown, the metabarcoding approach is much more appropriate than the use of traditional Sanger sequencing. I emphasise, however, that Sanger sequencing is still a useful method for unmixed samples as it is able to produce longer sequences and provide greater confidence in terms of taxonomic resolution.

DNA barcoding has been described as a 'highly optimised methodology' that is becoming a standard methodology for characterising biological systems (Evans et al. 2016). Recent studies have used DNA barcoding to create food webs, host-parasitoid relationships (Kaartinen et al. 2010), pollinator sources (de Vere et al. 2017), plant-fungus interactions (Toju et al. 2014) and to identify bacterial communities. This is in addition to its use in characterising single species taxonomically. It is a robust methodology and has been used on samples from a range of environments such as soil, sediment and water (Thomsen et al. 2012). Few researchers, however, have used these methods to identify insect herbivore interactions. There remain limitations with the variety of procedures and platforms that can be used to carry out sequencing, not least of which is the expense of the procedures.

For herbivore diets, there remains issues with accuracy when using DNA barcodes to identify an exact species of host plant. Problems can occur at each stage of the barcoding procedure, such as obtaining high concentration of DNA (extraction), difficulties with amplification (PCR stage), cleaning samples, errors that can occur when sequencing. After sequence processing, there is always a risk of mismatch of sequences to reference databases themselves not without errors of identification. The risk of these errors can be reduced in a number of ways. We used a very robust extraction method to reduce problems with our extractions, ran duplicate PCRs for each sample to increase the chances of amplification success, followed well-established protocols for the clean-up stages, and used a comprehensive plant reference database for the region and multiple plant markers to increase our chances of correct taxonomic identification. Although there is no single molecular approach that is suitable for the wide range of ecological studies, our study identifies a successful methodology for detecting plants from within the gut contents of insect herbivores and concludes, as do others (Avanesyan 2014; Garcia-Robledo et al. 2013; Jurado-Rivera et al. 2009), that DNA barcoding is becoming an integral tool for the broader understanding of ecology and biodiversity.

I have **assessed two gene markers for measuring dietary breadth across Orthoptera species occupying the same habitat (Aim 2, Chapter 4).**

Using DNA metabarcoding methodology, I describe the diets of two different genera of Orthoptera from a sub-tropical open eucalypt woodland habitat in southeast Queensland. I found that the use of two gene markers (rbcL and trnH-psbA) was effective but suggest that for increased accuracy and food web completeness, three markers should be used, as different plant markers are able to pick up slightly different plant types (Hollingsworth et al., 2009). Although based on relatively small sample sizes, my results show that both Orthoptera species had broad generalist diets. Overlap in plant species across the diets was low,

suggesting that there was little competition between the two orthopteran species in terms of food resources. If this habitat were to change and plant availability was reduced, however, it is possible that competition between species could occur (Behmer & Joern 2008; Chase 1996). Further research is needed in this field and will generate greater understanding of herbivory, species competition and niche-overlap in communities and ecosystems.

I have **described the effects of habitat degradation (logging of primary forests and conversion to oil palm plantations) in the tropics, on orthopteran assemblages (Aim 3 Chapter 5)**.

In Chapter 5, I show that habitat degradation in the tropics in the form of logging and the conversion of forest to oil palm plantations, altered orthopteran assemblages. Although there were no previous studies on Orthoptera and this precise type of land-use change, research on other insect taxa was accorded with our findings. Different insect communities respond in a variety of ways to habitat degradation, and although species richness, abundance and diversity of some groups may be little affected, community composition was altered. Oil palm plantations are poor substitutes for tropical forests, with reduced plant diversity and harsher micro-climates, and the ecological processes which operate within them are altered. Although there are more overlapping species found in primary forests and logged forests, than that of primary forests to plantations, logged habitats were also found to have reduced species richness with its consequent effects on overall ecosystem processes. As primary forests have been found to be the most species-rich terrestrial habitats on Earth, they vital for sustaining tropical biodiversity (Gibson et al. 2011) and therefore protecting these habitats is an essential goal in conservation and ecology.

Other than sustaining biodiversity, trees are an important source of carbon storage which can play a critical role in reducing the impacts of climate change. Tropical deforestation alone

contributed to almost 10% of global anthropogenic greenhouse gas emissions between 2001 and 2013 (Vijay et al. 2016). In Sabah, Malaysia, deforestation has been largely due to expansion of agricultural land, particularly for oil palm plantations (Turner, EC & Foster 2009). The conversion of primary forest to oil palm plantations has been found to reduce the total standing biomass (Mg C/ha) by 81 – 88%. However, the Government of Malaysia has stopped the conversion or clearing of any type of primary forest for the purpose of oil palm agriculture(Kho & Jepsen 2015). Carbon stocks in logged forests are influenced by the degree of degradation but total standing stock can be reduced by approximately 77% when converted to oil palm plantations. These estimations, however, vary based on method and should be interpreted with caution (Koh and Jepsen 2015).

Palm oil is the world's primary source of vegetable oil, and the expansion of oil palm agriculture is set to increase. Further investigations on the impacts of this expansion on different taxonomic groups, particular those that are less studied, such as Orthoptera, are critical. Earlier studies have found differences at the ordinal level (Gibson et al. 2011; Turner, EC & Foster 2009), and my results show differences at the family level. Further investigations are required to understand the consequences of compositional changes and the ecological mechanisms underlying the differing vulnerability of taxa to anthropogenic disturbance.

I have added to **the greater understanding of dietary differences of Orthoptera across habitats and species, particularly within degraded tropical habitats (Aim 4, Chapters 6).**

In Chapter 6, I use the same methodology as described in Chapter 1, to investigate the diets of Tetrigidae across an altered tropical landscape in Sabah, Malaysia. This is the first study to use metabarcoding for the diets of Tetrigidae. I showed that, although generally reported to be conservative feeders, when one is able to identify plant species from leaf litter, the

Tetrigidae include many different plant families within their diets. One of the major findings from this study is that species of Tetrigidae occupying oil palm plantations had very different diets to those occupying forests (both logged and primary).

To date, most ecological networks are constructed using non-molecular methods, requiring field observations, gut content analysis by microscopy or stable isotopes, or choice or no-choice feeding trials. Each of these methods is labour-intensive and often only low taxonomic resolution of host plants can be obtained. Molecular approaches have the ability to produce more accurate results and are transforming food web ecology (Roslin & Majaneva 2016). These methods can be used to monitor species biodiversity and ecosystem functioning and assess the resilience and robustness of interacting communities to biodiversity loss (Evans et al. 2016). Understanding species interactions and the diversity of diets across different arthropod taxa is particularly important in habitats being changed by human impacts to predict responses and ultimately protect biodiversity.

7. 3 Conclusions and future directions

The work in this thesis has touched on many large and complex topics that will inevitably benefit from further research.

Orthoptera are a large and ecologically important insect Order contributing to herbivory, decomposition and overall ecosystem functioning across most habitat types. Habitat degradation and loss for agriculture are major threats to Orthoptera, particularly as certain families are less resilient than others. With the scale of natural habitat loss worldwide, it is essential to assess as wide a range of taxa as possible (Lawton et al. 1998) so that their responses to change can be predicted. We suggest that further in-depth research should investigate the less-studied arthropod groups in landscapes threatened with anthropogenic change. These include the Orthoptera. To continue on from the community analyses from the

forest understorey level, sampling Orthoptera in the canopy would make an interesting comparison in terms of species composition and richness among habitat types. It would not be surprising if different species were found in the canopy as species richness has been recorded as higher in the canopy when compared to the understorey and leaf litter of a tropical forest (Basset et al. 2015) but this has not been explored across altered habitats. Additionally, an interesting research question to answer using DNA barcoding would be whether canopy Orthoptera are more or less specialised in terms of diets and more or less resilient to habitat loss than those in the understorey. The recent increased availability of canopy cranes as flexible access tools (Nakamura et al. 2017) holds great promise in this regard.

As emphasised throughout this thesis, the use of molecular tools is becoming an essential part of ecological research. Very few studies, however, have used DNA sequencing to identify interactions among insects and their host plants, and even fewer where host plants are previously unknown. Our techniques, described in Chapters 3, 4 and 6, show great promise for future studies, which can now begin to assess insect diets in natural communities. Further to this, future studies should move on from binary food webs, to create more complex networks with tri-trophic levels. With the rate of land-use change worldwide, particularly in biodiverse tropical landscapes, habitat management is a challenge that requires an in-depth and comprehensive understanding of how interactions operate within communities (Evans et al. 2016). If interactions are known, then how these interactions are related to ecosystem functioning and the responses to environmental change can be better managed.

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