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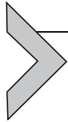

CHAPTER FIVE

Molecular Approaches for Studying Root Herbivores

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Abstract

The use of molecular techniques in insect ecology has expanded rapidly, allowing ever more challenging questions to be addressed. Compared to their aboveground equivalents, root herbivore molecular ecology has received less attention, despite essentially the same ecological questions being of importance in both the above- and belowground ecosystems. Studies so far have concentrated on economically important taxa, using mitochondrial and nuclear DNA sequencing and a variety of markers to investigate the species identity and relationships, population dynamics and dispersal, distribution, feeding behaviour and interactions with other organisms. Although this has proved useful for elucidating these aspects of their ecology, there remains a need to focus on the functioning of root

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herbivores in the soil ecosystem. Application of new and emerging technologies developed for aboveground systems will increasingly be applied to those belowground, allowing a focus on root herbivore biology and ecology in the context of ecosystem processes and systems ecology. For the foreseeable future, however, the use of molecular techniques is likely to remain dominated by the need to address pragmatic research questions about specific taxa, notably pests.

1. INTRODUCTION

A rise in the number and accessibility of DNA techniques and their application to entomology has led to investigation of, among other aspects, the genetic diversity, phylogeography and species identity and taxonomic relationships of a wide range of invertebrates. Numerous published studies have used molecular markers in tandem with data obtained in the field or laboratory to answer ecological questions, focusing on both applied (e.g. pest management and conservation biology) and theoretical aspects of the ecology of various species, but the vast majority of these are on aboveground insect herbivores, which are generally conspicuous, accessible and easy to manipulate both in the field and the laboratory using well-described methods. For example, only 10 of the 249 articles on insect herbivores published in the journals *Molecular Ecology* and *Molecular Ecology Resources* (formerly *Molecular Ecology Notes*) are on root-feeding taxa. This bias towards the study of aboveground herbivores has been noted (e.g. Blossey and Hunt-Joshi, 2003; Hunter, 2001) and the distribution, abundance, activities and effects of many important belowground species remain underresearched. This reflects a general situation where we know far more about above- than belowground herbivory. A large proportion of the studies that have been carried out on root antagonists focus on nematodes, for which researchers have adopted molecular approaches similar to those used in soil microbiology (e.g. Powers et al., 2011; Waite et al., 2003) to investigate their biology and ecology partially because their microscopic size and morphologically cryptic nature makes this particularly difficult. Many other taxa have been overlooked, yet molecular genetic methods have the potential to overcome some of the difficulties associated with the study of root herbivores and allow the investigation of areas of their ecology for which we currently lack a detailed understanding. This has become increasingly recognised by researchers in this field over the last decade and some

important advances have been made, but further opportunities remain, particularly as new methods are developed.

Most molecular genetic techniques revolve around the extraction of DNA from the organism of interest followed by polymerase chain reaction (PCR) to amplify DNA, allowing it to be subsequently manipulated and characterised (Freeland, 2005). Using primers, either universal or species-specific, to target different regions of DNA allows a large degree of flexibility in the questions that can be asked. Mitochondrial DNA (mtDNA) is often used because of its high mutation rate, ease of manipulation and lack of recombination and is therefore useful for investigating diversity within and between populations, for example, for indirectly inferring dispersal from gene flow and determining evolutionary relationships. Molecular markers are used extensively in ecology to quantify levels of genetic variation. Allozymes (enzyme polymorphisms) determined using isoelectric focusing/isoenzyme electrophoresis (separation of proteins using gel electrophoresis) and monoclonal antibodies are two of the first protein-based techniques used in ecological research. Restriction fragment length polymorphisms (RFLPs), based on variation in DNA sequences rather than proteins, and PCR–RFLP, a variation on this targeting specific regions of DNA, are now less common but can still be useful for certain applications. Microsatellites are often used in population genetic studies. These are stretches of DNA that consist of tandem repeats of 1–6 bp, and their use is based on allele size differences between individuals, caused by mutations arising during slippage in replication (Freeland, 2005). Other markers include randomly amplified polymorphic DNA (RAPD; Welsh and McClelland, 1990; Williams et al., 1990), for which PCR is used to generate fragments using a randomly selected 10-bp primer (Ritland and Ritland, 2000) and amplified fragment length polymorphisms (AFLPs), which use restriction digest of genomic DNA followed by amplification of a subset of fragments (Vos et al., 1995). These techniques do not require prior sequence knowledge and so development time can be short (though see Section 3.2), but RAPDs are no longer commonly used for population genetics because of lack of reproducibility and therefore reliability (Freeland, 2005). For many of the previously mentioned methods, the DNA sequence that is being targeted is not known, but DNA sequences obtained through Sanger sequencing (a widely used DNA sequencing method) can be used directly to quantify genetic variation via base-pair differences between individuals. Related to this, single-nucleotide polymorphisms are single-base-pair positions in a sequence that are variable

Table 5.1 Summary of molecular techniques, including target DNA region (where applicable) and their application to the study of root herbivore taxa

Application	Molecular technique	Target DNA region	Taxa	Review section
Predator–prey interactions	Species-specific PCR fragments	*COI*	Scarab larvae (*Melolontha melolontha*)	2.1
			Scarab larvae (*Phyllopertha horticola*)	
	qPCR	*COI/tRNA-Leu*	Corn rootworm (*Diabrotica* spp.)	
		COI	Scarab larvae (*M. melolontha/Amphimallon solstitiale*)	
Plant–herbivore interactions	Species-specific PCR fragments	*rbcL/trnL*plastid/*trn*T-F chloroplast (plants)	Wireworms (*Agriotes* spp.)	2.2
Endosymbionts	Mitochondrial (mtDNA) sequencing	*12S-N4* and *CB2H-C2R*	Corn rootworm (*Diabrotica barberi*)	2.3
		16S rRNA, *ftsZ* and *wsp* (*Wolbachia*)		
		16S rRNA	Wireworms (*Limonius canus/Agriotes lineatus*)	
		Metagenome sequencing	Cutworm (*Agrotis ipsilon*)	
Genetic diversity	Allozymes	Protein-based	Corn rootworm (*Diabrotica* spp.)	3
	RFLPs (PCR–RFLPs, T-RFLP)	*12S-N4* and *CB2H-C2R/ITS1*	Corn rootworm (*Diabrotica* spp.)	

	Microsatellites	–	Corn rootworm (*Diabrotica* spp.)	
			Gall-forming root aphids (*Pemphigus* spp.)	
			Ant-associated root aphids (multiple Aphididae)	
			Root weevil (*Diaprepes abbreviatus*)	
	AFLPs	–	Gall-forming root aphids (*Pemphigus* spp.)	
	RAPDs	–	Root weevil (*Diaprepes abbreviatus*)	
	Mitochondrial (mtDNA) sequencing	*16S rRNA* and *COI*	Root weevil (*Diaprepes abbreviatus*)	
		16S rRNA	Wireworms (multiple Elateridae)	
		COII	Scarab larvae (*Antitrogus parvulus*)	
	Nuclear (nuDNA) sequencing	*ITS1*	Corn rootworm (*Diabrotica barberi*)	
Species identification	Mitochondrial (mtDNA) sequencing	*Cytb*	Leatherjackets (*Tipula* spp.)	4.1
		16S rRNA/COI	Wireworms (multiple Elateridae)	
	RAPDs	–	Gall-forming root aphids (*Pemphigus* spp.)	
	RFLPs (PCR–RFLPs, T-RFLP)	*COII*	Scarab larvae (multiple Scarabidae)	
		16S rRNA	Wireworms (*Agriotes* spp.)	
	Isoelectric focusing	Protein-based	Leatherjackets (*Tipula* spp.)	
	Multiplex PCR	*COI*	Wireworms (*Agriotes* spp.)	

Continued

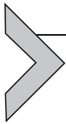
Table 5.1 Summary of molecular techniques, including target DNA region (where applicable) and their application to the study of root herbivore taxa—cont'd

Application	Molecular technique	Target DNA region	Taxa	Review section
Association of life stages	Mitochondrial (mtDNA) sequencing	*COI*	Gall-forming root aphids (multiple Eriosomatini)	4.2
		COI and *16S rRNA*	Scarab larvae (multiple Scarabidae)	
	Nuclear (nuDNA) sequencing	*28S rRNA*	Scarab larvae (multiple Scarabidae)	
Phylogenetic relationships	Allozymes	Protein-based	Corn rootworm (*Diabrotica* spp.)	4.3
	RFLPs (PCR–RFLPs, T-RFLP)	*NADH 4*	Corn rootworm (*Diabrotica* spp.)	
	Mitochondrial (mtDNA) sequencing	*COI, COII*	Corn rootworm (*Diabrotica* spp.)	
		COI	Gall-forming root aphids (multiple Fordini)	
	Nuclear (nuDNA) sequencing	*ITS1*	Corn rootworm (*Diabrotica* spp.)	
		EF-1 α/LWO	Gall-forming root aphids (multiple Fordini)	
Species distribution	RFLPs (PCR–RFLPs, T-RFLP)	*16S rRNA*	Wireworms, leatherjackets, Bibionid and Sciarid fly larvae	4.4
	Multiplex PCR	*COI*	Wireworms (*Agriotes* spp.)	
Quantification	Monoclonal antibodies	Protein-based	Slugs (*Deroceras reticulatum*)	4.5

These studies have been examined in further detail in the specified review section.

between individuals and provide another way of determining sequence variation at multiple loci. Quantitative polymerase chain reaction (qPCR) can be used to determine the amount of DNA present in a sample, whereby a molecular tag is incorporated into the PCR. When the tag binds to DNA, it fluoresces and this is recorded at each cycle as a fluorometric reading, which is proportional to the amount of DNA present. More recently, next-generation sequencing (NGS) approaches, which allow massively parallel, or deep, sequencing of DNA fragments, have allowed whole genomes or communities to be sequenced in a time- and cost-effective manner, paving the way for larger-scale studies and opening up new opportunities for the study of certain aspects of insect ecology.

The use of these markers depends upon both the question under study and practicality (time and resources) and as such a variety of methods have been used in different disciplines within root herbivore ecology (Table 5.1). This chapter aims to describe and summarise the applications of these techniques, drawing on studies already undertaken and providing suggestions for the focus of future work.



2. SPECIES INTERACTIONS

The nature of the soil environment, and the size of the organisms within it, makes it difficult to study the behavioural ecology of belowground insects without manipulation of conditions in usually less than realistic laboratory experiments. One area in which molecular ecological techniques have helped to overcome these practical issues is feeding ecology. Aboveground insect herbivores have been extensively studied, for example, in terms of host–plant interactions (e.g. Pieterse and Dicke, 2007; Whiteman and Jander, 2010) and predator–prey food webs (Hereward and Walter, 2012), but there are relatively few studies on soil insect herbivores specifically.

Soil organisms have been recognised to play a vital role in nutrient cycling and decomposition and as such have been studied in relation to their role in the soil food web. This has mainly been limited to the mesofauna, bacteria and fungi (through the use of stable isotopes, e.g. Crotty et al., 2011, 2012); however, researchers have started to consider species-specific interactions of the macrofauna in the soil food web (Eitzinger et al., 2013). This has been achieved by gut content analysis, using molecular probes that target specific regions of plant or herbivore DNA present in whole-organism

DNA extracts using PCR, without the need for extraction and dissection of the gut. These PCR products can then be subjected to gel electrophoresis and identified based on the size of the product produced or fluorescently labelled and analysed using fragment analysis using an automated sequencer. Traditionally, monoclonal antibodies (reviewed in Sheppard and Harwood, 2005) and isoenzyme electrophoresis (e.g. Amalin et al., 2000; Traugott, 2003) have been used for gut content analysis, but diagnostic PCR protocols are now more commonly adopted since they are cost- and time-efficient and use widely available equipment, and once the tool is developed, it can be applied by other researchers and/or modified to detect specific DNA targets, depending upon the question under study (Juen and Traugott, 2006).

2.1. Predator–prey interactions

As in studies of aboveground insects (Sheppard and Harwood, 2005), sections of the mtDNA *COI* gene have been targeted in the present studies. This region of DNA is commonly used for species identification through barcoding (see Section 4.1) and contains both conserved and variable regions, meaning universal primers (Folmer et al., 1994) and species-specific primers can be employed. As such, this makes it an easily manipulated target for use in gut content analysis. Juen and Traugott (2005) developed the first PCR-based approach for studying soil insect predator–prey dynamics using white grubs, also known collectively as canegrubs and chafers (Scarabidae larvae; *Melolontha melolontha*), in feeding experiments with larvae of the ground beetle *Poecilus versicolor*, a common above- (as adults) and belowground (as larvae) predator. They tested the effect of digestion time on detection of target DNA of different lengths, using primers designed specifically to amplify *M. melolontha* DNA. Importantly, for future studies, they found that DNA could be detected from even small amounts of predation (i.e. when larvae were fed a single egg) and that the primers were able to amplify DNA from feeding on both carcasses and fresh prey at similar levels. This was not correlated with rate of digestion and did not differ significantly between lengths of fragment targeted (ranging from 175 to 585 bp). It was therefore not possible to distinguish between scavenging and active predation—a possible limitation to this type of study. This protocol was applied in an investigation of the predator guild of another pest scarab, the garden chafer *Phyllopertha horticola,* but using newly designed primers specifically targeting this species (Juen and Traugott, 2007). *P. horticola* DNA was successfully detected in a variety of predators (mostly in Geophilidae and predatory beetle larvae), showing that the larvae are a

potentially important prey item in the soil food web and that these taxa could therefore be important natural enemies of this pest. This is especially relevant since earthworm DNA was only found in 2.7% of tested individuals, yet this taxon is known to be a widespread and highly abundant and important food source for soil predators.

Using a slightly different approach, Lundgren et al. (2009) considered predation of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) larvae using qPCR to detect *D. virgifera* DNA sequences in arthropod predator guts. Primers that amplified a segment of the *COI* and tRNA-*Leu* genes of *D. virgifera* were designed and used together with feeding experiments with various predators. This semiquantitative method not only detects prey DNA but gives an idea of the amount present. It was found that more predators were positive for *D. virgifera* DNA in the egg stage than the larval stage and predators differed in their propensity to consume *D. virgifera*. This suggests that timing might be an important factor in predation (i.e. longevity of egg and larval stages) and that a range of arthropod species (ground beetles, harvestmen, wolf spiders and predaceous mites) are important in regulating this pest, which has implications for agricultural management. However, the study mainly concerned surface-active predators caught in pitfall traps; focusing on predators within the soil column may provide a different picture. Using the same approach, a further study (Lundgren and Fergen, 2011) evaluated whether winter vegetation increased larval predation by predators aboveground and in the soil column. Twenty predator taxa from the soil column and 33 taxa from the soil surface were found to have consumed the larvae, with Carabidae being one of the most abundant in both, showing this pest has a diverse predator community, which suggests conservation efforts should be targeted at this level rather than at specific taxa. qPCR correlated well with pest damage only when a predation index was used taking into account DNA quantity, predator abundance and relative frequency of detection but, combined with predation intensity observations using restrained larvae, gave an overall view of these predator–prey interactions.

Eitzinger et al. (2013) recently developed a PCR assay for the detection of prey DNA in soil-dwelling centipedes (*Lithobius* spp.) from forest soils, targeting a range of invertebrate taxa at the family level, and others have developed assays for soil-dwelling predacious beetle larvae-consuming taxa at other trophic levels (e.g. detritivores: earthworms and Collembola; Eitzinger and Traugott, 2011), which could potentially be adapted for assessing interactions of root herbivores specifically.

As shown in aboveground predator–prey systems, there are various parameters that can affect the ability to detect and quantify prey within predators, such as predator identity and size of the target DNA molecule (Sheppard and Harwood, 2005). These effects are little known for root herbivores, but some aspects have recently been studied by Waldner et al. (2013), who tested prey detection intervals for DNA fragments of different sizes, meal size and relative biomass increase in four different soil-dwelling predators of white grub. Whereas meal size and predator biomass had no significant effect (as for similar aboveground studies), prey detection intervals were affected by the size of DNA fragments, for which smaller intraspecific differences were found for the medium-sized fragment in beetles, and differed between, but were similar within, taxa. These results imply that primer sensitivity and predator identity may affect observed outcomes. In terms of quantification, there are many interacting factors that lead to the final amount of food present in the gut, and as such, it is not possible to make inferences about the number of prey eaten, rather an indication of which species are involved and their relative predation capabilities (Lundgren et al., 2009).

2.2. Plant–herbivore interactions

Studies on the dietary choices of root-feeding insects have also only recently started to be explored. Staudacher et al. (2011b) developed a method for detecting the presence of wheat and maize DNA in wireworms (Coleoptera: Elateridae; click beetle larvae) using a similar protocol as the predation studies previously mentioned but targeting genes that are more relevant for plant identification (*COI* is not thought to be suitable since it evolves too slowly). General primers were developed to amplify fragments of *rbcL* and *trnL* plastid DNA, often used for plant DNA barcoding and also specifically for maize and wheat. The plants could be detected for up to 72 h postfeeding (the maximum tested), and the specific primers made it possible to determine which plant DNA was present because of differentially sized products, though further testing would be needed to determine the postfeeding limits of detection. Following on from this, Wallinger et al. (2013), using a multiplex PCR approach (Wallinger et al., 2012), determined that plant identity, but not wireworm species identity, and level of decay were important in the detection of plant DNA, with decayed material having a lower detection rate than fresh material and wheat detection being significantly decreased over time as compared to maize.

Only one study has applied a developed molecular method, in combination with stable isotopes, to specifically investigate aspects of the ecology of root herbivores, again using *Agriotes* wireworms as a model species (Staudacher et al., 2013b). Using plants that are more or as attractive to pests than the cultivated crop has been proposed to prevent wireworm damage (Landl and Glauninger, 2013; Vernon et al., 2000), and this study investigated wireworm feeding behaviour in the presence of increased plant diversity and whether this could protect the maize crop. The protocol described by Wallinger et al. (2012) was used for gut content analysis, while stable isotopes were used to assess the long-term diet. The presence of a range of plant species did in fact lure the wireworms away from the main crop, increasing its yield, while it was shown that the larvae were actively feeding on the other plant species, switching to these from the maize crop previously consumed. This not only confirms previous observations but also shows that using a mixture of noncrop plants planted at the same time as the crop potentially provides protection from damage. However, some plants were consumed more often than others and this differed between seasons, so further work using these techniques is needed to determine the most appropriate plant mix for optimum results.

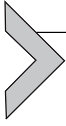
2.3. Endosymbionts

The importance of symbionts in insect–plant interactions is becoming increasingly recognised (Frago et al., 2012), partly due to the relative ease in which this can now be studied due to advances in molecular genetic and genomic techniques. Gut symbionts allow adaptation to different food plants, protection from predators or stress and may also promote speciation by causing reproductive and ecological isolation of host populations (Moran et al., 2008). *Wolbachia* species are intracellular, maternally inherited bacterial parasites that can cause cytoplasmic incompatibility (preventing infected males fertilising the eggs of uninfected females), feminisation of males and killing of male embryos. Using primers targeting *Wolbachia* genes, the presence of this endosymbiont was investigated in northern corn rootworm, *Diabrotica barberi* (Coleoptera: Chrysomelidae) populations in north central United States (Roehrdanz and Levine, 2007). Previous studies found two distinct mtDNA clades east and west of this boundary suggesting little mixing of populations (Roehrdanz et al., 2003) and since *Wolbachia* had been detected in populations from the east of this area, this was postulated as a possible cause. mtDNA (*12S-N4* and *CB2H-C2R* genes) was amplified

for the corn rootworm to gain mitochondrial haplotypes and primers targeting *Wolbachia* genes (*16S rDNA*, *ftsZ* and *wsp* genes) used to determine its presence in rootworm DNA extracts. Two different strains were detected on either side of the boundary, each associated with a different mtDNA clade, suggesting the strains are not compatible, but testing with nuclear DNA may help to determine whether *Wolbachia* is reducing gene flow and increasing population differentiation or other factors are also involved. Practically, if these delineated populations have different traits affecting their control (e.g. insecticide resistance), this could have an impact on pest management programmes.

Some endosymbionts have been considered for genetic manipulation as biocontrol agents, especially for root-feeding pests such as wireworms for which pesticide application is difficult and broad-spectrum pesticides are now limited. Microbial agents have been trialled for wireworms in the past with limited success, but a modified form of known symbionts in certain species may increase their efficacy. Lacey et al. (2007) carried out a survey, using *16S rRNA*, of gut bacteria associated with *Limonius canus* wireworms, which are often associated with potato damage, for this purpose. A number of potential control candidates were found, including species that have already been manipulated to produce insecticidal toxins and antagonistic rhizosphere-associated species, which could be added to seed dressings or used in a trap cropping approach (*Rahnella aquatilis* is common in the wheat rhizosphere). Other bacterial species have also been isolated and tested for their pathogenicity using the same approach for *Agriotes lineatus* wireworms, though testing in the field is needed to confirm their efficacy in control (Danismazoglu et al., 2012).

Metagenomic analysis of gut symbionts in three different herbivorous insects, grasshopper (*Acrida cinerea*; Orthoptera), cutworm (*Agrotis ipsilon*; Lepidoptera) and termite (*Nasutitermes* sp.; Isoptera: Termitidae), recently revealed that gut symbiont composition was related to their function in biomass degradation and nutrient biosynthesis (Shi et al., 2013). Furthermore, gut symbionts and insects coevolved based on insect food preferences in favour of optimal biomass degradation, nutrient utilisation and other species-specific factors related to lifestyle. For example, cutworm contained a higher diversity of symbionts than the other herbivores, possibly related to its generalist feeding habit. This deep-sequencing approach revealed many more symbionts than traditional techniques such as denaturing gradient gel electrophoresis followed by *16S rRNA* sequencing, including many unculturable (but taxonomically described) species.



3. GENETIC DIVERSITY

Investigating the genetic diversity within and between populations provides much insight into the biology and ecology of species and is an important contributor to the long-term conservation of populations, particularly under environmental change. Though it may be relatively straightforward to distinguish populations of aboveground insects, for which boundaries can be more obvious, the soil represents a continuous habitat, which makes it difficult to determine where one population ends and another begins or indeed whether organisms are spatially structured in the same way as they are aboveground. Dispersal is a key mechanism for maintaining genetic diversity and viable populations, and for pest species, knowledge of this is particularly relevant for devising management strategies, taking into account spatial and temporal aspects of species' biology.

At present, little is known of invertebrate movement within the soil. Though some sophisticated techniques have been employed to look at the *in situ* movement of soil-dwelling invertebrates, for example, X-ray microtomography (Johnson et al., 2004) and controlled laboratory and field experiments (Murray et al., 2010; Williams, 2012), this fails to provide information on the extent of movement within and between populations in the field. Using stable isotopes, Schallhart et al. (2011) attempted to track the movement of *Agriotes obscurus* wireworms (click beetle larvae) between fields, though none was detected (probably because there was a food supply present). However, since many root herbivores are insect larvae with aboveground adult stages, which can be assessed more easily, the majority of studies carried out to date have focused on adults. Few root-feeding taxa have been studied but, where they have, several techniques have been used to determine the population genetics of economically or ecologically important species.

3.1. Corn rootworm

Molecular markers have been utilised to investigate the population genetics of corn rootworm, which comprise a complex of *Diabrotica* species that are of particular economic importance in maize systems; larvae feed on crop roots belowground, while adults inhabit the aboveground plant. Early studies using allozymes (Krysan et al., 1989; McDonald et al., 1985) and PCR–RFLP (based on the nuDNA *ITS1* region; Szalanski et al., 1999) found low levels of differentiation in geographically isolated populations of western

corn rootworm (*D. v. virgifera;* WCR), Mexican corn rootworm (*D. v. zeae*; MCR) and northern corn rootworm (*D. barberi*; NCR), suggesting high dispersal ability and limited barriers to gene flow. In the PCR–RFLP study, it was also found that for some mitochondrial regions (*18S* and *5.8S rRNA*), there was no variation between subspecies, suggesting a recent common evolutionary history. Because of the lack of variation at these mitochondrial regions, microsatellite markers have since been developed for all three species (Kim and Sappington, 2004; Kim et al., 2008; Waits and Stolz, 2008). Kim and Sappington (2005) used these microsatellites for the WCR and found that there were high levels of genetic diversity but little differentiation between populations (though there was a significant correlation between geographic and genetic distances overall). The authors concluded that although it is possible these beetles have a high migration rate, it is more probable that populations have had insufficient time to become significantly genetically different since their expansion eastwards across the United States around 50 years previously. More recently, Chen et al. (2012) used microsatellites for WCR to genotype populations with high, intermediate and low levels of resistance to methyl parathion and aldrin, finding significant differentiation between populations with high and low levels of resistance and between geographically separated populations. As well as showing how genetic and biological species information can be combined to increase understanding of species' ecology, the long-term study of corn rootworm also shows how marker choice has evolved and is important in quantifying genetic variation at different organisational levels.

3.2. Root-associated aphids

Due to their status as major agricultural pests, much work has been carried out on the genetic diversity and evolutionary history of aphids and their association with crops (Hales et al., 1997). There are, however, root-associated species, which have attracted less attention, but are interesting because of their life cycle. The lettuce root aphid (*Pemphigus bursarius*), for example, sexually reproduces on black poplar and winged forms then migrate to members of the Compositae (including lettuce) as secondary hosts where they parthenogenetically reproduce on the roots. After several generations, winged forms emerge and they migrate back to the primary host (Miller et al., 2003). Microsatellites have been developed (Miller et al., 2000) and applied (Miller et al., 2003) to investigate the genetic structure of populations on the primary and secondary hosts. The population structure

of this species was found to be irregular. Several sample locations showed significantly different allele frequencies when compared, but there was no significant relationship between genetic and geographic distances. Excess homozygosity was observed at a number of sites (i.e. populations were not in Hardy–Weinberg equilibrium), and inbreeding and local bottlenecks, based on observational data of galls on poplar, which contained no aphids but sometimes anthocorid (Heteroptera: Anthocoridae) predators, were put forward as reasons for this spatial structure. In addition, there were allele frequency differences between populations on poplar and lettuce, which may be accounted for by secondary host races and occurrence of anholocyclic (viviparous females give birth to only viviparous females, clonally reproducing) and holocyclic (viviparous females produce oviparous females or males, which go on to sexually reproduce). In this case, the use of microsatellite markers increased the understanding of the population dynamics of this species, though further work is needed to determine the mechanisms, which account for the structure that was found. Since there are differences in the dispersal abilities of above- and belowground organisms, particularly those such as aphids that are wind-dispersed aboveground, the spatial scales of study and dispersal may differ and as such this should be taken into account in future studies.

Understanding the population dynamics of species with this type of life cycle becomes more complicated when two morphologically similar species are involved. In this case, Chen et al. (2009) used both RAPDs and AFLPs to differentiate species and determine the population dynamics and occurrence of *Pemphigus populitransversus* and *Pemphigus obesinymphae*, which have a primary poplar host and secondary cruciferous host, namely, cabbage, on which they are important pests in Texas. The occurrence of two separate species was confirmed (previously only determined using morphology) and they were found at different times of the year but co-occurred for extended periods, having implications for previous studies that assumed one, continuously present species. Most aboveground alate adults, determined morphologically, were *P. obesinymphae* and clearer RAPD banding patterns were found for this species in the soil, which the authors suggest could be down to their greater presence. However, many factors can affect the efficiency of the PCR and the increased band intensity may therefore be attributable to causes other than simply more individuals of this species being present. Methodologically, RAPDs proved to be superior to AFLPs; this technique was more efficient, cheaper and with higher numbers of polymorphic bands (many were missing from the AFLPs in tests with DNA of known

species). These molecular methods were particularly useful for determining the apterous soil stage of the species, which is more difficult to identify morphologically, but required fresh samples for the best results (those preserved in 100% ethanol contained less and lower-quality DNA).

Other root-associated aphid species also have interesting life histories, which have consequences for their genetic diversity and population structure that may be in contrast to aboveground species. The yellow meadow ant *Lasius flavus* found in grasslands in Europe tends a number of aphid species in exchange for honeydew (and also eats them). Ivens et al. (2011) developed 26 microsatellite markers for studying the effects of these mutualistic relationships on the population genetic structure of four species of ant-associated aphids and then applied them (Ivens et al., 2012b) to determine the extent of dispersal, mode of reproduction and potential for dispersal between nests by winged forms. All species were found to be anholocyclic with few alate individuals and there was a strong correlation between genetic and geographic distances for two species, which confirms previous observations and suggests that there is limited dispersal between nests. However, some dispersal was observed since some clonal lineages were spread along the sampling transect. As with *Pemphigus* species, sexual reproduction usually occurs on the primary host, but these were not present nearby, which could have had an effect on the results. Their presence in the underground nests all year may have had an impact too and is in contrast to their aboveground equivalents, which often maintain a holocyclic lifestyle, highlighting how it is important to study these belowground populations independently. A further study (Ivens et al., 2012a) looked into the spatial structure in more detail and found that more than half of the mounds contained a single species with a single clone, but where multiple clones did exist, they were spatially separated. As well as giving information on aphid biology, it gives much insight into the behaviour of these ants, which seem to eat the younger instars and maintain lower levels of honeydew-producing adults in a similar way that humans farm livestock.

3.3. Root weevil

The genetic diversity of other beetle species has also been investigated. The root weevil (*Diaprepes abbreviatus*) is a serious invasive pest introduced from the Caribbean to the United States in the 1960s, since spreading and becoming associated with more than 300 plant species (Ascunce et al., 2008). Larvae feed on roots and adults on leaves. Studies on populations in Florida

using esterase polymorphisms and RAPD–PCR markers (Bas et al., 2000) produced different results dependent on the marker used; esterase and protein can be changed by physiological analysis (they depend upon active genes) and optimum PCR conditions are critical to the success of RAPD markers, reducing their reliability for population genetic studies. Since then, eight microsatellite markers have been developed (though published studies using them are not yet available; Ernst et al., 2006).

Finally, *16S rRNA* and *COI* mitochondrial markers have been used to characterise populations and determine dispersal in Florida and Dominican populations, their supposed origin (Ascunce et al., 2008). There was low genetic diversity and one haplotype in each of six sampled populations, with low numbers of substitutions between the three haplotypes found in total, suggesting a recent introduction. That the same haplotype was found in the east and west, despite populations being discontinuous, suggests some form of human transportation on infected plants (movement has been shown to be slow and localised previously). The authors suggest that using fine-scale neutral markers such as microsatellites may help to resolve the differences seen using between mtDNA and esterase polymorphism and RAPDs, which suggest more widely differentiated populations than *COI* or *16S rRNA* sequences. None of the *COI* sequences for the Dominican samples matched those from Florida, suggesting these may have been imported from another Caribbean island. However, there was some uncertainty in the morphological identification and a high genetic distance between species varieties found in Dominica and Florida within this genus, which requires further analysis.

3.4. Wireworms

A similar study to that of Ascunce et al. (2008) has been carried out for wireworms in Canada (Benefer et al., 2013). However, unlike all previous studies on the genetic diversity of root herbivores, this focused on the damaging, soil-inhabiting larval stage rather than the aboveground adults. Wireworms are click beetle larvae that feed on crop roots, causing high levels of economic damage in some areas. There are several invasive species in Canada (including those in the genus *Agriotes*, which are the main UK pest species) for which information on the distribution and species identity is lacking. Phylogenetic analyses were conducted using mtDNA *16S rRNA* to assess genetic variation in populations across Canada. While there was low intraspecific variation for most of the species (low sample size prevented further analysis or strong conclusions to be drawn for these), there was relatively

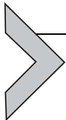
high variation between *Hypnoidus bicolor* individuals and a significant relationship between genetic and geographic distances, with genetic separation of populations from the east to west of Canada. Further investigation revealed that there may be two different, cryptic *Hypnoidus* species present. There was also some evidence for genetic variation related to geographic location for *Limonius californicus*, for which samples from British Columbia in the west were separated from those in Saskatchewan and Alberta in the mid to midwest areas. Since the distance between these locations is large (up to 1460 km), the opportunity for populations to mix is probably limited. High cryptic diversity and possible nuclear mtDNA (numts) were limitations to the study, but it provided preliminary data as a starting point for further investigation into pest species identity and distribution in Canada.

Like for root weevil, it is recognised that it would be advantageous to have fine-scale data on wireworm population genetics. Previous attempts had been made to develop microsatellite markers at Plymouth University (Benefer, 2011; J. Ellis, personal communication) for *A. lineatus*, *A. obscurus* and *A. sputator*, common European pests, without success; none were found to be polymorphic on initial screening. Benefer (2011) then developed AFLP markers to investigate population structure and dispersal of these species in the United Kingdom, but due to numerous methodological problems (including lack of reproducibility between runs), an optimised set of markers could not be produced. Work is now under way to develop microsatellite markers using NGS data for the same species (C. Benefer, personal communication), whereby fragment libraries were generated from known adult genomic DNA and all three sequenced on a 454 GS FLX platform, the data mined for microsatellite loci and primers designed using freely available software. This protocol cuts out the lengthy development time associated with traditional cloning techniques and produces hundreds of loci, which can be selected depending on their likely ability to amplify the target loci (taking into account primer and loci attributes). So far, 20–25 microsatellite loci per species have been developed using this protocol and are being screened across individuals.

3.5. Canegrubs

In a follow-up study to that of Miller et al. (1999) where pest scarab larvae were identified using PCR–RFLP and genetic variation was found to correspond with species distributions (see Section 4.1), Miller and Allsopp (2005) investigated the phylogeography of the scarab beetle *Antitrogus*

parvulus in southeastern Queensland. The females of this species are thought to be poor dispersers, mating and laying eggs close to the emergence site, while males are strong fliers. This type of mating behaviour and other ecological factors play a role in population structure, having implications for pest management, and as such, the effect of habitat fragmentation and maternal dispersal ability were focused on here, using cytochrome oxidase II (*COII*) sequences analysed by analysis of molecular variance (AMOVA), nucleotide divergence (as a measure of sequence divergence among individuals between populations), Mantel test for isolation by distance and neighbour-joining (NJ) phylogenetic analysis. Most of the genetic variation was accounted for at the regional or population level, while intrapopulation variation was low, and there was a highly significant relationship between geographic distance and gene flow, with migration limited by geographic distance. This was reflected in the NJ tree in which populations from the same regions were clustered together, sharing more than one haplotype. These results suggest that females do not move far, but when they do, they move to the next closest soil patch. Two populations were anomalous, with high gene flow compared to others, possibly due to other factors, for example, wind flow, playing a role in their dispersal. Other populations were found to be somewhat genetically distinct despite their close proximity due to geographic barriers. Soil-type preference and rainfall/moisture could also be important, as seen in similar species, which suggests knowledge of dispersal ability and ecological preferences may help in targeting management practices for scarab pests in this region.



4. SPECIES IDENTIFICATION AND PHYLOGENY

Some of the studies already mentioned have shown how important it is to be able to identify the species concerned correctly (see Section 3). This has been made possible for many insect taxa through the sequencing of diagnostic regions of DNA that are variable between species. Though other DNA regions can and are used, this DNA barcoding approach commonly refers to the use of a 648 bp region of the mtDNA *COI* gene. This has been somewhat controversial, mainly where it is applied to the identification of new species because of the unpredictable range of intraspecific variation (Blaxter et al., 2005; Hebert et al., 2003; Janzen, 2004; Taylor and Harris, 2012). Nevertheless, it has been extensively applied to a range of invertebrate taxa, again mainly aboveground, providing information on species ecology that was previously inaccessible. For soil-dwelling species, the

use of such barcoding methods provides a relatively quick, easy and affordable way of identifying morphologically similar or cryptic species that usually require a great deal of skill and time using microscopy and that for many immature stages is not possible at all, such as for many Coleopteran and Dipteran larvae, or microscopic and mesofaunal organisms. Species identification through the use of sequence data has been adopted for root-feeding taxa such as nematodes (from the early 1990s, e.g. Okimoto et al., 1991, and more recently, e.g. Floyd et al., 2002) including using metagenomic, NGS approaches (Porazinska et al., 2009). Others include springtails (Collembola; Porco et al., 2012; Yu et al., 2012) and earthworms (Huang et al., 2007; Porco et al., 2012). To date, root-feeding taxa have been identified by a combination of molecular markers and sequence data.

4.1. Identification of larval pests

There are 19 pest species of Melolonthini (Coleoptera: Scarabaeidae) in Australia whose larvae cause economic levels of damage to sugarcane by feeding on their roots, but of these, five are indistinguishable using morphological techniques and have similar behaviours and overlapping distributions. Therefore, cytochrome *c* oxidase II subunit gene (*COII*) sequences were used to develop a diagnostic tool for species identification (Miller et al., 1999). Firstly, sequences from known adults, which are more easily determined, were used to produce a phylogenetic tree. This information on species relationships and mtDNA variation (base-pair differences) was used to select restriction enzymes producing diagnostic RFLPs for use in PCR–RFLP for analysis using gel electrophoresis, negating the need to sequence larvae. The phylogenetic analysis also revealed genetic differentiation of populations of one species (*Lepidiota negatoria*), which could be related to biogeographic boundaries to gene flow, and no differentiation for another species (*Antitrogus consanguineus*), suggesting uninterrupted gene flow. As well as providing a tool to identify important pest species that could be useful in pest management, this study also suggests that the sequence data may be more broadly useful for assessing species' biology, ecology and behaviour.

Crane fly larvae, or leatherjackets (Diptera: Nematocera), were some of the first root herbivore species to be subjected to molecular identification using isoelectric focusing, based on the position and number of protein bands (Humphreys et al., 1993). *Tipula oleracea* and *T. paludosa* are pests of cereal crops and grassland and this method was used to assess the species responsible for the recent (at the time) destruction of winter cereals in

Northern Britain, since larvae of these species are difficult to differentiate using morphological characteristics and time-consuming to rear to adults. A survey of Northern Ireland and Scotland revealed that *T. oleracea* was rare in grassland, possibly due to different moisture and vegetation preferences to *T. paludosa*, and that it might only become damaging when adult dispersal is restricted (eggs are usually more widely dispersed due to better female flight ability). This technique had not been used since this survey, for any published study at least, and has now been replaced by the use of sequence data. Rao et al. (2006) used mitochondrial cytochrome B (*cytB*) to distinguish these same two leatherjacket species in Oregon, the United States. Although there are other *Tipula* species present in this state, these particular species are invasive, with *T. paludosa* becoming a pest of pastures, lawns and golf courses but with less information on the host plants of *T. oleracea*. In contrast, the native species, which look very similar, are thought to feed on organic matter rather than the grass itself. Indeed, of all the samples identified using the technique, only the invasive species were found in urban landscapes (excluding one *T. tristis*), while only native species were found in cultivated grass. The invasive species were also found in cultivated peppermint with very few native species. The authors suggest further work would be needed to ascertain whether the native species are actively feeding on the crop, but the tool could help to identify the invasive species that are likely to cause damage, preventing indiscriminate pesticide application on finding leatherjackets. This method (Rao et al. 2006) is relatively laborious and costly when compared with other PCR-based techniques and *Tipula* research would benefit from the development of a different method.

The use of molecular techniques for the study of wireworm identity, distribution and phylogeography has increased rapidly in recent years. Ellis et al. (2009) developed a terminal restriction fragment length polymorphism (T-RFLP) approach to identify three UK pest species (*A. obscurus*, *A. sputator* and *A. lineatus*). As with the other larval pests mentioned so far, these species are almost impossible to identify without a great deal of time and expertise. Firstly, primers were developed for the mitochondrial *16S rRNA* region and adults of each species from locations in the United Kingdom, Europe and Canada sequenced. Based on this sequence data, restriction enzymes were chosen for their ability to produce fragments of different sizes dependent on species identity. A PCR in which the reverse primer is fluorescently labelled was carried out, then digested with the chosen enzymes and subjected to fragment analysis on an automated sequencer, differentiating it from the similar PCR–RFLP method, which uses gel electrophoresis to

discriminate DNA fragments. This method was subsequently used by Benefer et al. (2010, 2012) to look further into the distribution of these species (see Section 4.3) and the *16S rRNA* sequencing protocol used to identify Canadian wireworm species (Benefer et al., 2013; phylogenetic analyses described in Section 4.4). While there are thought to be approximately 30 economically important species in Canada, lack of a robust identification technique has limited studies on the distribution and occurrence of pest species or aspects of their biology and ecology, which could provide useful information for control. Importantly, this study showed that identification to morphospecies was not always reliable due to identification errors (morphological characters were difficult to differentiate) and cryptic species. In a similar scenario to that of chafer communities (Ahrens et al., 2007; Section 4.2), although adults are fairly easy to distinguish, the larvae for some species have not been described, and due to their long life cycle (3–5 years depending on climate), it is not practical to rear these to adults for the majority of biological and ecological projects. The use of *16S rRNA* sequences may therefore be useful as a first step in linking adult and larval life stages (including eggs) and initiating morphological description of larvae.

Using a PCR-based approach, Staudacher et al. (2011a) identified further pest species within the genus *Agriotes* that are of agricultural importance. This technique focused on the *COI* region, first amplified using universal primers or newly designed primers in some cases (for degraded DNA) using adult samples from across Europe. Once general sequences were obtained for 20 *Agriotes* species, a set of primers were developed targeting nine of the most important species for use in a multiplex PCR assay and tested using the original 20 species plus other soil invertebrates found together with *Agriotes* populations. It was then applied to over 900 *Agriotes* larvae, 83% of which produced diagnostic bands when using gel electrophoresis. Of those that did not produce bands using the multiplex PCR, several were found to be related *Adrastus* spp., which are morphologically very similar. The study also found that there was a high genetic similarity between *A. proximus* and *A. lineatus*, which also have only minor morphological differences as adults and are attracted to the same sex pheromone in the field. Further analysis of both the pheromone composition and *COI* sequences of these two species has been carried out using a different section of the *COI* region (Vuts et al., 2012), finding again very high sequence similarity between the two species (>99%) and also a highly similar pheromone profile suggesting a very close relationship between the species. Since sex pheromones are used for monitoring adult presence and activity in the field as a surrogate for wireworm

presence in the soil, it is important to understand the relationship of these species, and further work is currently underway on overlapping populations.

The *COI* region has also been used to identify wireworm species in the Midwestern United States, where a complex of species causes damage to maize (Lindroth and Clark, 2009). Larvae were collected from the Midwestern and Eastern United States and identified using dichotomous keys and comparison to museum specimens. Sequences comprising the majority of the *COI* gene were obtained for eleven of the 15 known economically important species. For the *Melanotus* species, museum specimen sequence data were compared to unknown larvae from four species that could not be distinguished and also compared with the full wireworm dataset, allowing identification based on their grouping with known species in maximum likelihood analysis. The ability to identify these species provides insight into the environments in which they are found (e.g. soil temperature and moisture preferences), allowing targeting of these areas for pest control.

4.2. Association of life stages

Aphids with morphologically different life stages that exploit taxonomically different host plants are typically difficult to assign to the correct species. Zhang et al. (2008) used *COI* sequences from Eriosomatinae aphids found on *Gramineae* (grass) roots, as the secondary host, and those found on *Ulmus* (elm) species, as the primary host, to identify species and associate the different life stages, thereby using them as a diagnostic tool. Using sequence data already available from adult specimens, a phylogeny was produced and the unknown morph sequences clustered with *Tetraneura chinensis*, confirming the species identity. The authors then described in detail the morphology of the secondary morph, allowing inclusion in future identification keys.

Ahrens et al. (2007) sequenced both mitochondrial (cytochrome oxidase subunit 1; *cox1* and 16S ribosomal RNA; *rrnL*) and nuclear (*28S rRNA*) DNA from chafer (see Sections 2.1 and 3.5) communities in order to associate adult and larval life stages from tropical lowlands of Nepal. Although adult taxonomy is well developed and identification is possible (albeit by taxonomic experts), new species are being discovered and the relation of the root-feeding larvae, which have limited morphologically characterising features, to the adults aboveground is unknown. These authors used more in-depth phylogenetic analyses to align sequences to species since intraspecific variation can make this difficult when using absolute methods of

sequence divergence and especially for unknown, large assemblages of species. Combined mitochondrial and nuclear gene trees (using maximum likelihood) and statistical parsimony, whereby populations are subdivided into subgroups, and population aggregation analysis, which combines populations that are uniform for a particular character state, percentage differences (*p*-distance) and AMOVA and species delimitation methods for estimating species boundaries from the tree were used to assign species membership. This resulted in 24 species of which 19 could be associated with adults and identified using Linnaean names (nearly 93% of unknown larvae were identified to species). The *rrnL* and *cox1* networks produced using the statistical parsimony analyses were congruent, and there was much lower divergence for the *28S* sequences, which has implications for gene marker choice in such studies. As for the aphid study, the use of these techniques for determining species and then searching for diagnostic morphological characters of both larvae and adults is advocated. In terms of relating this to species biology, the species assemblages found above- and belowground can depend upon larval mortality, which varies both spatially and temporally; in particular, larvae and adults were often not found in the same season, which can confound attempts to associate larvae with adults. With a sampling scheme that takes these factors into account, it may be possible to use such DNA-based taxonomy to further investigate spatiotemporal patterns and geographic variation.

4.3. Phylogenetic relationships

As well as simply for species identification, a number of studies have used this DNA sequence data to produce molecular phylogenies for some taxa for further information on their evolutionary relationships. This can provide information on the rates and pattern of evolution and species diversification, providing insight into species ecology. Aboveground, this has been much used to investigate plant–herbivore interactions (e.g. Jurado-Rivera et al., 2009; Pinzon-Navarro et al., 2010) and the taxonomy of specific genera (e.g. Bell et al., 2004; Ortiz-Rivas et al., 2009). However, fewer studies have focused on community genetics, coevolution and plant–herbivore interactions in the belowground context (though see Hiltpold et al., 2013; Rasmann and Agrawal, 2011).

Diabrotica (corn rootworm) species have already been discussed in terms of predator–prey interactions (Section 2.1) and genetic diversity (Section 3.1), but studies have also considered their phylogenetic

relationships. Little was known of phylogenetic relationships within this genus due to their morphological similarity. Allozymes have been used to determine that these species are represented in two distinct groups (*virgifera* and *fucata*) (Krysan et al., 1989) and molecular markers (mtDNA *NADH 4* with PCR–RFLP) were developed for differentiation of southern corn rootworm, WCR and NCR (Szalanski and Powers, 1996). Nuclear (*ITS1*) and mtDNA (*COI* and *COII*) genes have since been used to construct the phylogeny of *Diabrotica* species (Clark et al., 2001; Szalanski et al., 2000), which were in support of allozyme and morphological data with the same *virgifera* and *fucata* groupings observed. As adults are found in ecosystems with permanent perennial grasses, the *virgifera* group has been inferred to have an ancestor that might have evolved as a grass roots specialist (Branson and Krysan, 1981; Krysan and Smith, 1987), and the authors point out an interesting route to go down would be to assess phylogenetic relationships of larval associations of the *virgifera* group with their preferred hosts and female host–plant oviposition preferences, taking the focus to plant–herbivore interactions and evolutionary relationships.

Using a combination of nuDNA (translation elongation factor 1a; *EF-1* α) and mtDNA (*COI*), morphological and biological characters, Zhang and Qiao (2007) examined the evolution of gall morphology in Fordini aphids from China and Israel. Using samples collected on different primary hosts (*Pistacia* or *Rhus* species; the secondary hosts are grass roots or mosses), phylogenetic relationships of species were assessed, and it was found that host–plant affinity was an important character in subtribe division, with separate clades in the phylogenetic tree for *Pistacia*- and *Rhus*-feeding species. Gall traits also differed between species and over time seem to have become more adapted to manipulating the host plant, also reflected in the phylogenetic tree. This study focused on only the aboveground primary host. In the past, failure to take into account the secondary host morphs has led to taxonomic difficulties, with researchers creating synonymies. Further investigation (Ortiz-Rivas et al., 2009) using transfer experiments (of larvae from winged primary host morphs to wheat shoots), morphometric data of the wingless aphids reproducing on the roots and sequences from nuclear long-wavelength opsin, *EF-1a* and mtDNA, found no correlation of the phylogenetic relationships with primary host specialisation, but some aspects of gall morphology (gall capacity) were related to species position in the phylogenetic tree, as found previously. As in other studies of gall-forming aphids (see Section 3.2), it was difficult to distinguish secondary host morph species

using morphology, but this was possible using nuDNA and mtDNA sequences. The data brought into question the characterisation of some species and suggested further ecological and biological data are needed to inform gall-forming aphid taxonomy.

4.4. Distribution

Studies on the spatial distribution of root-feeding organisms have been somewhat limited. While it is often found that organisms have patchy distributions within the soil, related to a range of biotic, chemical and physical factors, interactions between taxa and the scale of sampling are largely unknown. Benefer et al. (2010) investigated the spatial distribution of four root-feeding insect larvae pests (wireworms, leatherjackets, Sciarid (Diptera: Sciaridae) and Bibionid (Diptera: Bibionidae) flies) and their possible interactions over different sampling scales. Wireworms were identified to three species using T-RFLP (Ellis et al., 2009; see Section 4.1), while the other insects were defined using morphological characteristics, where possible. By separating the wireworms to species, differences in their abundance, composition and spatial distribution became apparent; for example, more variance in *A. obscurus* distribution was accounted for at the field scale than the site scale (locations sampled within fields) and their associations with other wireworm species changed from the field (largest) to the core scale (smallest). This has implications for grouping the three *Agriotes* wireworm UK pest species together in ecological studies as has traditionally been the case, assuming they are similar in biology and ecology (mainly because of a lack of reliable identification method; see Section 4.1). The same pattern was true of Bibionid larvae, separated to two species morphologically, but Sciarid larvae and leatherjackets could not be identified morphologically and so a similar result may be apparent when species are considered individually.

Other wireworm studies have considered their occurrence and above–belowground distribution in agricultural land, using molecular identification techniques. Staudacher et al. (2013a) assessed *Agriotes* distribution in relation to climatic and soil parameters in Austria using multiplex PCR (Staudacher et al., 2011a; see Section 4.1). This also included newly developed primers for *Adrastus* spp., which are morphologically very similar to *Agriotes*. Six out of 14 known species were identified from 85 sites, with more than 50% of sites containing more than one species with species-specific differences in their distribution and abundance apparent across the country. Altitude,

annual precipitation, pH and water permeability was correlated with occurrence, with differences being found between species suggesting different environmental preferences, accounting for their distribution on a national scale. Ninety-two of the 1242 individuals were found to be *Adrastus* species. That not all known *Agriotes* species were found and some morphologically similar samples were from different genera suggests it is important to have knowledge of the species present in the soil, since the presence of wireworms with this general morphology does not necessarily mean damage will occur. The relation to climatic and soil data allowed distinction of two ecological groups, confirming some previous observations and providing information that could be used together with other species-specific factors to predict their presence and in different agricultural regions.

Few studies have considered how populations of above- and belowground stages of root herbivores are distributed, despite there being many ecologically and economically important species that spend their larval stage in the soil. As already discussed, UK *Agriotes* wireworms were historically considered together as a pest complex, without differentiation of individual species. Benefer et al. (2012) again used T-RFLP (Ellis et al., 2009) to identify three species of wireworm in the soil to their conspecific adult male click beetles trapped aboveground using sex pheromone traps. Sex pheromone traps are used to monitor adult male presence and activity and assume that this reflects wireworm distribution in the soil. However, no *A. lineatus* wireworms were identified from soil samples despite adults being the most numerous species captured and present in all fields. Twelve of 72 wireworm samples subjected to T-RFLP failed to produce the expected fragment sizes, and subsequent sequencing at the *16S rRNA* region revealed them to be 'non-*Agriotes*'—there was no conclusive match to the three UK *Agriotes* species or any other wireworm species sequenced at *16S rRNA*. Phylogenetic analyses (Benefer, 2011) suggested that one of these unknown species may be related to *Athous haemorrhoidalis*, an occasional pest, and *Denticollis linearis*. This result shows that the relationship between aboveground adults and belowground larvae is not necessarily straightforward and that sex pheromone traps may therefore be misleading in terms of the proportion and distribution of species encountered, confirming the deductions of Blackshaw et al. (2009) and Blackshaw and Hicks (2013). In addition, other species may be involved in damage if found in large enough populations. Using multivariate analysis, different associations were found between wireworms and environmental variables when considered as a group or separated

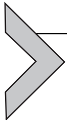
to species, emphasising, as per Staudacher et al. (2011a), that although these species are closely related, they may have different ecological and biological preferences that shape their distributions and should be considered separately.

4.5. Quantification

Soil sampling and extraction techniques (e.g. soil coring followed by heat extraction) are widely used to obtain samples for further analysis using a range of sampling designs. Although there are inherent biases and practical drawbacks to their use (as with any sampling method, whether above- or belowground), this remains the most practical way to estimate root herbivore abundance in the field, though acoustic monitoring techniques have been used to map soil insect populations (e.g. Brandhorst-Hubbard et al., 2001; Zhang et al., 2003). One molecular ecological technique that has been used is monoclonal antibodies and enzyme-linked immunosorbent assays (ELISA), for quantifying slug density in the soil (McKemey et al., 2006). Slugs are particularly labour-intensive to sample, the traditional method involving collecting blocks of soil from the field and slowly flooding to drive them to the surface. Monoclonal antibodies were tested and selected based on their ability to detect slug proteins, but not those of other invertebrates, from soil samples, and then experiments were carried out using ELISA to calibrate slug biomass, comparing slug protein equivalents against known biomass, and field experiments comparing flooding and the ELISA-based technique. The two techniques produced similar estimates of slug density in the field, proving its utility in studies aiming to determine slug densities in general, though not species-specific estimates. One complication was that its sensitivity varied by soil type; though it worked well in natural soils, it failed in commercially available humus-rich soil, possibly due to high levels of organic matter. Despite this, the technique has the potential to overcome some of the difficulties associated with sampling these pests and could be applied to other root herbivores.

Another molecular approach to quantification involves the use of the number of DNA sequence reads per individual derived from NGS data. However, there have been mixed results from studies so far, with some authors finding little correlation between the number of sequence reads and the number of individuals of a species (Binladen et al., 2007; Deagle et al., 2013; Porazinska et al., 2009), while others have shown that read abundance generally reflected the number of individuals per species (Porazinska et al., 2010). This could be associated with taxon-specific

variation in copy number per cell, tissue cell density or environmental persistence or due to technical factors during amplification, for example, using tagged primers, stringent bioinformatics filtering methods or sequencing platform (Deagle et al., 2013), and would need to be properly evaluated using controlled experiments and data analysis for application in root herbivore studies. Since this technology and associated quantification issues are only recently being evaluated, our understanding of the factors involved should improve as further studies on a wider range of taxa are carried out.



5. SUMMARY AND FOCUS FOR FUTURE WORK

To date, a variety of molecular techniques, including DNA sequencing of mitochondrial and nuclear genes, markers such as microsatellites, AFLPs, RFLPs and RAPDs and qPCR or a combination of these, have been used to study root herbivore ecology. Particularly when combined with other observational and experimental methods, the data have proved useful in elucidating species identity and relationships, population dynamics and dispersal, distribution, feeding behaviour and interactions with other root herbivores, predators, symbionts and plants. The majority of research has been carried out on belowground larval stages of economically important species, particularly Coleoptera (Scarabidae, Elateridae and Chrysomelidae), with most studies focusing on the aboveground adult stage, which is involved in dispersal, ultimately responsible for the distribution of the root-feeding stage in the soil and more practical to obtain. In common with their aboveground counterparts, root-feeding aphids have been the subject of a number of molecular studies due to their interesting, from an evolutionary point of view, and complicated two-host life cycle. Other less extensively covered taxa include Lepidoptera (*Agrotis* cutworms), Curculionidae (*Diaprepes* root weevils), Tipulidae (*Tipula* crane fly larvae—leatherjackets), Bibinoid and Sciarid fly larvae and a mollusc (*Deroceras reticulatum* slugs, not an insect but included as an important non-root herbivore and as explanation of a potentially useful method).

In general, the literature reviewed here shows that root herbivory research lags behind that for aboveground herbivores in the application of molecular approaches. Although the full range of available techniques has been used and in similar ways to that of aboveground herbivores, it is clear that the taxa coverage is much smaller (only studies on 10 taxa, though comprising several species, were identified during the literature search; Table 5.1) and that in some cases this is still in a method development or

optimisation phase. For example, protein-based gut content analysis techniques have been used to study predator–prey interactions in aboveground herbivores since the late 1980s and DNA approaches since the 1990s, while they have only been applied to that of root-feeding taxa relatively recently (Juen and Traugott, 2005). In addition, we found few published studies that had applied the developed methods to test specific hypotheses. Despite the relatively recent adoption of molecular techniques for some taxa (e.g. Elaterid wireworms), there has been a surge in studies using these types of methods to answer questions on species' ecology, which was not possible using traditional techniques, for example, species-specific relationships between adult and larval distributions in *Agriotes* wireworms, the interaction between *Wolbachia* parasites and corn rootworm population dynamics and differences in distribution of native and invasive Tipulid pest species. While this has opened up new areas of research in many cases, extension of these studies and application of new and emerging technologies are likely to further increase the practicality and value of this research.

The dominance of (relatively few) pest studies has led to a level of pragmatism in the questions that have been addressed; the focus has been on pest management-related issues rather than research to understand the functioning of root herbivores within the soil ecosystem (but see Hiltpold et al., 2013), linking back to the research lag mentioned earlier. This means that some clearly important questions have not yet been addressed. For example, most root herbivores have patchy distributions, but the mechanisms underlying this are poorly understood. The development of molecular approaches to identify siblings is an essential precursor to understanding the role of adult females in oviposition site selection as a biotic factor, hence allowing the effect of abiotic influences to be better addressed. It is also desirable to be able to distinguish the sex of root herbivores in spatiotemporal studies, and here, there is a challenge to develop a molecular approach to substitute for laborious dissections. Dispersal, scaling and metapopulation dynamics are also topics that have received scant attention in root herbivore research. Direct observation is rarely possible in the soil and our current knowledge is largely based on inferences derived from statistical analyses but, as the work of Benefer et al. (2012) has shown, such conclusions can be influenced by the sampling method. Genetic studies are likely to be less susceptible to this effect and, we suggest, will drive these topics forward in the future.

A further current limitation to root herbivore research is that sampling and extraction methods are shaped by old technologies (e.g. Tullgren funnels) and have little relevance to the spatial scale at which the herbivores

interact with the soil system. It is also desirable to be able to sample at the spatial scales relevant to specific taxa. Here, lessons can be learned from soil microbiologists and the recovery of whole-soil DNA for NGS. For this to be effective for the study of root herbivores necessitates robust sequence data being available for target species and the ability to scale extraction methods. For some herbivores, such as nematodes, samples are inevitably bigger than the space occupied by the animal and extension of the semiquantitative methods used (Porazinska et al., 2010) to larger herbivores and much bigger soil samples would be desirable.

At a broader level, there are potential applications in community ecology and above–belowground interactions that extend current studies and put root herbivore biology and ecology in the context of ecosystem processes and systems ecology. NGS techniques now make it possible to investigate the genetics of whole communities of organisms (metagenetics). Gut content analyses have a much bigger contribution to make to understanding the role(s) of root herbivores in food web and system functioning, and such methods could be applied to the analysis of root–herbivore interactions in soil food webs (extension of feeding ecology studies, Pompanon et al., 2012), species-specific surveys of endosymbiont diversity and links with root–herbivore functional diversity, large-scale phylogenetic analysis using several genetic markers across many related species for use in root–herbivore host–plant coevolutionary studies and fast and cost-effective isolation of genetic markers for use in phylogeographic and population genetic studies (e.g. Bai et al., 2010; Perry and Rowe, 2011). Other applications such as transcriptome characterisation, allowing genes expressed and their functions in different life stages or species to be assessed (Ekblom and Galindo, 2011), could be useful in understanding the genetics behind variation in traits between individuals and species. Similarly, gene expression profiling, often used to assess responses of plants to herbivory, could provide the other side of the picture and enable understanding of responses of root herbivores to environmental cues including parasites and temperature. The use of such newly emerging methods for nonmodel organisms would allow a view of the functional aspect of root herbivore ecology.

The application of molecular methods has spread across the biological sciences and enabled researchers to address ever more challenging questions. These are early days in their adoption for the study of root herbivores, but we can anticipate their increasing use as awareness of their potential and the development of molecular skills expands. It is, however, likely that this expanded use will continue to be dominated by the need to address pragmatic research questions about specific taxa, notably pests.

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