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THE MOLECULAR AND BEHAVIOURAL ECOLOGY OF CLICK BEETLES (COLEOPTERA: ELATERIDAE) IN AGRICULTURAL LAND

By

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THE MOLECULAR AND BEHAVIOURAL ECOLOGY OF CLICK BEETLES (COLEOPTERA: ELATERIDAE) IN AGRICULTURAL LAND

CARLY MARIE BENEFER

The larvae (wireworms) of some click beetle genera inhabit the soil in agricultural land and are crop pests. In the UK, a pest complex of *Agriotes* species, *A. obscurus*, *A. sputator* and *A. lineatus*, has been identified as the cause of the majority of damage. However, studies on their ecology are lacking, despite knowledge of this being important for the development of sustainable risk assessment and pest management strategies, in part due to the morphologically cryptic nature of wireworms. The ecology of economically important click beetle species was investigated, focusing on UK *Agriotes* species.

The relationship between sex pheromone trapped male *Agriotes* adults and wireworms, identified using a molecular tool (T-RFLP), was influenced by sampling method, and some environmental variables significantly correlated with species distributions. Scale of sampling influenced the observed distribution of wireworms and other soil insect larvae. Other wireworm species were trapped together with *Agriotes* species, but mitochondrial 16S rRNA sequences could not be matched to those of other UK species. Sequences from Canadian wireworm samples revealed possible cryptic species. Differences in adult movement rates were found in laboratory tests (*A. lineatus* > *A. obscurus* > *A. sputator*). Molecular markers (AFLPs) were developed to assess dispersal in adult male *Agriotes* but further protocol optimisation is required.

The results show the importance of identifying wireworms to species for assessing adult and wireworm distributions, since the *Agriotes* pest complex may not be present

or as widespread as previously assumed. Sex pheromone trapping of adults may not be appropriate for risk assessment as the relationship between aboveground adult and belowground wireworm species distribution is not straightforward. The differences observed in *Agriotes* species' ecology have implications for the implementation of pest management strategies. The techniques used here can be applied in future studies to provide information on other economically important click beetle species worldwide.

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Chapter 1   Introduction

1.1 Click beetles as agricultural pests

Species in the large family known as the click beetles (Coleoptera: Elateridae) are found worldwide in a variety of habitats including woodland, woodland margins, pasture and arable land. Most species are not considered problematic, but the polyphagous larvae (wireworms) of certain genera are major pests of a variety of arable and horticultural crops, causing damage to crop quality and/or yield (Anon, 1983). Traditionally associated with areas of permanent grassland, some species are becoming an increasing problem in all arable rotations with no history of long-term grassland (Parker & Howard, 2001). Estimates of crop damage from the UK are lacking, but figures from Canada and Australia suggest crop loss or insecticide application for wireworm control costs farmers £300,000 – £500,000 per year (Samson & Calder, 2003; Vernon, 1998). With increasing consumer demand for high quality produce and the public's growing environmental awareness, sustainable and reliable methods of control are needed. In order for these to be developed and applied appropriately, monitoring systems need to be in place to assess the relative distribution and abundance of damage-causing species, and the behavioural factors that affect this distribution. Therefore, better knowledge of both adult click beetle and wireworm ecology is needed.

Previous studies identified a complex of three wireworm species – *Agriotes obscurus* (Linnaeus), *A. lineatus* (Linnaeus) and *A. sputator* (Linnaeus) - as most commonly occurring in the UK, and hence these species have since been considered responsible for the majority of damage to crops. Although research on these pests has been carried out since the early 1900s, when large areas of permanent pasture were

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ploughed up for arable farming during the first and second world wars, the ecology of these species is still poorly understood. Little is known of the distribution and abundance of adult click beetles in relation to wireworms of the same species, mostly due to *Agriotes* wireworms being morphologically cryptic, but also because of the difficulty of relating the aboveground adults to the belowground larvae, which are of a different generation. These problems have hindered studies on *Agriotes* ecology, and thus the development of control strategies. Therefore, this thesis is concerned with improving the ecological knowledge of these UK *Agriotes* species in particular, but also other economically important Elateridae pest species from the UK, Europe and Canada.

1.2 Wireworm damage to crops

Many crops are attacked by wireworms, but the seriousness of the damage depends on a number of factors including the plant species, growth stage, vigour when attacked, and plant density (Anon, 1983). Root crops such as swede, sugar beet and potato are attacked as seedlings, causing serious loss of plants if attack occurs in the later stages of the crop after thinning (Anon, 1983; Miles, 1942b). Wireworms cause severe injury to potato tubers, leaving tunnels and small round holes on the surface which reduces quality rather than yield and makes the crop unmarketable to consumers (Anon, 1948). Other pests such as slugs, millipedes or bacterial rot may then use these tunnels, further increasing the damage (Parker, 2005). Attack on cereal crops is characteristic and easily recognisable, causing wilting and patchy germination (Miles, 1942b). Autumn and spring sown oats and wheat are more susceptible than barley and rye, and damage to maize can be particularly serious due to its low plant density (Anon, 1983). Other crops such as Brassicas, leeks, French beans, tomatoes

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and carrots are also attacked at both the seedling stage and in later development,

although once established serious damage is rare (Anon, 1983; Cockbill et al., 1945;

Griffiths, 1974; Miles, 1942a).

1.3 Biology of click beetles as agricultural pests

1.3.1 Species distribution

Some 60 species of click beetle are known to occur in Britain, but agricultural pest

species complexes differ with location (Anon, 1983). *Agriotes* species are found in

Europe, Asia and North America (Hill, 1987). Previous surveys suggest that *A. lineatus*,

A. obscurus and A. sputator are the main species occurring in agricultural land in the

UK (Anon, 1948), with *A. lineatus* more dominant in the south and *A. obscurus* more

abundant in parts of Northern England and Scotland (Gratwick, 1989). Similar genera

e.g. *Ctenicera* (Latreille), *Hypolithus* Eschscholtz, *Ectinus* Eschscholtz and *Melanotus*

Eschscholtz are pests in Canada, the USA and Asia (Chalfant et al., 1992; Doane, 1977;

Doane et al., 1975; Elberson et al., 1996; Hill, 1987; Vernon et al., 2010). In both the UK

and the USA it seems that no one species can be regarded as the main pest (Radcliffe

et al., 1991).

1.3.2 Morphology

Agriotes adults are brown or black, elongate beetles around 10-20mm in length (Hill,

1987). *Agriotes lineatus* is the largest of the three UK species, followed by *A. obscurus*

and *A. sputator*. Adults are distinguishable due to differences in their size and the

shape and colour of the thorax and abdomen (Figure 1.1).
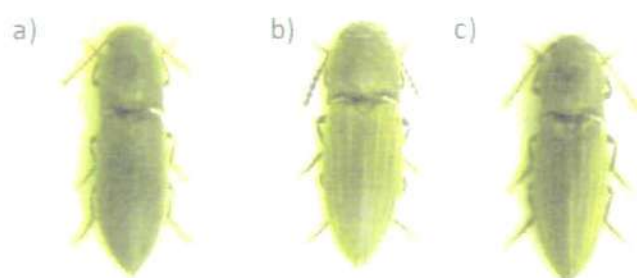
Figure 1.1 Adult a) *A. sputator*, b) *A. obscurus* and c) *A. lineatus* (Dusanek & Mertlik, 2007b; Dusanek & Mertlik, 2007c; Dusanek & Mertlik, 2007a).

Wireworms are translucent white and approximately 1.5mm long when they hatch, but grow to around 25mm in length and turn a shiny golden brown colour (Anon, 1983). There are three pairs of short legs behind the head, which is dark brown with biting mouthparts, and the body is smooth, segmented and cylindrical (Anon, 1983) (Figure 1.2 and 1.3). There are two principal distinguishing characters of *Agriotes* larvae; the presence of a tooth on the inner edge of the mandible and two eye-like pits near the base of the ninth abdominal segment, thought to be sensory organs (Miles, 1942b) (Figure 1.2). Although it is possible to identify *Agriotes* larvae to species by microscopy, this method is problematic. The identification of first instar wireworms is 'difficult or even impossible' using the keys available (van Emden, 1945) as little is known about this stage of the wireworm life cycle. The separation of species based on larval characters is complicated and for many species, including *Agriotes*, identification is based on the mandibular structures which can become worn down during feeding. Identification is time consuming, particularly with large numbers of samples, difficult, and a considerable amount of expertise is required to make a reliable identification.
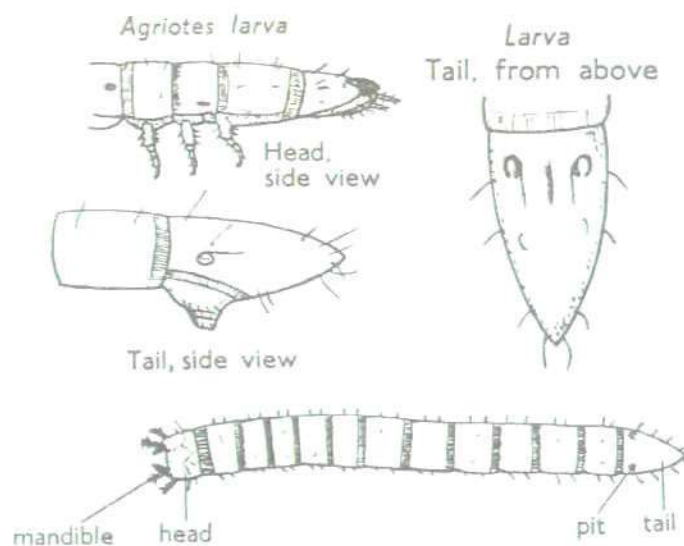
Figure 1.2 *Agriotes* wireworm anatomy (Jones & Jones, 1984)
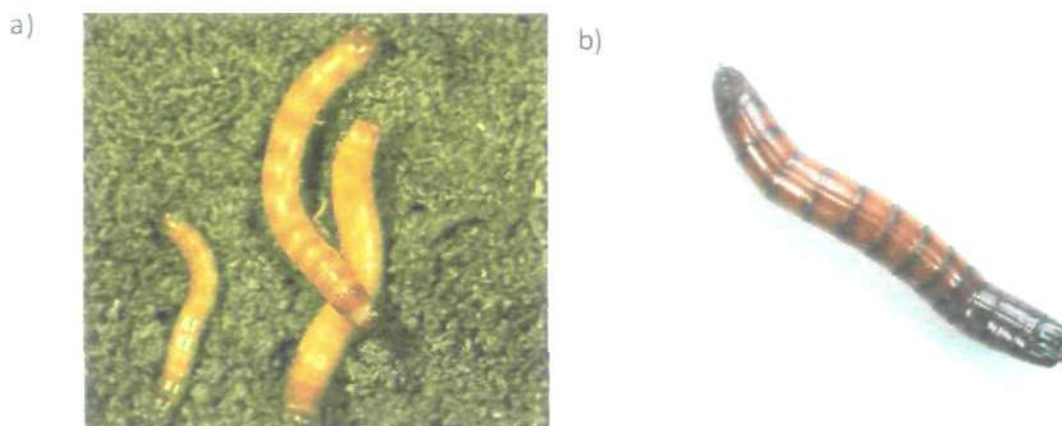
Figure 1.3 a) *Agriotes* wireworms and b) a *Conoderus* wireworm (Anon, 2005; Dorling Kindersley, No date supplied)

Molecular techniques offer a reliable, fast and relatively easy method of identification and have been used to identify cryptic insects of ecological importance e.g. *Anopheles* Meigen mosquitoes (Wilkerson et al., 1993) and bumblebees (Ellis et al., 2006; Murray et al., 2008). A PCR-based terminal-restriction fragment length polymorphism (T-RFLP) method has been developed for *A. obscurus*, *A. lineatus* and *A. sputator* which can be carried out using a genetic analyser (Ellis et al., 2009). Other recent methods include a

multiplex PCR approach using the mitochondrial cytochrome oxidase I gene (COI) (Staudacher et al., 2011), and direct sequencing analysis of the COI region (Lindroth & Clark, 2009).

1.3.3 Life History

Emergence of *Agriotes* adults from over-wintering cells in the UK corresponds with the rise in temperature in spring (Brian, 1947; Roberts, 1921) (Figure 1.4) and oviposition takes place from May to July (Cohen, 1942; Evans & Gough, 1942). Soil moisture and humidity (Cohen, 1942; Furlan, 1996; Furlan, 2004) and the widespread cover of vegetation provided by the presence of grasses could be a factor in adult habitat preference and movement, though female food preference may also be involved (Cohen, 1942; Gough & Evans, 1942; Roberts, 1921). Wireworms hatch in June and July and move downwards through the soil (Roberts, 1919). Development into the adult varies between species and geographic location, but around 1-3 instars are completed each year (Hill, 1987). At lower latitudes or in warmer seasons development may only take 2-3 years (Furlan, 2004), but in the UK 3-5 years is usual (Parker & Howard, 2001). Wireworms reach maturity in July to September and bury around 10cm below the surface where they build pupal cells (Miles, 1942a; Figure 1.4), forming the adult in around 4 weeks (Roberts, 1919). Adults over-winter in the soil until the following spring unless disturbed, in which case they move up through the soil and find shelter elsewhere (Gratwick, 1989).
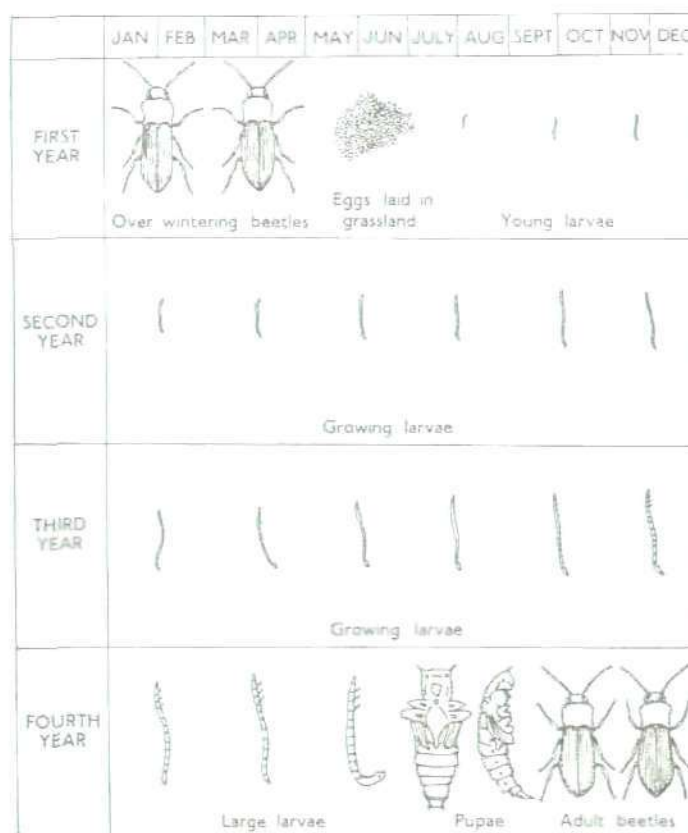
Figure 1.4 The life history of *Agriotes* species (Joy, 1932)

1.3.4 Seasonal occurrence and activity periods

Wireworms become part of the 'effective' wireworm population at the end of their

first year (Miles, 1942b). They have short, distinct periods of feeding two or three

times a year with regular periods of inactivity lasting several months (Evans, 1944).

Agriotes wireworms are predominantly herbivorous, although some *A. obscurus* larvae

and *Adrastus* Eschscholtz and *Athous* Eschscholtz species feed on animal prey

(Traugott et al., 2008). The most intense feeding occurs in March to May, coinciding

with the seedling stage of many crops, and September to October, when most crops

are mature in the UK (Anon, 1983; Jones & Jones, 1984). There are interspecific

differences in seasonal abundance in the USA (Cherry, 2007; Seal, McSorley &

Chalfant, 1992), which suggests there may be species differences in pupation times

that could affect the timing and extent of crop damage. In the UK the three main *Agriotes* species are thought to develop at the same time causing periods of increased crop damage. However, due to lack of species differentiation in past studies there may be differences that are not yet apparent. Adults are active on the soil surface in spring when they feed and mate. Although adults may feed on the leaves of crops they do not cause a large amount of damage (Cohen, 1942), and there is evidence that animal food may be preferred (Mesnil 1930, cited in Cohen 1942). In general, click beetles are active for around 5 months and do not live for longer than 1 year; males tend to die soon after mating (Cohen, 1942).

1.3.5 Factors affecting wireworm activity

Wireworm movement in the vertical soil profile tends to coincide with changes in soil moisture, humidity and temperature. Wireworm activity increases in dry substrate and decreases dramatically in very moist substrate, probably due to muscular inhibition (Campbell, 1937; Lees, 1943a). When food is available there is less movement, possibly because food itself is a source of moisture. Conversely, Evans (1944) found that no food was eaten below 15% soil moisture content, indicating other factors may be involved in wireworm feeding behaviour. Gravity was found to be unlikely to affect a downward movement response in larvae (Lees, 1943a). Wireworms are unable to survive long periods of desiccation and tend to move to areas of greater humidity in the soil (Lees, 1943b).

Preferred temperatures change as the season changes, indicating that wireworms are well adapted to temperature fluctuations (Campbell, 1937). *Agriotes* larvae can survive at temperatures between -10 and -15°C (Lagenbuch 1932; Gueniat 1937, cited in Falconer 1945), at least for periods of a few hours, but other studies have found that

larvae cannot survive more than 4 hours at -7°C, and not more than 6 days at -3°C
(Falconer, 1945). Controlled lowering of temperature, as would occur in the field,
increased wireworm resistance to low temperatures (Falconer, 1945). Although a
possible limiting factor in vertical wireworm distribution elsewhere in Europe where
these low temperatures may occur, this is unlikely to be an important factor in the UK
but has not been studied in the field. The highest temperature at which wireworms are
able to maintain activity is 35°C to 36°C; higher temperatures result in paralysis and
then death (Falconer, 1945).

1.3.6 Spatial distribution

Studies in the 1940's showed a marked difference in the estimated levels of infestation
between and within regions and between the north and south of the UK (Anon, 1948).
Since then there have been limited studies on the UK distribution of wireworms in
agricultural land, though maps of the distribution of each Elateridae species in Britain,
based on adult records, are available (Mendel & Clarke, 1996). Similarly few studies
have assessed wireworm and adult species distribution at the field and agricultural
landscape scale, partly due to difficulties in relating adult and larval distributions at the
species level.

1.3.6.1 Spatial distribution of wireworms

Salt and Hollick (1946) found that Agriotes wireworm distribution was non-random,
with some plots being heavily infested while others close by were much less so, and
environmental factors correlated with wireworm distribution were different in the two
fields studied. Highly infested areas were almost exclusively A. sputator, whereas areas
of lower infestation were a mixture of A. sputator and 'A. lineatus-obscurus' (which
could not be reliably distinguished from one another). More recently the distribution

of *Agriotes ustulatus* Schaller was found to be correlated with vegetational cover and was seemingly not affected by soil moisture (Toepfer et al., 2007). This suggests there may be interspecific differences in tolerance to environmental conditions that may lead to diverse levels of infestation at the field level. Another recent study found no evidence of wireworm spatial structure at one study site, but significant aggregations at another with evidence of patches and gaps (Blackshaw & Vernon, 2008).

There is evidence that fields in the south and west of England are more highly infested and contain patches of wireworms than fields in the north of England, in which wireworms are randomly distributed, indicative of a link between high infestation levels and congregations of wireworms in patches (Finney, 1941). The age structure of wireworms may also affect their spatial distribution, where aggregations are higher for small larvae. Larger larvae are better able to disperse from the site of oviposition leading to less aggregation with age (Doane, 1977). This could partly explain the 'patchiness' in damage often observed within fields. Results from other studies have been variable, with some finding an aggregated spatial arrangement (Williams, Schotzko & McCaffrey, 1992) and others little or no spatial pattern (Hicks, 2008).

1.3.6.2 Spatial distribution of adult click beetles

Few studies have focused on adult distribution at the field scale or attempted to link this with the distribution of wireworms of the same species. However, the relationship between pheromone trap catches of adult males and wireworms (as a complex) has been shown to vary between sites (Blackshaw & Vernon, 2008), but further study is needed in this area. There is some evidence that both adult male *A. lineatus* and *A. obscurus* distribution is highly aggregated at the landscape scale, and that there are significant associations between the two species (Blackshaw & Vernon, 2006). At the

field scale there is variation in the spatial pattern of adult *A. obscurus* and *A. lineatus*, with associations differing between sites (Blackshaw & Vernon, 2008).

1.3.7 Dispersal of adult click beetles

Much research has been carried out on insect migration by flying, for example using radar equipment (Chapman, Reynolds & Smith, 2003), aerial netting (Reynolds et al., 1999) and suction traps (Campbell & Muir, 2005) but dispersal has been overlooked for many root feeding pests, including click beetles (Murray et al., 2010). Walking is thought to be the main mode of adult click beetle dispersal (Roebuck, Broadbent & Redman, 1947), at least for short range movements. Field studies suggest that *Agriotes* males and females make mostly short journeys close to the place of emergence, but large scale directional movement does occur (Brian, 1947; Roebuck, Broadbent & Redman, 1947; Schallhart et al., 2009; Sufyan, Neuhoff & Furlan, 2007). There are interspecific differences in the estimated walking speed (per day) of male UK *Agriotes* species; *A. lineatus* is the fastest, followed by *A. obscurus* and then *A. sputator* (Hicks & Blackshaw, 2008). Most of the limited number of studies on click beetle dispersal have used direct observation in the field to obtain movement rates and 'maximum' dispersal distances (one study used pheromone trapping followed by carbon stable isotope analysis; Schallhart et al., 2009), which has led to contradictory results. Differences in movement rates may have implications for the infestation of new fields, and so further studies to determine whether there are underlying differences in movement behaviour and factors that affect this e.g. male response to sex pheromones may corroborate already existing evidence. Assessment of dispersal over larger scales, possibly via the use of molecular markers such as microsatellites, amplified fragment length polymorphisms (AFLPs), Single Nucleotide Polymorphisms (SNPs) or direct sequencing

of mitochondrial DNA (mtDNA) to infer dispersal via gene flow between populations, has been carried out for other insects e.g. *Aedes aegypti* Linnaeus mosquitoes (using AFLPs; Ravel et al., 2001), Queensland fruit fly, *Bactrocera tryoni* Froggatt (using microsatellites; Yu et al., 2007) and *Halobates* Eschscholtz spp. of oceanic bugs (using mtDNA sequence data; Anderson et al., 2000) and may be an alternative to field studies for click beetles, which can be adversely affected by a number of factors.

All three UK *Agriotes* species have been observed in flight, mainly in moist, humid conditions both in daylight and after sunset, but flight activity varies between individuals (Brian, 1947; Fryer, 1941). Studies in Canada suggest that flight does occur for many species but at a low frequency for most (Boiteau, Bousquet & Osborn, 2000). Beetles have been observed in flight following disturbance or capture (Crozier, Tanaka & Vernon, 2003), which suggests that the situations in which flight would be induced as compared to walking or feeding are different (Brian, 1947). It is also possible that males use flight to locate females, and that females take to flight to find an appropriate place for oviposition (Crozier, Tanaka & Vernon, 2003). However, no specific studies on flight in *Agriotes* have been made and data available so far are observational.

1.4 Infestation risk assessment

In order to effectively manage wireworms it is important to be able to assess the level of infestation so that the appropriate control method can be used. For a highly infested field it may be economical not to plant a crop, or to plant a less susceptible crop, rather than risking the loss of most plants to wireworm damage. For wireworm counts to be of use in assessing risk of infestation, populations need to be determined

accurately using methods that are manageable in terms of time and effort, resulting in

the ability to ascertain damage to crops precisely (Yates & Finney, 1942).

1.4.1 Site history and characteristics

In general wireworm infestations are higher in heavier soils than light soils, possibly

due to soil being moister in the summer months (Anon, 1948). Aggregations may

occur where favourable habitat, perhaps dependent on soil type, ends and there are

environmental boundaries to further colonization (Blackshaw & Vernon, 2008).

However, other studies report soil bulk density to be lower in infested fields (Parker &

Seeny, 1997).

One of the best ways to assess whether wireworms are present is to review the history

of the fields in terms of the length of time in grassland (Parker & Howard, 2001).

Grasslands provide a suitable habitat for wireworms due to the abundance of food,

stable moisture and temperature conditions, limited disturbance, and vegetation cover

for adult food and shelter (Miles, 1942a). Although the wireworm population is often

reduced following ploughing, even fields which have been converted from grassland

for a number of years can remain highly infested (Anon, 1948). Fields which have been

in grass for 10 years or more have a one in two chance of being infested, increasing to

a three in four chance for permanent pasture (Parker & Seeny, 1997).

1.4.2 Soil sampling

In this method approximately twenty 10cm by 15cm cores are taken from fields of

between 4 and 8ha. The cores are examined by hand sorting or a liquid separation

method, and the numbers of wireworms found recorded and converted into numbers

of wireworms per hectare (Anon, 1948). Although this gives an estimate of the

wireworm population of a field, the method is subject to sampling errors where a patchy distribution can lead to estimates of infestation that are too high or too low, which can have an effect on the management decisions taken in terms of which crops to grow (Yates & Finney, 1942). In addition, soil cores that sample to a depth 15cm may underestimate the number of wireworms in the vertical profile of the soil, depending on the season of sampling, as significantly more wireworms can be found at 15-30cm depth (Salt & Hollick, 1944).

1.4.3 Bait traps

Bait traps are useful in determining if wireworms are present before crop planting begins. Cereal-baited traps have been reported to be equally or more effective than soil cores at detecting wireworms in most cases (Parker, 1996) and corn/wheat bait, in comparison to other bait types, is reported as being most effective at estimating wireworm populations (Simmons, Pedigo & Rice, 1998). If just one wireworm per trap is found on average after one week there is a high probability of damage (Rice & Simmons, 1999). Leaving bait traps for longer periods of time results in more wireworms being trapped (Parker, 1996), but many factors may affect the effective range e.g. soil moisture and temperature, and so they are not reliable enough to predict wireworm damage to subsequent crops and basing management decisions on bait trap results should be carried out with caution (Parker, 1996).

1.4.4 Adult trapping using sex pheromones

Sex pheromone traps for a number of the main pest species within the genus *Agriotes* have been used in Italy (Furlan et al., 2001b; Toth et al., 2002), Hungary (Toth et al., 2002), Canada (Vernon, 2004), the UK (Hicks & Blackshaw, 2008), Croatia (Ivezic et al., 2007), the Netherlands (Ester, van Rozen & Griepink, 2001) and Portugal and Bulgaria

(Toth et al., 2008). The tufted apple bud moth (*Platynota idaeusalis* Walker)

pheromone has been used to detect *Melanotus* spp. in North America (Simmons,

Pedigo & Rice, 1998) and the sex pheromone for *Melanotus okinawensis* Ohira was

recently used for mass trapping of adult males as a control strategy in Japan (Arakaki

et al., 2008).

The method is based on the principle that as the Elateridae only seem to disperse

locally (although this has not yet been fully investigated) the numbers of adults

trapped can give an indication of the local population of wireworms in the soil (Parker

& Howard, 2001). The intention is that this information can then be used to forecast

future outbreaks and devise and target control measures, thus reducing the

application of pesticide (Toth et al., 2002).

Correlations have been found between adults caught using pheromone traps and

below ground wireworms (unidentified) (Furlan et al., 2001a; Ivezic et al., 2007), but

other studies on *Agriotes* species in Europe have found variability in the response of

some species to the sampling method used (Blackshaw & Vernon, 2008). Significant

differences in recapture rates of adult *A. obscurus*, *A. lineatus* and *A. sputator* were

reported in a recent mark-release study, showing differences in species responses to

sex pheromone traps and that trap spacing is important to avoid interference (Hicks &

Blackshaw, 2008). Differences have also been found in the species caught between

walking (YATLOR) and flying (VarB and YATLOR funnel) traps in Europe, suggesting

species variability in terms of dispersal and also that both trap types may need to be

operated in order to fully assess the damage causing species present (Ivezic et al.,

2007). Due to the effects of trap spacing, timing of operation and the difficulty of

differentiating wireworm species, as well as the variability in results obtained across

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different studies, using pheromone traps to monitor the wireworm infestation level needs further study before they can be used as a reliable risk assessment method (Blackshaw & Vernon, 2008).

1.5 Methods of control

The traditional pest management approach employed before the 1960's involved finding a suitable pesticide and using this intensively on the pest and its food source (Milne, 1965). This led to further complications and even aggravated the problem in some cases, causing pesticide resistance e.g. the Colorado potato beetle *Leptinotarsa decemlineata* Say and DDT (Webber, 2003), or the emergence of new pest species e.g. the substitution of *Anopheles* mosquito species following intensive DDT spraying (Moore, 1967). Pest control now relies upon a mixture of chemical, biological and cultural practices, and knowledge of the pest species' ecology is paramount to the development of sustainable pest management strategies. Wireworms are particularly difficult to control due to their habitation of the soil, long life-cycle and incomplete knowledge of certain aspects of their ecology.

1.5.1 Cultural

Cultural practices involve modifying the soil environment to render it unsuitable for wireworms. Although the best way of avoiding crop damage is to simply not plant crops in infested fields (Parker & Howard, 2001) this is not always an option and relies upon dependable information from risk assessment procedures.

Ploughing of grassland and cultivation can reduce wireworm numbers and change the composition of the population from that of old grassland (large numbers of small larvae and decreasing numbers of larger larvae) to uniform numbers of larvae from

each size-group (Salt & Hollick, 1944; Salt & Hollick, 1949). Some wireworms are directly killed by ploughing but cultivation may also reduce numbers by exposing wireworms to birds and altering the soil structure, making it unsuitable for both wireworms and adult oviposition (Salt & Hollick, 1949).

Cover crops are widely used for improvement of soil organic matter, nutrients and nitrogen, weed control, and in pest management (Sullivan, 2003). When planted in the preceding season or with a food crop, they provide an alternative source of food for the pest and can also attract beneficial predators for biological control. Cruciferous plants, such as mustard and cabbage, contain glucosinolates that when hydrolysed form biologically active compounds that can control soil pests (Parker & Howard, 2001). There is evidence that infestations are lower in mustard treatments (Frost, Clarke & McLean, 2002). Further research is needed to determine whether these plants contain enough glucosinolates to be toxic to wireworms, and to identify the cover crops that would be most suitable in deterring wireworm damage.

Crop rotation can prevent wireworm attack. Linseed is tolerant to wireworm damage (Cockbill et al., 1945), and carrots and field beans have been shown to work well as catch crops in organic rotations with potato, where infestation levels were significantly lower than in rotations in which an undersown crop was used (Schepl & Paffrath, 2003).

Flooding for wireworm control has been successful in the laboratory for *Melanotus communis* Gyllenhal and *Conoderus falli* Lane (Genung, 1970; Hall & Cherry, 1993) but ineffective in the field. *Agriotes lineatus* and *A. obscurus* wireworms were found to die more quickly in saline soil at high temperatures than at lower temperatures in other

soil types, indicating flooding during the summer months may provide more effective control (van Herk & Vernon, 2006).

1.5.2 Biological

The fungus *Metarhizium anisopliae* Metschnikoff has been implicated as an effective biological control agent for wireworms in Canada (Kabaluk et al., 2001), Switzerland (Keller & Schweizer, 2001), and has also been noted to attack adults in Italy and the UK (Parker & Howard, 2001). Large doses of the fungus over a long period of time are needed to produce high mortality (Kabaluk et al., 2001), but *M. anisopliae* seed treatment has been shown to increase plant yield, density and foliage area (Kabaluk & Ericsson, 2007a). There are several behavioural and environmental factors that may affect the infection of wireworms (Kabaluk & Ericsson, 2007b; Kabaluk et al., 2007), and for development of the fungus as a control agent the problems of targeting wireworms and production and formulation of the isolate, as well as the expense, need to be overcome (Kabaluk, 2007).

Entomopathogenic (insect parasitic) nematodes have been used as biological control agents for soil insect pests since the 1970s, but they have not been successful for wireworm control (Eidt & Thurston, 1995). The wireworm *Ctenicera destructor* Brown is not susceptible to *Steinernema feltiae* Filipjev and *Heterorhabditis bacteriophora* Poinar infection (Morris, 1985) and *Conoderus falli* is only slightly susceptible to *Steinernema carpocapsae* Weiser (Creighton, Cuthbert & Reid, 1968). Other wireworms show high resistance when exposed to these nematodes (Eidt & Thurston, 1995). Morphological features of the wireworm mouth and anus, such as dense, branched hairs in the spiracular orifice and preoral cavity, may be physical deterrents to nematode infection (Eidt & Thurston, 1995).

1.5.3 Chemical

Chemicals to control wireworms need to be easily incorporated into the soil and persistent, particularly when crops that are attacked later in life are grown, such as potato. There are now few insecticides that can be used to treat wireworm as many that were used previously, e.g. aldrin and gamma-HCH, contained organochlorines and are now banned due to environmental and health concerns (Grove, Woods & Haydock, 2000). Most of the insecticides registered for wireworm control in potato are carbamates and organophosphates. The organophosphates chlorpyrifos, ethoprop, fonofos, phorate and diazinon, when applied as a pre-plant broadcast or in-furrow at-planting treatment, give the best control of wireworms (Kuhar et al., 2003). The organophosphates thionazin and Bayer 5019 are as good at controlling wireworms as the organochlorine aldrin (Griffiths & Bardner, 1964), and there is evidence that they increase stands of barley. However, they are not as effective at protecting potatoes (Griffiths, Scott & Lofty, 1969). Chlorpyrifos and diazinon now have restricted use in the USA and all other organophosphates are under scrutiny in the USA and Europe due to their toxicity to humans and non-target organisms. Trials with newer insecticides have been carried out in the USA using pyrethroids, fipronil and neonicotinoids, but so far have only given moderate suppression of wireworms (Kuhar et al., 2003). Commercial application rates of the nematicide fosthiazate, although not approved for wireworm control, produced low wireworm mortality but activity was considerably reduced (Grove, Woods & Haydock, 2000).

1.6 Priorities for future research

Further knowledge of the ecology of both wireworms and click beetles is required before risk assessment and pest management methods can be developed and applied appropriately. As such, two main priority areas for future research identified from the literature are: a) investigation of the relationship between adult and larval species distributions, and factors affecting these and b) assessing interspecific movement rates and dispersal.

The risk assessment methods currently in use have limitations and are not fully reliable in identifying the extent of wireworm infestation. One of the inherent problems of using adult sex pheromone trapping as a risk assessment method has been the inability to relate adult and wireworm populations at the species level. Jansson and Seal (1994) argue that wireworm identification is needed in order to target control at the damage causing species in a given region. Knowledge of the wireworm species that are present would enable an assessment of the relationship between adult and wireworm distribution and the factors affecting this distribution, which up until now have not been accurately assessed. Adult dispersal is also an inadequately studied area in click beetle ecology, despite it being important to understand how new areas become infested. Species specific studies on adult movement, in particular the response to sex pheromones, and dispersal combined with modern molecular genetic studies, as suggested by Parker and Howard (2001), may aid the understanding of how wireworm populations are maintained at the field and farm-scale levels. Although studies are underway into new insecticides for the treatment of wireworms none have yet been found to be fully effective and reliable (Parker & Howard, 2001). However, with

research in these priority areas it may be possible in the future to target and adapt existing control strategies to the damage causing species present in different areas. This thesis will investigate these aspects of economically important click beetle species' ecology, focusing particularly on UK *Agriotes* species but including other pest genera within the Elateridae. Chapter Two aims to determine the relationship between adult and wireworm *Agriotes* species, and the effect of environmental variables and sampling method on the distribution observed. Chapter Three investigates the effect of other factors (soil insect larvae and scale) on *Agriotes* wireworm species distribution. Chapter Four examines other wireworm species found in agricultural land in the UK and Canada, in particular the identification of non-*Agriotes* species found together with *Agriotes* populations in the UK, and genetic diversity and phylogenetic relationships between click beetle/wireworm species. Chapter Five focuses on the movement behaviour of adult male *Agriotes* species and the response to sex pheromones, and Chapter Six describes the development of amplified fragment length polymorphism (AFLP) markers for the study of click beetle dispersal in agricultural land.

Chapter 2   The efficacy of click beetle and wireworm sampling methods for

assessing aboveground-belowground species distributions in relation to abiotic

variables

2.1 Introduction

Until relatively recently the aboveground and belowground components of the

terrestrial ecosystem have been considered separately, but there is growing evidence

that interactions between the biodiversity in these two spheres play an important role

in community and ecosystem processes (Schroter et al., 2004). Numerous studies have

investigated interactions between above- and belowground invertebrates in terms of

their influence on plant dynamics e.g. succession and species diversity (Binns, Nyrop &

van der Werf, 2000; De Deyn et al., 2003; van Ruijven et al., 2005), induced plant

defence strategies (Erb et al., 2008; Wackers & Bezemer, 2003), and their indirect

influence on each other in terms of growth, survival, oviposition and host plant

selection due to their effect on the quantity and quality of resources that plants

produce (Soler et al., 2009; Wardle et al., 2004). However, few studies have

considered how the spatial distributions of species with life stages in both spheres are

linked despite many species of ecological and economic importance having both

above- and belowground life cycle stages, in this case click beetles, but others include

crane flies (*Tipula paludosa* Meigan and *T. oleracea* Linnaeus), some Carabidae (e.g.

Pterostichus melanarius Illiger), and clover root weevil (*Sitona lepidus* Gyllenhall;

Murray et al., 2010).

A recent review of aboveground – belowground ecology (van der Putten et al., 2009)

identified 'improving the sustainability of agricultural crop and grassland systems' as a

key area for future work. The significant economic implications associated with the activity of root herbivore pest species dictate that it is vitally important to understand the distributions, influencing factors and relationships between all life stages in order to assess risk of future damage, and to target control measures at the correct species, thus reducing pesticide application and increasing sustainability. In addition, in order to predict the effects of global changes on pest species ranges and distributions, such as those associated with climate and land use, accurate information on their ecology in their current habitats and ranges is required.

Assessing the risk of infestation, and potential crop damage, is an important part of effectively managing belowground wireworms, and there are currently three methods used to estimate their presence and/or abundance within fields; soil core sampling, bait traps and adult sex pheromone traps. Soil core sampling was widely used in surveys carried out in the 1940's in the UK, but is labour intensive (it is recommended that 20 soil cores are taken per 4-10ha field; Anon, 1948), can underestimate abundance where populations are low (Yates & Finney, 1942) and time-consuming processing has to be carried out in a laboratory. Bait traps have been developed as a possible alternative, since they can be processed relatively quickly in the field. A number of designs have been tested, using vegetable and cereal baits, and have been found to be as effective, if not better, than soil cores at detecting wireworms (Parker, 1996). However, it can be difficult to separate wireworms from germinating bait, and the trap catches cannot be used for predicting subsequent crop damage since the effective range of the trap can be affected by a number of site-specific variables, such as soil conditions, availability of alternative food sources and temperature (Parker, 1996). Therefore, compared to soil core sampling there are quantitative limitations

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since the bait traps only provide a relative measure of activity abundance (Tingle, 2002).

Female sex-pheromones have now been identified for a number of economically important *Agriotes* species in Europe and Canada, including *A. obscurus*, *A. sputator* and *A. lineatus* (Toth et al., 2003), and traps developed to capture adult males (Furlan et al., 2001b; Vernon, 2004). Sex pheromone traps have been suggested for mass trapping of adults as a control measure (Arakaki et al., 2008; Milonas et al., 2010), but their main application has been for monitoring. Since adults are only thought to disperse locally, mainly by walking, adult trap catches have been used as an indicator of wireworm presence/absence as an alternative to direct wireworm sampling. However, there are limitations in relating adult trap catches to wireworm (as a complex) distribution assessed using soil core or bait trap sampling (Furlan *et al.*, 2001b; Blackshaw & Vernon, 2008). Only with the recent development of a genetic method to identify *Agriotes* wireworms (Ellis et al., 2009) has it been possible to study wireworm species distribution, and as such no published studies to date have been able to relate adult *A. obscurus*, *A. lineatus* and *A. sputator* to wireworms of these species.

Wireworms often have a patchy distribution, resulting in crop damage which is variable between infested fields (Blackshaw & Vernon, 2006; Salt & Hollick, 1946). A further limitation to soil and bait trap sampling is that these patchily distributed populations may be missed, and therefore insecticide may be applied anyway as an insurance treatment, or susceptible crops planted in these fields may be damaged (Parker & Seeny, 1997). Previous studies have considered the effect of abiotic variables in influencing wireworm distribution, and factors such as soil moisture

(Campbell, 1937; Evans, 1944), humidity (Lees, 1943b) and temperature (Campbell, 1937; Evans, 1944; Falconer, 1945) have been shown to affect wireworm behaviour. Knowledge of the variables that affect species distributions in the field are required to inform management practices. Parker and Seeny (1997) attempted to produce models to predict wireworm (as a complex) presence/absence based on a limited number of individual field characteristics with little success, and further site-specific factors to the small selection studied may be involved. There may also be differences in species distributions related to different abiotic variables which are not apparent when wireworms are grouped as a pest complex.

With these issues in mind, in this study two aspects of adult and wireworm distributions were investigated:

1. The suitability of soil core, bait trap and sex pheromone sampling methods for assessing the relationship between *A. obscurus*, *A. sputator* and *A. lineatus* adult and wireworm distributions

2. The influence of a range of environmental, chemical, physical and cultural abiotic variables on adult and wireworm species distributions.

In addition, to determine whether it is appropriate to group wireworms as a species complex for risk assessment and management purposes, separate analyses were conducted, firstly with adults as separate species and wireworms grouped as a species complex, and secondly with both wireworms and adults as separate species.

2.2 Materials and methods

2.2.1 Wireworm and click beetle sampling

Wireworm and click beetle sampling and abiotic data collection was carried out by Hicks (2008). Wireworms were collected from organic fields in the South Hams, Devon, UK, between February and April 2004 using both soil cores (10cm deep, 10cm wide) and bait traps (1:1 wheat-barley seed mixture) (Parker & Howard, 2001). Twenty soil cores were sampled from each of 99 fields in a 'W' formation (Parker & Howard, 2001), and 10 bait traps were then placed inside every other hole left by the soil cores and collected one week later. However, warm weather conditions meant that the seed bait germinated and grew rapidly making the traps difficult to extract and sort. Therefore only bait traps in fields sampled early in the season (the first 41) were recovered. The bait itself and the surrounding soil were hand sorted for specimens, though the majority of wireworms were found in the surrounding soil. The contents of the soil cores were processed using wet sieving within 48 hours of sampling, and recovered wireworms were frozen at -20°C prior to identification. From 7th May to 12th August 2004 adult males were trapped using sex pheromones for each species in Yatlor traps (Furlan et al., 2001b; Toth et al., 2003) from the centre of 92 of the 99 fields (seven fields were excluded for practical reasons). Traps were emptied weekly and replaced in the same position. Click beetles and wireworms were stored at -20°C within 12 hours of sampling.

2.2.2 Abiotic data

The abiotic data collected are listed in Appendix 1. Soil chemical properties were determined from soil cores taken randomly in each field (3cm diameter, 25cm deep,

20 per field). Samples were air dried, bulked and analysed using standard methods (Anon, 1986). Cultural attributes were obtained from interviews with the farmers, and environmental properties were taken from Ordnance Survey maps (Appendix 1; Hicks, 2008).

2.2.3 DNA extraction and species identification

DNA from wireworms and adult males was extracted using a standard salt/chloroform method (Rico, Kuhnlein & Fitzgerald, 1992). Wireworms of the species *A. obscurus, A. sputator* and *A. lineatus* were identified using terminal restriction fragment length polymorphism (T-RFLP) which targets the mitochondrial 16S rRNA gene, using adult males (identified as *A. obscurus, A. sputator* or *A. lineatus* using morphological characteristics) as positive controls, following the protocol of Ellis *et al.* (2009).

2.2.4 Direct sequencing

In cases where no restriction fragment was produced (12 samples), samples were sequenced directly. Cycle sequencing reactions, using a Big Dye terminator cycle sequencing kit (Applied Biosystems), contained 4μl ready reaction mix (1/8th final concentration), 3.5μl sequencing buffer (1X), 1μl forward primer (0.25μM), PCR products (1.5μl) and H_2O (10μl). Twenty five cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes were carried out. DNA was precipitated using a standard ethanol/EDTA precipitation protocol (Applied Biosystems, 2002) and re-suspended in 20μl Hi-Di formamide (Applied Biosystems). Samples were sequenced on a 3130 Genetic Analyser (Applied Biosystems, California, USA).

2.2.5 Data analysis

2.2.5.1 Sample groups

Adult male data were complete for 90 fields but due to missing samples and/or sample information wireworm identification data were only available for 19 out of the 24 fields in which they were found in total (59 out of 72 wireworms collected in total). Identified adult and wireworm samples were divided into groups based on sampling method for the analyses. These groups are defined as 'soil core 90', 'soil core 49', 'soil core 41', 'bait trap 41' and 'bait trap and soil core 41' (Table 2.1) and will be referred to as such throughout the rest of the chapter.

Group	Definition
Core 90	Wireworms trapped using soil cores, and adults trapped using sex pheromone traps for the total 90 fields surveyed
Core 49	Wireworms trapped using soil cores, and adults trapped using sex pheromone traps for the 49 fields in which bait traps were not used
Core 41	Wireworms trapped using soil cores, and adults trapped using sex pheromone traps for the 41 fields in which bait traps were also used
Bait 41	Wireworms trapped using bait traps, and adults trapped using sex pheromone traps for the 41 fields in which soil cores were also used
Bait and core 41	Wireworms trapped using both soil cores and bait traps, and adults trapped using sex pheromone traps for the 41 fields in which both methods were used

Table 2.1 Descriptions of sample groups used in the data analysis

2.2.5.2 Multivariate analysis

To determine the effect of sampling method on the relationship between wireworm and adult distributions, separate analyses were carried out for each sample group (Table 2.1) together with abiotic data. Prior to each analysis, Variance Inflation Factors (VIF's) were used to identify high levels of co-correlation between abiotic variables. A backward selection procedure was used, in which variables with a VIF >50 were

removed one variable at a time, starting with the highest VIF value. VIF values were then recalculated for the remaining variables and the process repeated until they were <50 (Zuur, Leno & Smith, 2007). "Percentage clay" was also removed since this variable is redundant when "percentage sand" and "percentage silt" are also included (Hicks, 2008).

Redundancy Analysis (RDA) was performed on each data set to visualise the relationship between species and abiotic variables using species abundance data which was standardised, and transformed using Hellinger distance to prevent the high number of double zeros (fields in which neither species are found) influencing the outcome of the analysis (Zuur, Leno & Smith, 2007). To avoid the problems associated with stepwise regression models (Whittingham et al., 2006), forward selection of the abiotic variables significantly influencing species distributions was applied only where a global test (Monte Carlo significance test on all canonical axes (all species) using a full model with 1000 permutations) was significant, and then a corrected coefficient of multiple determination was used together with a significance level of $p<0.05$ (Blanchet, Legendre & Borcard, 2008).

Abiotic variables out-numbered samples (fields) in analyses with the groups 'core 41', 'bait 41' and 'core and bait 41', and so a selection had to be made before Redundancy Analyses could be carried out. Therefore, the significant abiotic variables determined by forward selection of 'core 90' and 'core 49' data were used in the RDA for 'core 41', 'bait 41' and 'core and bait 41' without further forward selection.

VIFs and RDAs were obtained using Brodgar v.2.6.6 (Highland Statistics Ltd., 2006), and forward selection was carried out using the package 'packfor' in R v. 2.12.1 (Blanchet, Legendre & Borcard, 2008; R Foundation for Statistical Computing, 2010).

RDA correlation biplots, in which the angles between species lines represent

correlation between species (Zuur, Leno & Smith, 2007), were produced for each

analysis using all abiotic data, but for clarity only the variables found to be significant

in the forward selection were visualised.

2.2.5.3 Sequence data

Sequences (254 - 312 bp fragments of mitochondrial 16S rRNA) from wireworms

unidentified from the T-RFLP method were edited and aligned using BioEdit v. 7.0.9.0

(Hall, 1999; Thompson, Higgins & Gibson, 1994), and compared to published

sequences from this region (Ellis et al., 2009) for *A. obscurus* (three haplotypes,

GenBank accession numbers EU285484, EU285482 and EU285483), *A. sputator* (two

haplotypes, EU285485 and EU285480) and *A. lineatus* (1 haplotype, EU285481). For

sequences that did not match any of the above species, genetic distances were

calculated using the *p*-distance method in MEGA4 (Tamura et al., 2007). In this model

the number of nucleotide differences is divided by the total number of nucleotides

compared. No correction is made for multiple substitutions at the same site, biases in

substitution rate or differences in evolutionary rates among sites (Tamura et al., 2007),

so estimates of genetic distance may be conservative, but are not likely to be over-

estimated. Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) searches of

these sequences were also made at http://blast.ncbi.nlm.nih.gov/Blast.cgi using

megablast, which is designed to find long alignments between very similar sequences.

2.3 Results

2.3.1 Proportion and abundance of species

Overall, for sex pheromone trapped adult males, A. lineatus (7,287 individuals) was the most abundant of the three species, followed by A. obscurus (6,337 individuals) and A. sputator (4,359 individuals). Of the 72 wireworms recovered from the soil cores and bait traps, T-RFLP revealed that A. obscurus were the most abundant (35 individuals) followed by A. sputator (22 individuals), but no A. lineatus larvae were found. Twelve samples consistently produced an undigested fragment at around 400bp ('non-Agriotes') and were investigated further by direct sequencing. Three samples could not be identified due to failure of the PCR and restriction digest (these samples were not included in subsequent analyses).

For the 56 wireworm samples that were successfully analysed by T-RFLP or direct sequencing, the proportions of species trapped are not consistent between methods, or between adults and wireworms within species (Table 2.2). For example, for soil core data over 41 fields, A. obscurus wireworms comprise 90% of all wireworms trapped, whilst 'non-Agriotes' species make up the final 10% and no A. sputator wireworms were trapped. In addition, adults of A. sputator and A. lineatus are present despite no wireworms of these species being found. Yet, when bait traps over 41 fields are considered, A. sputator and A. obscurus wireworms are found in similar proportions and no other species of wireworm are present.

Although eigenvalues were calculated for all axes, only axes 1 and 2 are interpreted here since they explain the majority of the variation in the adult and wireworm data (Table 2.3; Zuur, Leno & Smith, 2007). The total amount of explained variation in species distributions is variable between analyses e.g. only 42% of the variation is accounted for using the 'core 41' group with wireworms separated to species, whilst 90% and 91% of variation is explained using the 'core 49' with wireworms as a complex and separated to species (Table 2.3).

The RDA biplots (Figure 2.1 and Figure 2.2) reveal that there are differences in the relationship between adults and wireworms depending on the trapping method. For wireworms separated to species, 'non-*Agriotes*' wireworm were found in soil cores but not in bait traps in the same fields, and similarly no *A. sputator* wireworms were found in soil cores but were present in bait traps from these fields (Figure 2.2a, b). When soil core and bait trapped wireworms are combined, species distributions' change again, with *A. obscurus* and *A. sputator* wireworms and *A. sputator* adults closely associated with each other, but *A. obscurus* adults dissociated from these species (Figure 2.2e). There is a closer relationship between *A. obscurus* adults and wireworms, and *A. sputator adults* and wireworms when the 'core 49' group is considered compared to the 'core 90' group (Figure 2.2 c,d). Changes in the relationship between the wireworm complex and adult species are also apparent in different analyses (Figure 2.1).

In addition, separating wireworms to species instead of grouping as a complex reveals more information in terms of the relationship between adult and wireworm distribution. For example, in the RDA for 'bait 41' (Figure 2.1b) wireworms as a complex are associated with *A. obscurus* adults and 'Yrsconvert' and 'additive index',

whereas when separated to species *A. sputator* and *A. obscurus* wireworms are associated with different abiotic variables and are somewhat dissociated from one another (and more closely aligned to adults of the same species; Figure 2.2b).

Of the original 40 abiotic variables used for forward selection (39 for 'core 49'; % sand had a VIF > 50 and was removed), 15 variables in total (for wireworms as a complex and separated to species combined) were found to significantly influence adult and wireworm distribution, comprising 8 cultural, 2 environmental, 3 physical and 2 chemical variables. For wireworms separated to species in 'core 49', there were fewer significant variables than when grouped as a complex, but the variables themselves were the same (Table 2.4), while for wireworms separated to species in 'core 90' similar numbers of variables were found to be significant, though some differed between analyses with wireworms grouped in a complex or separated to species. In particular, cultural variables related to the time the field has been in grass e.g. 'grass duration', 'Yrsconvert' and 'Yrsingrass', are associated with wireworms as a complex and *A. obscurus* and *A. sputator* wireworm distribution, but to a lesser extent with the 'non-*Agriotes*' species of wireworm (Figure 2.1 and Figure 2.2).

Species	All data		Core 90		Core 49		Core 41		Bait 41		Core and bait 41	
	Adult	Wireworm	Adult	Wireworm	Adult	Wireworm	Adult	Wireworm	Adult	Wireworm	Adult	Wireworm
A. sputator	24	30	24	5	34	9	15	0	15	46	15	27
A. obscurus	35	54	35	52	22	18	47	90	47	54	47	71
A. lineatus	41	0	41	0	44	0	38	0	38	0	38	0
non-Agriotes	-	16	-	43	-	73	-	10	-	0	-	2

Table 2.2. The proportion of the total catch (%) for A. sputator, A. obscurus, A. lineatus adults and wireworms and 'non-Agriotes' wireworms, based on data for all the fields surveyed ('all data') and for each group used in the analyses ('core 90, 'core 49, 'core 41', 'bait 41' and 'core and bait 41').

	Core 90		Core 49		Core 41		Bait 41		Core and bait 41	
Axis	Complex	Species	Complex	Species	Complex	Species	Complex	Species	Complex	Species
1	34	23	46	31	47	30	53	33	53	30
2	23	18	22	19	21	9	12	7	14	7
3	14	10	21	17	3	2	6	5	4	3
4	1	9	1	14	0	2	0	4	0	2
5	-	4	-	9	-	0	-	0	-	1
6	-	0	-	1	-		-		-	0
All axes	71	66	91	90	72	42	71	50	71	43

Table 2.3. Eigenvalues (the percentage of explained variation) for each axis in the Redundancy Analysis for each group, for wireworms as a species complex and separated to species.
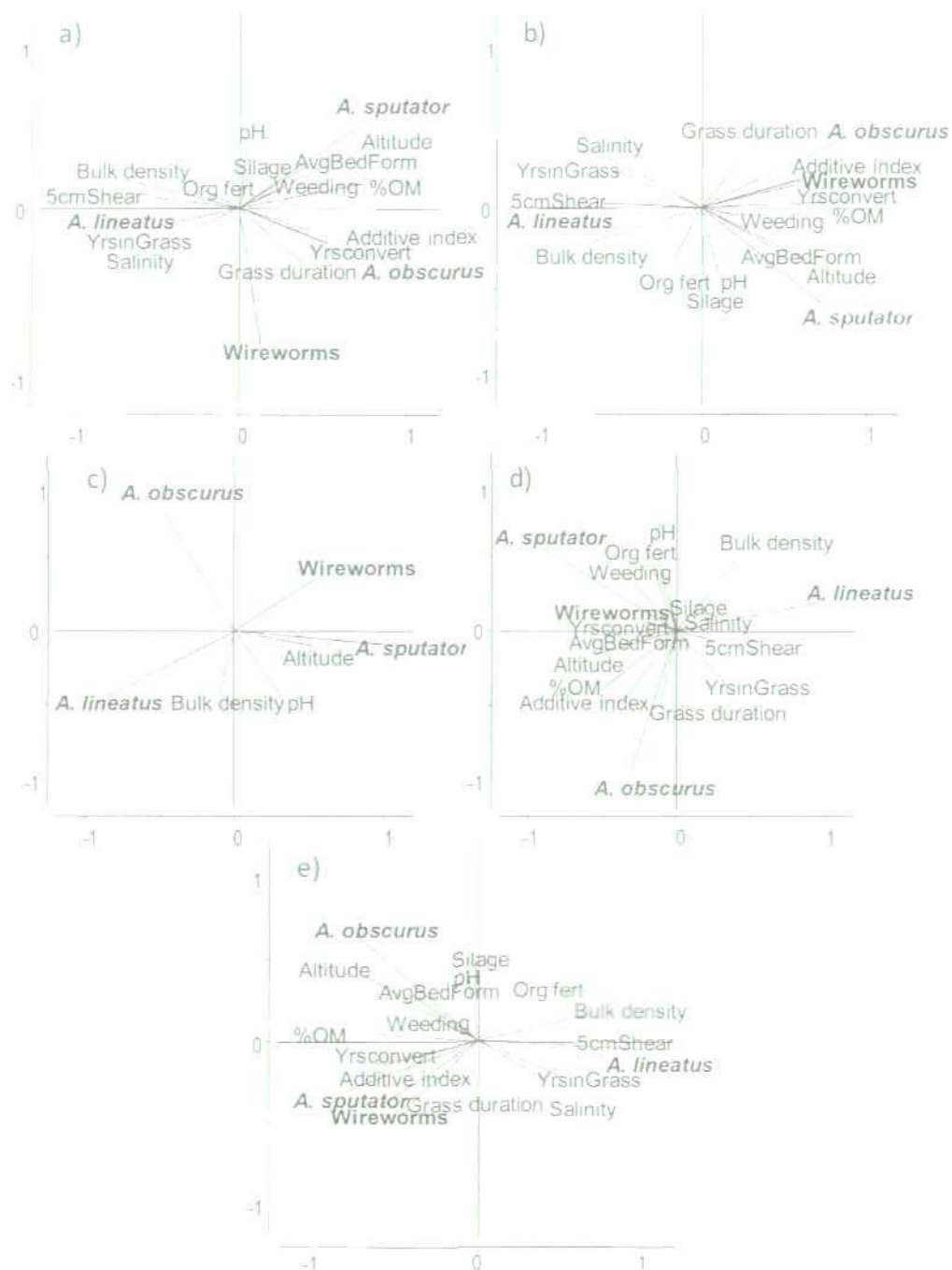
Figure 2.1. Redundancy analysis correlation biplots of axes 1 and 2 for wireworms as a species complex in a) 'core 41', b) 'bait 41', c) 'core 49', d) 'core 90' and e) 'core and bait 41'. Abiotic variables found to be significant in forward selection ($p < 0.05$) are visualised. See Appendix 1 for definition of abiotic variables.
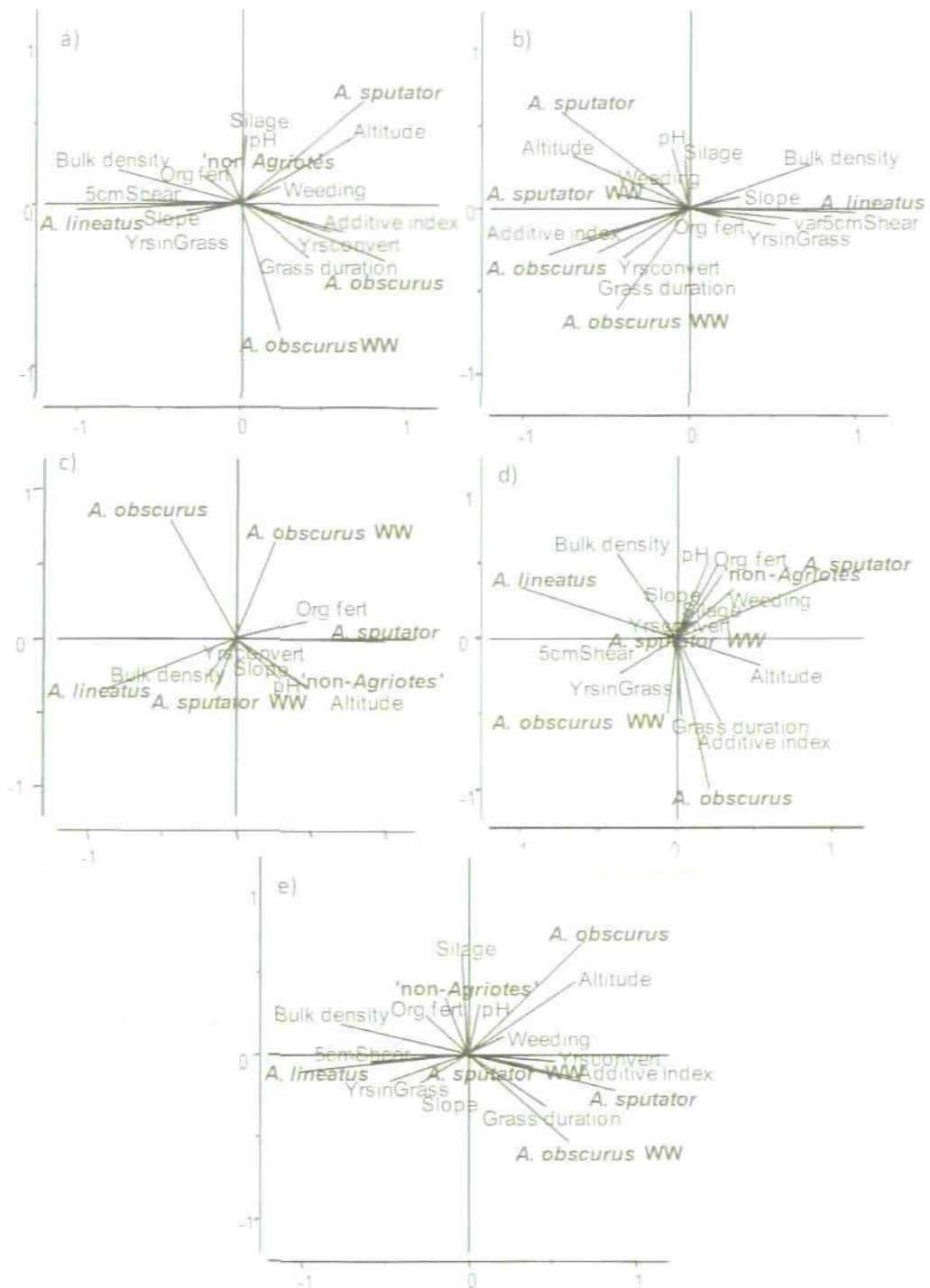
Figure 2.2. Redundancy analysis correlation biplots of axes 1 and 2 for wireworms separated to species in a) 'core 41', b) 'bait 41', c) 'core 49', d) 'core 90' and e) 'core and bait 41'. Abiotic variables found to be significant in forward selection ($p < 0.05$) are visualised. See Appendix 1 for definition of abiotic variables.

a)

Variable	Order	R_2	R_2cum	Adj R_2Cum	F	p
Altitude	1	0.10	0.10	0.08	5.35	3.01×10^{5}
Slope	4	0.07	0.17	0.13	3.79	1.21×10^{2}
Org fert	20	0.06	0.23	0.18	3.50	2.38×10^{2}
Yrsconvert	19	0.04	0.27	0.21	2.59	1.87×10^{2}
Bulk density	14	0.04	0.32	0.24	2.79	1.21×10^{2}
pH	5	0.05	0.36	0.27	2.99	7.79×10^{3}

b)

Variable	Order	R_2	R_2cum	Adj R_2Cum	F	p
Altitude	1	0.15	0.15	0.13	8.21	4.01×10^{6}
Bulk density	14	0.07	0.22	0.18	4.14	3.11×10^{5}
pH	5	0.06	0.28	0.24	4.08	3.46×10^{3}

Table 2.4. Abiotic variables found to be significant in forward selection ($p < 0.05$) for a) wireworms grouped as a species complex, and b) wireworms separated to species using the group 'core 49'. See Appendix 1 for definition of abiotic variables.

a)

Variable	Order	R^2	R^2cum	AdjR^{2}Cum	F	p
Bulk density	15	0.13	0.13	0.12	13.09	5.96×10^{-10}
Org fert	22	0.08	0.21	0.19	9.20	4.49×10^{7}
Altitude	1	0.07	0.28	0.25	7.94	3.49×10^{6}
pH	5	0.05	0.32	0.29	5.71	4.86×10^{-4}
AvgBedForm	38	0.03	0.35	0.31	3.31	1.12×10^{-2}
GrasDura	18	0.03	0.38	0.33	3.32	1.09×10^{-2}
Yrsconvert	21	0.03	0.40	0.35	3.57	7.21×10^{-3}
5cmShear	10	0.03	0.43	0.37	4.18	2.57×10^{-3}
%OM	17	0.02	0.45	0.39	2.92	2.13×10^{-2}
YrsinGrass	20	0.02	0.47	0.41	3.21	1.34×10^{-2}
Silage	19	0.03	0.50	0.43	4.59	1.29×10^{-3}
Weeding	31	0.03	0.53	0.46	4.43	1.70×10^{-3}
Additive index	26	0.03	0.55	0.48	4.42	1.73×10^{-3}
Salinity	9	0.02	0.57	0.49	2.95	2.05×10^{-2}

b)

Variable	Order	R^2	R^2cum	AdjR^{2}Cum	F	p
Bulk density	15	0.09	0.09	0.08	9.07	1.89×10^{9}
Org fert	22	0.07	0.16	0.14	7.00	3.51×10^{7}
Slope	4	0.06	0.22	0.19	6.11	3.35×10^{6}
Altitude	1	0.05	0.27	0.23	5.65	1.09×10^{3}
pH	5	0.03	0.30	0.26	3.85	9.12×10^{-4}
Additive index	26	0.03	0.33	0.28	3.77	1.10×10^{7}
Grass duration	18	0.03	0.36	0.30	3.73	1.21×10^{3}
Yrsconvert	21	0.02	0.38	0.31	2.43	2.54×10^{2}
YrsinGrass	20	0.02	0.39	0.33	2.38	2.81×10^{1}
5cmShear	10	0.02	0.42	0.34	3.13	5.07×10^{3}
Silage	19	0.02	0.44	0.36	3.05	6.21×10^{7}
Weeding	31	0.03	0.47	0.38	3.87	8.84×10^{4}

Table 2.5. Abiotic variables found to be significant in forward selection ($p < 0.05$) for a) wireworms grouped as a species complex, and b) wireworms separated to species using the group 'core 90'. See Appendix 1 for definition of abiotic variables.

2.3.3 'Non-Agriotes' wireworms

Sequences from the 12 'non-*Agriotes*' wireworms and those of *A. obscurus*, *A. sputator* and *A. lineatus* did not match when aligned. Within these samples there were two groups of similar sequences: group one consisted of 10 individuals (overall average distance between haplotypes (*p*-distance) 0.1%, following the removal of 12 ambiguous sites), whereas group two contained two individuals with identical haplotypes (following the removal of 7 ambiguous sites). The between group distance (mean of all sequences within a group) for the *Agriotes* species and group one was 21% and for *Agriotes* species and group two was 13%. Between groups one and two the overall average difference was 23% (Table 2.6), compared to an overall average difference for an alignment of the three UK *Agriotes* species in this study of 3.7%. There were 'highly similar' matches with other click beetle species in BLAST. Group one sequences were similar to those of *Denticollis linearis* (Linnaeus) (*P*-distance up to 21%) and *Athous haemorrhoidalis* (Fabricius) (*P*-distance up to 27%), whereas *A. obscurus*, *A. lineatus*, *A. sputator* and *A. sordidus* were similar to sequences in group two (*P*-distance up to 10%). However, subsequent alignments with these species in BioEdit were not similar enough to provide a reliable identification, and as of yet they are unknown (the morphology could not be checked since unfortunately the last abdominal segment, a key identification feature, was used for DNA extraction).

	Agriotes	Group 1	Group 2
Agriotes		2.6	2.2
Group 1	21		2.8
Group 2	13	23	

Table 2.6. The percentage base differences per site from averaging over all sequence pairs (p-distance) between 'non-Agriotes' wireworms in group 1 and group 2, and the Agriotes species. Standard error estimates are shown above the diagonal in italics and were obtained by a bootstrap procedure (1000 replicates) in MEGA4. Gaps and missing data were eliminated from the dataset (complete deletion option) (Tamura et al., 2007).

2.4 Discussion

2.4.1 The proportion and abundance of species

By identifying both click beetle life-stages, this study has shown that there may not always be a straightforward relationship between aboveground adult male and belowground wireworm distribution. This has important implications in cases where the monitoring of a pest is carried out on a different life stage to that causing the damage. For Agriotes click beetles, the proportion and distribution of adult male species trapped, at least with sex pheromone traps, may give a very misleading picture of the proportion and distribution of wireworm species in the soil; although A. lineatus were the most abundant of the three adult male species trapped, no A. lineatus wireworms were identified and an unexpectedly large proportion of 'non-Agriotes' wireworms were found. Clearly, when wireworms are grouped as a complex these important differences are not apparent.

Other species are known to occur together with *Agriotes* populations, but they are not usually found in large numbers where *Agriotes* species dominate. Since species in other genera e.g. *Athous* and *Ctenicera,* have been recorded as attacking potatoes, turnips and oats (Anon, 1948), the high proportion 'non-*Agriotes*' wireworm species found in this study may have implications for their pest status (see Chapter 4 for a detailed analysis of the non-*Agriotes* species found in this study).

2.4.2 Comparison of sampling methods

As well as there being differences in the overall proportions of species trapped, differences were also observed in the abundance and distribution of wireworm species trapped using different methods. In particular, there were clear differences in the species trapped when soil core and bait trap samples from the same field are considered separately (Table 2.2 and Figure 2.2a, b). Although cereal baiting techniques have been found to be equivalent in efficacy to soil-cores in detecting wireworms, little is known of individual species movement and response to bait traps. In addition, if there are interspecific differences in the vertical distributions of wireworms, possibly due to dispersal ability or response to environmental factors, then the 10cm soil core may not have sampled those found in the lower layers of the soil; previous studies have recovered further wireworms from 30cm to 60cm (Furlan, 2004) and so the species trapped may be dependent on the sampling method used. Differences in adult male and wireworm species distributions were also discovered between analyses with 'core 49' and 'core 90' data, so although it is possible that the patterns exposed here may differ according to the region studied, scale of study may also influence the relationship between wireworm and adult species.

Adult male abundance between species as measured in sex pheromone traps could indicate differences in species responses to sex pheromones and dispersal ability (Blackshaw & Vernon, 2008; Hicks & Blackshaw, 2008), or interspecific differences in female oviposition preferences. Although the most numerous adult trapped, *A. lineatus* adults were somewhat dissociated from 'non-*Agriotes*' wireworms and adult species in all analyses. Alternatively, the lack of *A. lineatus* larvae may be due to a large change in species composition in the fields sampled; oviposition resulting in the adult males trapped in this study occurred approximately 4 years previously, whilst most of the wireworms trapped were approximately 2-3 years old. Any generational differences, however, would be difficult to verify, since adult males were only trapped during one season. The generational difference between adults and wireworms trapped in the same period is another limitation of using adult trap counts as a surrogate for wireworm sampling.

These results suggest that using a single sampling method will not truly reflect the wireworm species and distribution within the soil, which as the main vectors of damage we need to be able to model and predict. They also clearly highlight the need for accurate species identification, since even closely related species may have complex and decoupled life-stage distribution patterns, and therefore the previously used risk assessment practice of using the total number of individuals from all species caught in sex pheromone traps may not be appropriate. Such patterns, affected by individual species ecology, have wider consequences for community composition and related soil processes.

Direct sampling of wireworms, using both soil core sampling and bait trapping, rather than inferring the presence and distribution of wireworms from adult males trapped in

sex pheromone traps, would give a more accurate representation of the species

present. This way, if the species involved in crop damage are known to be present in a

particular field, appropriate decisions can be made about management before crop

planting begins. A method to identify all wireworm species present would therefore be

valuable (see Chapter 4). It is realised that soil core/bait trap sampling is a costly,

labour intensive method. However, the expense associated with the use of accurate

risk assessment methods might avoid the economic loss following crop damage

brought about by inaccurate information from pre-planting risk assessment methods.

Sex pheromone traps may have a potential use in pest management through mass

trapping of adult males when a known wireworm population is present, and has been

shown to reduce isolated populations of *Melanotus okinawensis* Ohira when a high

density of traps are used, but information on the dispersal extent of the UK *Agriotes*

species and testing in the field would be required to assess the viability of this in UK

agricultural land.

2.4.3 Distribution in relation to abiotic variables

It is recognised that there is uncertainty in the predictive value of RDA models when

the underlying ecology of the species is not well understood. As previously mentioned,

Parker and Seeny (1997) found that multiple and generalised linear regression models

had poor predictive accuracy for wireworm infestation. Despite the limitations

associated with these methods, in this case similarities to other studies in terms of the

variables associated with *Agriotes* distribution have been found. The RDA and forward

selection of abiotic variables uncovered notable visual interspecific differences in the

distribution of both adult males and wireworms, which appear to be correlated with

specific abiotic variables (Fig. 2.2). These include a positive association with variables

related to grass duration for wireworms (*A. obscurus* in particular) (Miles, 1942b;

Parker & Seeny, 1997), soil bulk density (Parker & Seeny, 1997) and weeding ('non-

Agriotes' wireworms, Figure 2.2d; *A.sputator* adults and wireworms, Figure 1.2b)

(Parker & Howard, 2001; Seal, McSorley & Chalfant, 1992), reinforcing these previous

findings on the possible effects of abiotic factors on wireworm distribution. The

prevalence of significant cultural variables suggests that in terms of management,

action to avoid damage by wireworms can be taken relatively easily, e.g. not planting

crops in fields which have recently been converted from grassland or are weedy

(female click beetles usually lay their eggs in patches of grass/weeds to prevent

desiccation; Parker & Howard, 2001). However, since these preventative methods are

already well-known (Parker & Howard, 2001), this limits the value of these models for

informing management practices. The variability of biotic and abiotic factors at

different spatial and temporal scales means that models that include biological

parameters such as dispersal and oviposition, in addition to abiotic variables, are likely

to have better predictive capacity (van der Putten et al., 2009).

Chapter 3   The spatial distribution of wireworms and other phytophagous insect

larvae in grassland soils

3.1 Introduction

Soil communities have been described as the 'poor man's tropical rainforest', due to

the relatively high level of biodiversity and large proportion of undescribed species

they contain, and the limited information that is known on community structure and

dynamics (Giller, 1996). Ninety per cent of insects spend at least some part of their

lifecycle in the soil (Klein, 1988), having an influence on, for example, the diversity of

plant communities, competitive interactions among plants and the yield of agricultural

systems (Hunter, 2001). Despite this, information on the distribution and abundance of

soil dwelling insects is lacking.

Physical, chemical and biotic factors are known to determine the presence, size and

survival of invertebrate populations within the soil, causing a patchy distribution

(Curry, 1987). However, spatial scale, although long recognised by ecologists as an

important component influencing species distributions (Levin, 1992; Wiens, 1989), has

been somewhat neglected in ecological studies. It is well recognised that spatial scale

of sampling and analysis affects the observed distributions, but it has often been seen

as a complicating factor rather than included as an explanatory variable in its own

right, and as such multi-scale experiments are rare (Sandel & Smith, 2009). Spatial

structuring, through environmental and community processes, plays a functional role

in ecosystems and in order to understand this, modelling spatial patterns at multiple

spatial and temporal scales is critical (Borcard & Legendre, 2002; Borcard et al., 2004).

Owing to the relatively recent growth in spatial statistical techniques there are now

many methods of incorporating spatial location into studies to determine how space affects species presence and/or abundance (Borcard, Legendre & Drapeau, 1992; Cocu et al., 2005; Coomes, Rees & Turnbull, 1999; Legendre & Fortin, 1989; Lichstein et al., 2002). In addition, information on the relative importance of biotic, environmental and spatial factors to individual species distributions would be useful in terms of managing pests and biodiversity, and may allow better predictive models to be produced. A combination of methods that describe the spatial distribution and underlying patterns in the data, such as indices of dispersion and multivariate techniques, together with methods that use the spatial location (e.g. geographical coordinates) of samples as an indication of spatial pattern, such as deviance partitioning, might enable a better overall understanding of species distributions to be obtained.

In this study, wireworms and other taxa commonly found in abundance in grassland - leatherjackets (crane fly larvae – Diptera: Tipulidae), sciarid fly larvae (Diptera: Sciaridae) and bibionid fly larvae (Diptera: Bibionidae) - were sampled from grassland soil. These taxa, when present in arable land, can cause considerable damage to crop roots and so research has mainly focused on their role as agricultural pests. Previous studies in the UK have attempted to determine the factors affecting the distribution of wireworms (Parker & Seeny, 1997; Salt & Hollick, 1946) and leatherjackets (McCracken, Foster & Kelly, 1995), but without inclusion of either spatial, scale or biotic variables, or all three, and studies on interactions between macro-invertebrates in agricultural land and the influence of other species on the distribution of soil insect larvae are lacking. A good understanding of pest distribution, and the factors affecting this, is essential to the development of sustainable management strategies. This will enable development of control methods that can be targeted at damage causing

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species at the appropriate scale. For soil living pests, this is particularly important since incorporation of pesticide into the soil can be inefficient, often even with high rates of application, and many of the persistent chemicals which were effective have now been withdrawn from use (Grove, Woods & Haydock, 2000).

With these issues in mind, the aims of the study were:

1. To assess the effect of scale of sampling, and the contribution of space, biotic interactions and scale to soil insect larvae distribution.

2. To determine whether there are any interspecific relationships between taxa, and how these relationships vary with the scale of sampling.

3.2 Methods

3.2.1. Study site and sampling

As part of a study on the use of water traps to predict the size of leatherjacket populations soil samples were collected from 26 sites over six grass fields from Seale Hayne Farm, South Devon, UK between 15th January and 31st March 2008. Apart from one field which was a permanent ley, all fields were in grass for at least 3 years before sampling, previously being in an informal rotation with cereals and maize. Soils were brown earths of the Highweek and Trusham series. At each site, samples were collected from the intersections of 24 radii at 15° intervals with concentric circles at 5, 10, 20 and 40m from a central point. Soil cores were collected using a 5cm diameter plastic pipe, which was pushed to a depth of approximately 10cm. In total 96 soil samples were collected from each site, with the exception of four sites which were

truncated due to sample points overlapping field boundaries and/or a stream, which equates to an area of $4.86m^2$ of soil sampled across the study site.

3.2.2 Extraction and identification of larvae

Larvae were recovered from soil cores using heat extraction (Blasdale, 1974) and placed into separate tubes containing 70% ethanol. Leatherjackets were assumed to be *Tipula paludosa* Meigen, as no other species had been captured as adults using water traps placed at the sites previously; Humphreys et al. (1993) found this assumption to be correct for the areas surveyed in their study. Bibionid larvae were identified as either *Bibio johannis* L. or *Dilophus febrilis* L. based on the posterior spiracles, using a light microscope (Brindle, 1962). Sciarid larvae could not be identified to species by their morphology and so remained unidentified and grouped as 'Sciaridae'. Wireworm DNA was extracted using a standard salt/chloroform protocol (Rico, Kuhnlein & Fitzgerald, 1992) and resuspended in 0.01X TE buffer (0.1 mM Tris HCl; 0.01 mM EDTA). A terminal restriction fragment length polymorphism (T-RFLP) technique (Ellis *et al.*, 2009) was used to identify wireworms of the species *Agriotes obscurus* L., *A. sputator* L. and *A. lineatus* L. Other species were grouped together as 'non-*Agriotes*'. There were also instances in which no restriction fragment was produced, possibly due to degraded DNA. These samples have been grouped together as 'unknown wireworms'.

3.2.3. Data analysis

Ecological data often tend to be spatially autocorrelated, where observations from nearby locations are more similar than would be expected by chance, driven by various

environmental and biotic processes (Kissling & Gudrun, 2008; Legendre & Fortin, 1989; Lichstein *et al.*, 2002). This lack of independence violates the assumptions of most traditional statistical tests, resulting in inflation of Type I errors which can affect the interpretation of results. Moran's autocorrelation coefficient (*I*) was calculated using Spatial Analysis in Macroecology (SAM) software v. 3.1 (Rangel, Diniz-Filho & Bini, 2006) for each species, using geographic distances with 21 distance classes (the default) and default distance class size with equal distances. Due to the low abundance of the taxa sampled, the significance of the Moran's *I* values were not tested, as when applied to data with many double zeros the degree of autocorrelation may be overestimated (Legendre and Fortin, 1989). Instead, the correlograms, in which autocorrelation values are plotted against distance classes and the Moran's *I* values alone were checked for spatial autocorrelation. It was determined that no spatial autocorrelation was present and as a result standard statistical tests were used for the analysis.

The variance to mean ratio (VMR) was used as an index of dispersion to determine the distribution of individual taxa at each scale, using species abundance data (Taylor, 1961). This gives an idea of the spatial *distribution* and underlying patterns in the data, based on the frequency distribution of number of specimens per sample unit. However, it is not an indication of spatial *pattern*, which uses the spatial location (e.g. geographical coordinates) of samples rather than count data (Binns, Nyrop & van der Werf, 2000).

Non-metric multidimensional scaling (NMDS) was computed using species presence/absence data in Brodgar v.2.5.7 (Highland Statistics Ltd., 2006), to visualise the relationships between all taxa at each scale. Jaccard's coefficient, an asymmetrical

binary coefficient which excludes double zeros (i.e. where the fields, sites or cores being compared both contain no taxa) was used as a measure of association between species (Legendre & Legendre, 1998). The optimal number of dimensions, or axes m, was determined by selecting the ordination with minimum STRESS, a measure of deviation from monotonicity (Kruskal, 1964); the number of axes was plotted against the STRESS values for each value of m. A clear change in stress (the elbow effect) indicates the optimal value of m (Zuur, Leno & Smith, 2007). For the field scale $m = 4$, STRESS = 0.0093, for the site scale $m = 3$, STRESS = 0.0247 and for the core scale $m = 4$, STRESS = 0.068.

Due to the high number of zero counts geostatistical methods were not used to assess spatial pattern as this would result in violation of the assumptions of these tests. Instead, partial linear regression, using a Generalized Linear Model (GLM) with a binomial distribution for taxa presence-absence data and logistic link function, was used to partition the deviance explained by space (latitudinal and longitudinal coordinates), biotic influences (presence/absence data for all other species) and scale for each taxon (Legendre & Legendre, 1998; Lobo, Lumaret & Jay-Robert, 2002). GLM allows for distributions other than the normal distribution and is also not constrained by the assumption of linearity between dependent and independent variables (Lobo, Lumaret & Jay-Robert, 2002). This makes it a useful technique for analysing ecological data, which is often non-normally distributed. Scale, as a nominal variable, was split further into 'field' (numbers 1-6) and 'site' (numbers 1-26) to determine the proportion of deviance explained by the scale of sampling. Eight components of the total explained deviance were calculated: effect of spatial variation, effect of scale, effect of biotic influences, combined effect of space and scale, combined effect of

space and biotic influences, combined effect of scale and biotic influences, the joint
effect of all three types of variables and the unexplained variation – variation not
accounted for by the explanatory variables included in this study. These analyses were
carried out using Brodgar v.2.5.7 (Highland Statistics Ltd., 2006).

3.3 Results

3.3.1 Abundance and composition of taxa

Bibio johannis was the most numerous species identified, followed by Sciaridae and
leatherjackets which were found in similar proportions (Table 3.1). *Dilophus febrilis*
was the least numerous dipteran species. The wireworm species were dominated by *A.
lineatus*, whilst *A. obscurus* and *A. sputator* were found in similar proportions and the
non-*Agriotes* wireworms comprised a relatively small proportion of all taxa (Table 3.1).
In addition there were a number of individual wireworms that could not be identified,
possibly due to degraded DNA (Unknown WW; Table 3.1).

Taxa	Number	%	Population density/m2
B. johannis	112	28	23
Sciaridae	83	20	17
Leatherjackets	80	20	75
D. febrilis	56	14	12
A. lineatus	35	9	7
A. sputator	15	4	3
A. obscurus	13	3	3
Unknown WW	7	2	1
Non-*Agriotes*	5	1	1

Table 3.1 Number of individuals of each taxon, their percentage of the total 406
insects obtained from 2474 soil cores over 26 sites in 6 fields, and the population
density based on an area of $4.86m^2$ (the total area of all soil cores collected).

3.3.2 Variance/mean ratio

The variance/mean ratio generally decreased from the field scale to the core scale

(Table 3.2). *Agriotes obscurus, A. sputator, A. lineatus* and leatherjackets were

aggregated at the field and site scales (VMR >1) but had a more random distribution at

the core scale (VMR = 1). The 'non-*Agriotes*' wireworms showed a random distribution

at all scales, whereas Sciaridae, *B. johannis* and *D. febrilis* were aggregated at all scales.

Taxa	Field scale	Site scale	Core scale
A. obscurus	6.4	2.9	1.1
A. sputator	8	6	1.1
A. lineatus	8	2.5	1
Non -Agriotes	1.6	1.3	1
Sciaridae	22.2	18.9	20.9
B. johannis	64.1	17.6	2.1
D. febrilis	27.8	30	19
Leatherjackets	12.6	3.3	1.3

Table 3.2 The variance/mean ratio for taxa at the field, site, and core scale.

3.3.3. Non-metric multidimensional scaling

NMDS ordination biplots (Figure 3.1) revealed that the distribution of taxa in relation

to each other changed between the field, site and core scales. Within the graphical

configuration of the biplot, taxa close to each other co-exist in the same fields (a), sites

(b) or cores (c) (Zuur, Leno & Smith, 2007). Notably, *A. obscurus* and non-*Agriotes*

wireworms, and, separately, leatherjackets and Sciaridae are found in close proximity

across all scales. The co-existence of other wireworm species is varied across scales,

while *B. johannis* and *D.febrilis* are somewhat dissociated at the broader field and site

scales, but are relatively closely associated at the core scale.
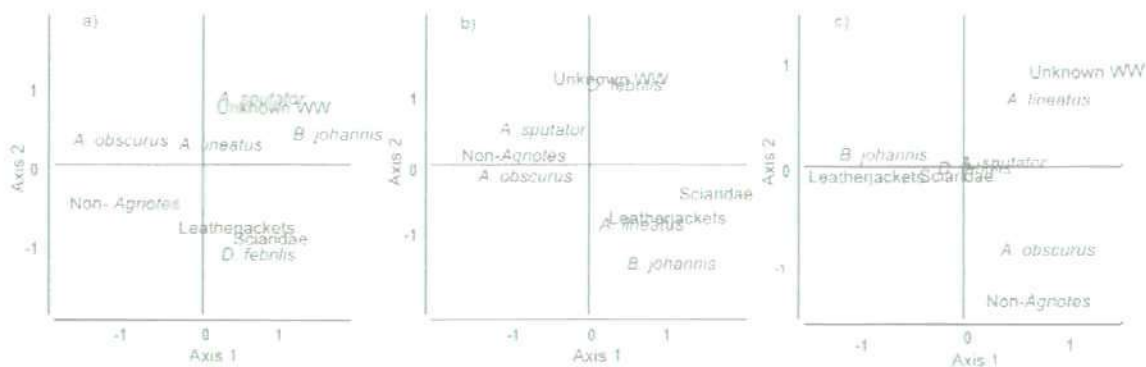
Figure 3.1 NMDS ordination biplots (axes 1 and 2) for (a) field scale (b) site scale and

(c) core scale. The distances between species represent relative similarity. 'Non-

Agriotes' refers to wireworms that are not one of the three UK *Agriotes* species (*A.*

obscurus, A. sputator and *A. lineatus*) and 'unknown WW' are wireworms for which no

restriction fragment was produced. Bibionid flies were separated to species (*D. febrilis*

and *B. johannis*), Sciaridae are grouped together as a species complex and

leatherjackets are assumed to be *Tipula paludosa*.

3.3.4. *Deviance partitioning*

The combined amount of deviance explained by the explanatory variables ranged from

12% (leatherjackets) to 43% (*A. sputator*) and the majority of the variation was

unaccounted for (Table 3.3). Of the explained deviance, scale was the most important

individual component for all taxa, comprising between 10% and 36%, while spatial and

biotic variables had a minor influence on most taxa. The biotic and scale variables

combined explained slightly more variation than the scale and spatial variables

combined, and the combined effect of biotic and spatial variables was relatively small

for most taxa. The importance of field and site in terms of explained deviance varied

between taxa; the presence of *A. obscurus*, non-*Agriotes*, *B. johannis* and *D. febrilis*

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was more affected by field than site, whereas *A. sputator*, *A. lineatus*, unknown

wireworms and Sciaridae distribution was more related to the site variable. Field and

site explained an equal amount of deviance for leatherjackets, which was relatively low

compared to the other species, suggesting scale does not considerably influence

leatherjacket distribution. Core scale data were not included due to the high number

of cores which contained no taxa. Only 14 out of 2474 cores contained two different

taxa, which is not significantly different from the six number of occasions of co-

occurrence predicted by a chi-square test (χ_2 = 11.76, *df* = 35, p >0.05), indicating the

taxa are distributed randomly in relation to each other at this scale and population

density.

	A. obscurus	A. sputator	A. lineatus	Non-Agriotes	Unknown WW	Sciaridae	B. johannis	D. febrilis	LI
Biotic	9	6	4	13	4	3	1	2	2
Scale (All)	30	36	16	26	29	17	30	32	10
Scale (field)	23	12	5	17	11	8	21	18	5
Scale (Site)	7	24	10	9	18	9	9	14	5
Space	6	27	1	1	11	2	30	12	2
Biotic and Scale	36	40	18	38	32	18	30	34	12
Biotic and space	16	34	5	15	15	5	2	15	3
Scale and space	33	39	18	27	31	19	30	34	10
All variables	38	43	20	39	34	21	30	36	12
Unexplained	62	57	80	61	66	79	70	64	88

Table 3.3 The percentage of total explained deviance obtained through deviance partitioning in the dependant variable (species presence/absence) between the three groups of explanatory variables and their combinations with each other, and the deviance unexplained by the variables in this study, for each taxa. See text for definitions of deviance categories.

3.4 Discussion

3.4.1 Abundance and composition of taxa

Only low numbers of insect larvae were recovered during this study when compared with other reports (Table 3.1). Sciaridae and Bibionidae are reputed to be among the most abundant families of soil dwelling dipteran larvae (Frouz, 1999), though Blackshaw and D'Arcy-Burt (1997) state that Bibionidae only occur 'sporadically and at low population density' in agricultural grassland. Wireworms and leatherjackets can occur in high abundances in grassland soil (e.g. Buckle, 1923; Gongalsky et al., 2009; Miles, 1942b; Staley et al., 2007), however, it is likely that large populations are the exception rather than the rule (see Anon, 1948; Blackshaw, 1983). Little is known of species-specific wireworm distribution in grassland in the UK and Europe due to the morphologically cryptic nature of wireworm species. The fact that differences in abundance have been observed here between closely related species underlines the need to separate taxa to species, particularly for potential pests, in order that the damage causing species can be identified. The growing use of molecular techniques, such as DNA barcoding (e.g. Ball & Armstrong, 2006), restriction fragment length polymorphism (RFLP) (e.g. Carew, Pettigrove & Hoffmann, 2003) and T-RFLP (e.g. Ellis et al., 2009) may aid identification of other problematic taxa.

3.4.2. The effect of scale, spatial and biotic variables on distribution of taxa

The limited importance of biotic and spatial variables, in terms of deviance explained, and the high amount of unexplained variation (between 57% and 88%, Table 3.3) indicates that other unmeasured factors are more important for determining the distribution of the taxa studied. As well as the effects of scale-dependent

environmental factors, reproductive and stochastic effects, which are more difficult to model, may play a role (Giller, 1996). The importance of scale for all taxa implies the effects of chemical, physical and environmental factors, which although not measured directly in this study, are known to differ between fine and broad scales. In turn, differences in taxon preference for these factors affects the distribution observed at different scales of investigation.

Aggregation of wireworms, leatherjackets, *B. johannis*, *D. febrilis* and Sciaridae has also been observed in other studies over different scales, and linked to biotic and environmental factors, including soil type, texture and moisture (Cherry & Stansly, 2008; Jackson & Campbell, 1975), amount of organic matter (D'Arcy-Burt & Blackshaw, 1991; Salt & Hollick, 1946), presence of grass species and other soil insects (Salt & Hollick, 1946), distribution of oviposition sites (Coulson, 1962; Doane, 1977; Frouz & Paoletti, 2000), host plant selection (Jackson and Campbell, 1975) and aggregation pheromones (Blackshaw and D'Arcy-Burt, 1997). In addition, larval dispersal ability may also affect species' distribution. For example, although early instar wireworms remain aggregated, late instar wireworms are better able to disperse, resulting in less aggregation with age (Salt and Hollick, 1946; Doane, 1977). This process is likely to affect larval distribution at smaller scales, and could explain the random distribution of the wireworm species, which were late instar, at the core scale in this study (Table 3.2). Similarly, Sciaridae larvae (and other soil dwelling Diptera) have been described as 'almost sessile' (Frouz, 1999). Thus, the lack of larval dispersal and possibly adult choice of oviposition site, may be contributing to their aggregation at all scales (Table 3.2).

An important outcome of the study is that larval distribution differs between species, likely caused by a variety of scale and species-specific factors. For example, scale contributed most to the explained deviance for all wireworm species, but there was variation in the importance of field and site between species (Table 3.3). Similarly, space explained an equal amount of deviance as scale for *B. johannis*, but not *D. febrilis*, indicating possible differences in the biology of these species, especially since pure spatial variation may be associated with biological processes not linked to environmental factors, e.g. growth, predation, competition and social aggregation (Borcard, Legendre & Drapeau, 1992). However, scale and biotic and spatial variables were ineffective at explaining leatherjacket distribution, with 88% of deviance unaccounted for. The equal deviance explained by the field and site scales suggests that scale, and the factors acting at these scales, do not have as much of an influence as, for example, reproductive factors or density dependant feedback mechanisms, such as cannibalism (Blackshaw & Coll, 1999; Blackshaw & Petrovskii, 2007).

3.4.3. Interspecific interactions across sampling scales

Whereas at large scales species-specific habitat requirements or environmental factors are likely to affect species distribution in relation to each other, at finer scales the effects of competition and predation may have more of an impact (Wiens, 1989). Furthermore, each taxon may have its own characteristic scale. As a result, interactions between two species could depend on the area over which it is measured (Sandel & Smith, 2009). The close association of *D. febrilis* and *B. johannis* with leatherjackets observed at the site and core scales (Figure 3.1) has been detected in previous studies (D'Arcy-Burt, 1987), but here the dissociation of *D.febrilis* from these taxa at the field

scale contradicts this. It has been noted that patches of damage caused by *Bibio*

species often occur near hedgerows and are related to the occurrence of adult mating

swarms, suggesting larval distribution is determined by female behaviour. However,

damage associated with *D. febrilis* is more widely distributed throughout a field

(D'Arcy-Burt, 1987; D'Arcy-Burt and Blackshaw, 1991), indicating possible differences

in species feeding and/or habitat preferences. Likewise, similarities in biology, for

example moisture preferences, may explain the similar distribution of Sciaridae and

leatherjackets. The dissociation of wireworm species (apart from *A. sputator*) from the

bibionids at the core scale may be related to the effects of predation; it has previously

been noted that in captivity *Agriotes* spp. attack and kill bibionid larvae (Andrianov,

1914). Published studies on species-specific wireworm distributions are lacking,

however the results in Chapter 2 suggest there may be associations at the field scale

between some species and not others, possibly linked to differences in abiotic

variables between fields. This reiterates the importance of identifying individual

species and not grouping related organisms together, as has commonly been the case

with the *Agriotes* 'pest complex'.

The few taxa sampled here represent only a small proportion of the total biodiversity

in soil. Although competitive interactions may contribute to variation in species'

distributions, predators also play a role. There are known to be large numbers of

predators which feed on soil taxa, but their effect on populations in the field is little

known (Giller, 1996), with soil food web models concentrating on detritus food webs,

despite the fact that root herbivores are important in soil structure and transport

processes (Brussaard, 1998). Depending on the questions being addressed, another

way to describe interactions between soil biota may be through the use of functional

groups, using ecological rather than morphological similarities (Giller, 1996). This would overcome the problem of lack of species differentiation in studies on soil communities. However, although this may be appropriate for studies on ecosystem processes and the roles of invertebrates in maintaining soil fertility and decomposition, for example, for others a more detailed approach is needed. In particular, for pest management and control information on species-specific interactions is important for developing selective methods that will target pest species.

Chapter 4   Species identification, genetic diversity and phylogenetic relationships

of economically important Elaterid species

4.1 Introduction

Early distributional studies of wireworm species reported on their relative abundance

across the UK based on adult data (Anon, 1948; Roberts, 1919), but although species

lists (Duff, 2008) and maps for the Elateridae (Mendel & Clarke, 1996) are available,

there is little recently published data on adult and wireworm species found in arable

land around the country. More recently, molecular methods of wireworm

identification have proved useful for assessing the relationship between above-

belowground life stages and species distribution (e.g. using mitochondrial DNA

(mtDNA) based T-RFLP; Chapter 2 and Chapter 3). Indeed, the large proportion of

other species of wireworm found in the same population as *A. obscurus, A. lineatus*

and *A. sputator* (and the lack of *A. lineatus*) in Chapter 2 raises questions about

whether the wireworm pest complex in the UK is made up of only three *Agriotes*

species. Although these other species make up a small number of the wireworms

trapped in the study in Chapter 3, during three years of using T-RFLP (Ellis et al., 2009)

to identify wireworm samples, further 'non-*Agriotes*' wireworms have also been found

in soil core samples from Cambridge. Other species of wireworm are known to damage

crops e.g. *Athous* Eschscholtz spp. (Anon, 1948; Roberts, 1919) and *Ctenicera cuprea*

Fabricius (Anon, 1948; Broadbent, 1946; Edwards & Evans, 1950), but other genera of

unknown pest status also occur in agricultural land e.g. *Adrastus* Eschscholtz, *Dalopius*

Eschscholtz, *Hypnoidus* Dillwyn, *Selatosomus* Stephens and *Prosternon* Latreille (Parker

& Howard, 2001).

In addition to the identification of cryptic life-stages, mtDNA is particularly suitable for studying closely related species in phylogenetic analyses, as well as population subdivision, geographic and species boundaries (Hillis, Mable & Moritz, 1996) due to its haploid status and high rate of evolution (Behura, 2006). In insects, the ability to readily amplify loci using universal primers for highly conserved mitochondrial genes is also an advantage (Behura, 2006). Studies using mtDNA have been undertaken on the evolution of bioluminescence and neoteny in the superfamily Elateroidea using COI and 28S rRNA (Bocakova et al., 2007), and on the evolution of bioluminescence in Elateridae using 28S rRNA (Sagegami-Oba et al. 2007). Mitochondrial DNA has also been used to assess relationships within Elaterid sub-families using 16S rRNA (Vahtera, Muona & Lawrence, 2009) and cytochrome oxidase I (COI) has been used to assess the relationships between economically important species in the genus *Melanotus* Eschscholtz in the Midwestern United States (Lindroth & Clark 2009) and between nine of the most abundant *Agriotes* larvae in Central Europe (Staudacher *et al.*, 2011).

However, considered as a whole, published research on the classification and phylogenetics of Elateridae worldwide, for both molecular and morphological data, is lacking. For example, in Canada there are around 30 economically important species, including *A. obscurus* and *A. lineatus* which were introduced from Europe approximately a century ago and are now serious crop pests (Vernon, 2004), as well as those in the genus *Ctenicera* Latreille, *Limonius* Eschscholtz, *Melanotus* and *Hypnoidus* Dillwyn (Vernon et al., 2010). One such species, *Hypnoidus bicolor* Eschscholtz, is emerging as a major agricultural pest (W. Van Herk, pers. comm.) but little is known of its biology and ecology. As is the case in the UK and Europe, there are a number of complexes comprising species that are morphologically difficult to identify, but for

which DNA sequence data is lacking. Knowledge of these species, and an efficient method of identification, would be useful for future studies in assessing relationships between wireworm pest and non-pest species, allowing more accurate investigation into species' ecology and effective measures of control (Lindroth & Clark, 2009). Similarly, knowledge of population genetic structure and phylogeography of elaterid species is also lacking, if not completely absent (at least in published form). Yet, understanding these aspects of population structure could yield useful insights into the management of species (for example population genetic studies could provide estimates of the scale over which gene flow occurs (e.g. Krafsur & Endsley, 2002; Ravel *et al.*, 2001; Schultheis, Weigt & Hendricks, 2002).

Since sequencing the 16S rRNA region of mitochondrial DNA had already been carried out successfully in previous studies (Chapter 2 and Chapter 3), this region was also used for the current study.

The objectives of this study were:

1. To identify the non-*Agriotes* wireworms trapped in agricultural fields in Devon (described in Chapters 2 and 3), and other samples obtained in the UK

2. To assess intra- and interspecific genetic variation in Canadian wireworm species, focusing on the relationship between genetic and geographic distance in *H. bicolor* which may give some insight into the genetic structure of populations and distribution of haplotypes over varying geographic distances.

3. To investigate the phylogenetic relationships between all Elateridae species for which 16S rRNA sequence data is available

4.2 Methods

*4.2.1 UK click beetle species and identification of non-*Agriotes *wireworms*

Non-*Agriotes* wireworms had been previously identified in the agricultural landscape by terminal-restriction fragment length polymorphism (T-RFLP) and subsequent sequencing (Chapters 2 & 3). However, the last abdominal segment, a key identification feature, (the whole sample for small wireworms or repeated DNA extractions) had been used in DNA isolation for most of these samples. Consequently, to try and identify matching 16S rRNA sequences, further adult samples or whole non-*Agriotes* wireworms were collected from sites within the UK by searching grassland areas and placing pitfall and forage traps in agricultural fields (Table 4.1). Some non-*Agriotes* species were also trapped in *Agriotes* sex pheromone traps. Species were identified morphologically by Darren Mann, Oxford Museum of Natural History (except *Melanotus villosus* Geoffroy, which was supplied already identified by Duncan Allen, Plymouth City Museum and Art gallery, but was checked by myself). DNA extraction, PCR and sequencing were carried out as outlined in Chapter 2.2.3. Full sequences could not be obtained for some species due to ineffective sequencing using the reverse primer. Although several attempts were made to clean these sequences using gel extraction of DNA and PCR products, Exo-SAP IT (Affymetrix, Inc., Ohio) for PCR products, and the use of new reagents and primers, in these cases only forward sequences were obtained.

Species	Location	GenBank Accession number
Agriotes acuminatus Stephens	Newton Abbot, Devon	-
Agriotes pallidulus Illiger	Okehampton, Devon	-
Athous campyloides Newman	Newton Abbot, Devon	-
Selatosomus aeneus Linnaeus	Dartmoor, Devon	-
Melanotus villosus Geoffroy	Plymouth, Devon	-
Ctenicera cuprea Fabricius	Dartmoor, Devon	-
Hemicrepidius hirtus Herbst	Cambridge	-
Adrastus pallens Fabricius	Cambridge	-
Agriotes lineatus Linnaeus	GenBank	EU285481
Agriotes obscurus Linnaeus haplo 1	GenBank	EU285484
Agriotes obscurus Linnaeus haplo 2	GenBank	EU285482
Agriotes obscurus Linnaeus haplo 3	GenBank	EU285483
Agriotes sputator Linnaeus haplo 1	GenBank	EU285485
Agriotes sputator Linnaeus haplo 2	GenBank	EU285480
Agriotes sordidus Illiger	GenBank	HM990670
Athous haemorrhoidalis Fabricius	GenBank	DQ198648
Stenagostus rhombeus Olivier	GenBank	DQ198653
Agrypnus murinus Linnaeus	GenBank	DQ198645
Ampedus balteatus Linnaeus	GenBank	DQ198646
Aplotarsus incanus Gyllenhal	GenBank	DQ198647
Denticollis linearis Linnaeus	GenBank	DQ198651
Panspaeus guttatus Sharp	GenBank	DQ198652
Selatosomus cruciatus Linnaeus	GenBank	EU128148

Table 4.1 UK click beetle species available for sequencing and/or alignment with the unidentified non-*Agriotes* wireworms

Forward and reverse sequences were edited and contiguous sequences ('contigs') produced using the contig assembly programme (CAP) in BioEdit v. 7.0.9.0 (Hall, 1999; Huang, 1992). Sequences were aligned using Clustal W (Thompson, Higgins & Gibson, 1994) with the non-*Agriotes* sequences and other UK species obtained from GenBank (Table 4.1). Genetic distance within and between groups of non-*Agriotes* species and all other available UK species were estimated by *P*-distances, since for closely related

sequences this measure is similar to more complex distance measures such as Tamura-Nei, but has a smaller variance (Nei & Kumar, 2000).

4.2.2 Intra and interspecific genetic variation in Canadian wireworm species

Wireworm samples were collected from 45 sites in Canada and one site in the USA (Figure 4.1) in 2010, preserved in 100% ethanol and identified morphologically to species (where possible) by W. Van Herk (Agriculture and Agri-Food Canada, Agassiz, BC, Canada). Although 400 individuals were available in total, only 139 (including representatives from each species in each location) were selected for the present study due to resource and time limitations. DNA extraction, PCR and sequencing were carried out as per section 2.2.3 in Chapter Two. Forward sequences were obtained for 109 samples (30 sequences contained too much missing data to use reliably in further analyses), and reverse sequences were also obtained for 31 individuals from each species in each location in order to analyse phylogenetic relationships across all species. Where applicable, forward and reverse sequences were edited and contiguous sequences ('contigs') produced as for the UK species (section 4.2.1).

Location	Map number
Dawson Creek, BC	1
Kamloops, BC	2
Oliver, BC	3
Boardman, Oregon	4
Manning, AB	5
Fairview, AB	6
Tangent, AB	7
Wembley, AB	8
Neerlandia, AB	9
Fort Sasktachewan, AB	10
Vegreville, AB	11
Drumheller, AB	12
Calgary, AB	13
Parkland, AB	14
Claresholm, AB	15
Fort Macleod, AB	16
Lethbridge, AB	17
Taber, AB	18
Meota, SK	19
North Battleford, SK	20
Domremy, SK	21
Wakaw, SK	22
Saskatoon, SK	23
Milden, SK	24
Drake, SK	25
Gull Lake, SK	26
Swift Current, SK	27
Strasbourg, SK	28
Moose Jaw, SK	29
Avonlea, SK	30
Drinkwater, SK	31
Indian Head, SK	32
Montmartre, SK	33
Fillmore, SK	34
Inglis, MB	35
Ste Rose, MB	36
Elkhorn, MB	37
Brandon, MB	38
Teulon, MB	39
Boissevain, MB	40
Altona, MB	41
Ridgetown, ON	42
Parkhill, ON	43
Brussels, ON	44
Orangeville, ON	45
Le Gardeur, QC	46

Figure 4.1 Sampling locations of Canadian wireworm species. The coloured markers represent the state (yellow = British Columbia, BC; red = Alberta, AB; Turquoise = Oregon, USA; green = Saskatchewan, SK; blue = Manitoba, MB and purple = Ontario, ON), and numbers represent towns.

Pairwise *P*-distance was calculated using MEGA 4 (Tamura et al., 2007) to assess the intraspecific genetic distance between individuals. The number of haplotypes (H_N), haplotype diversity (H_d) and nucleotide diversity (π) for each species was calculated using DnaSP v.5 (Librado & Rozas, 2009). The relationship between geographic and genetic distance was further investigated in *Hypnoidus bicolor* (N=40) via the Mantel test using the isolation by distance web service (IBDWS; Jensen, Bohonak & Kelley, 2005) with PhiST as a measure of genetic distance (1000 randomisations). Missing data and gaps were ignored and negative distances were set to zero. A minimum spanning network (MSN), which aims to construct the shortest possible tree of haplotypes, was calculated using Network (fluxus-engineering.com; Bandelt, Forster & Röhl, 1999), with a median joining (MJ) network algorithm and default epsilon (a weighted genetic distance measure).

4.2.3 Construction of phylogenetic trees

Three types of phylogenetic tree were constructed using a distance based method (Neighbour Joining, NJ), and two character based methods (Maximum Parsimony, MP, and Maximum Likelihood , ML) to determine the likely phylogenetic relationships for species in each data set:

1. All UK species and the 'non-*Agriotes*' wireworms

2. All Canadian wireworm species, using all forward sequence data

3. All Elateridae species for which 16S rRNA sequence data were available

Neighbour joining, maximum parsimony and maximum likelihood are just three of a number of methods used for phylogenetic inference. It has been suggested that NJ should only be used as a starting tree for further searches using branch swapping

under minimum evolution or additive-tree criteria (Swofford et al., 1996), but selecting a method depends upon a set of criteria including efficiency, robustness and computational speed, as well as the data under study (Hall, 2004). These methods are commonly used in phylogenetic studies (including those carried out for other Elateridae; Lindroth & Clark, 2009; Sagegami-Oba, Oba & Ôhira, 2007; Staudacher *et al.*, 2011). 16S rRNA sequence data for all other elaterid species not sequenced directly in this study were downloaded from GenBank (Table 4.2) and used to construct trees for all Elateridae species in addition to those in Table 4.1. Problems were encountered in selecting a suitable outgroup. Species from the 'false click beetle' family Eucenemidae, which is closely related to the Elateridae in the superfamily Elateroidea, have been used in other studies (e.g. Vahtera, Muona & Lawrence, 2009) and were trialled as outgroups in this study initially, but they were incorporated into the tree. Similar problems occurred in the trees of all Elaterid species with the *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) outgroup, which is more distantly related to the Elateridae, but when rooted manually this was positioned correctly and so was chosen as an outgroup for all trees, as per Lindroth and Clark (2009).

All trees were constructed in MEGA5 (Tamura et al., 2011). For the NJ tree *P*-distance was used as a measure of genetic distance and was tested by bootstrap with 1000 replicates. The complete deletion option was chosen for missing data, substitutions included transitions and transversions and the pattern among lineages was homogenous with uniform rates among sites.

MP tree support was tested by bootstrapping with 1000 replicates, and the additional options of Close-Neighbour-Interchange (CNI) with search level 1 with 100 random addition trees were chosen (to produce the initial tree to search against).

Prior to constructing ML trees JModelTest (Posada, 2008) was used to find the most appropriate model of nucleotide substitution (selected using AIC criterion); TIM3+G for data set 1, GTR+G for data set 2 and TIM3+G for data set 3. A parsimony tree was built using a heuristic search as an initial tree. Tree-bisection-reconnection (TBR) was used as a branch swapping algorithm. Tree support was tested using bootstrap (500 replicates due to time constraints).

Condensed NJ, MP and ML trees were computed with a cut-off value of 50% (i.e. any branches with a bootstrap support of <50% were collapsed).

Species	Accession no.	Reference
Acteniceromorphus kurofunei Miwa	AB375497	Sagegami-Oba et al. (2008)
Actenicerus aerosus Lewis	AB375485	Sagegami-Oba et al. (2008)
Actenicerus athoides Kishii	AB375486	Sagegami-Oba et al. (2008)
Actenicerus giganteus Kishii	AB375487	Sagegami-Oba et al. (2008)
Actenicerus kiashianus Miwa	AB375488	Sagegami-Oba et al. (2008)
Actenicerus kidonoi Ohira	AB375489	Sagegami-Oba et al. (2008)
Actenicerus naomii Kishii	AB375491	Sagegami-Oba et al. (2008)
Actenicerus odaisanus Miwa	AB375492	Sagegami-Oba et al. (2008)
Actenicerus orientalis Candèze	AB375493	Sagegami-Oba et al. (2008)
Actenicerus pruinosus Motschulsky	AB375495	Sagegami-Oba et al. (2008)
Actenicerus suzukii Miwa	AB375494	Sagegami-Oba et al. (2008)
Actenicerus yamashiro Kishii	AB375496	Sagegami-Oba et al. (2008)
Alaus oculatus Linnaeus	DQ402091	Unpublished
Balgus eschscholtzi Laporte	EU128165	Vahtera et al. (2009)
Balgus obconicus Bonvouloir	EU128160	Vahtera et al. (2009)
Balgus rugosus Blanchard	EU128159	Vahtera et al. (2009)
Balgus schnusei Heller	EU128167	Vahtera et al. (2009)
Corymbitodes gratus Lewis	AB375498	Sagegami-Oba et al. (2008)
Corymbitodes rubripennis Lewis	AB375499	Sagegami-Oba et al. (2008)
Cussolenis attenuatus Fleutiaux	EU128156	Vahtera et al. (2009)
Cussolenis curtus Fleutiaux	EU128154	Vahtera et al. (2009)
Cussolenis mutabilis Bonvouloir	EU128153	Vahtera et al. (2009)
Pterotarsus histrio Guerin	EU128150	Vahtera et al. (2009)
Pyrophorus divergens Eschscholtz	EF398270	Arnoldi et al. (2007)
Thylacosternus nigrinus Bonvouloir	EU128169	Vahtera et al. (2009)
Diabrotica virgifera virgifera LeConte	AY533631	Outgroup

Table 4.2 Elateridae species with GenBank accession numbers used in phylogenetic analyses of all available 16S rRNA sequences.

4.3 Results

4.3.1 UK species

4.3.1.1 Proportions and abundance of UK wireworm species

Of all the wireworms identified throughout the study period, *A. obscurus* were the most abundant (111 individuals), followed by *A. sputator* (73 individuals) and *A. lineatus* (39 individuals). Twenty seven non-*Agriotes*, four *Hemicrepidius hirtus* Herbst,

two *Athous haemorrhoidalis* Fabricius and one *Adrastus pallens* Fabricius wireworm(s) were also present (Figure 4.2).

Pitfall and forage traps in agricultural fields failed to capture any click beetle species, and all those used in the study were found by physically searching in grassland areas, obtained in *Agriotes* sex pheromone traps or from other researchers.

Of the UK species collected as adults or wireworms, only the reverse sequence was successfully obtained for *Agriotes acuminatus* Stephens, and the DNA extracted from *Adrastus pallens* was insufficient for subsequent successful PCR. These species were therefore not included in subsequent analyses.
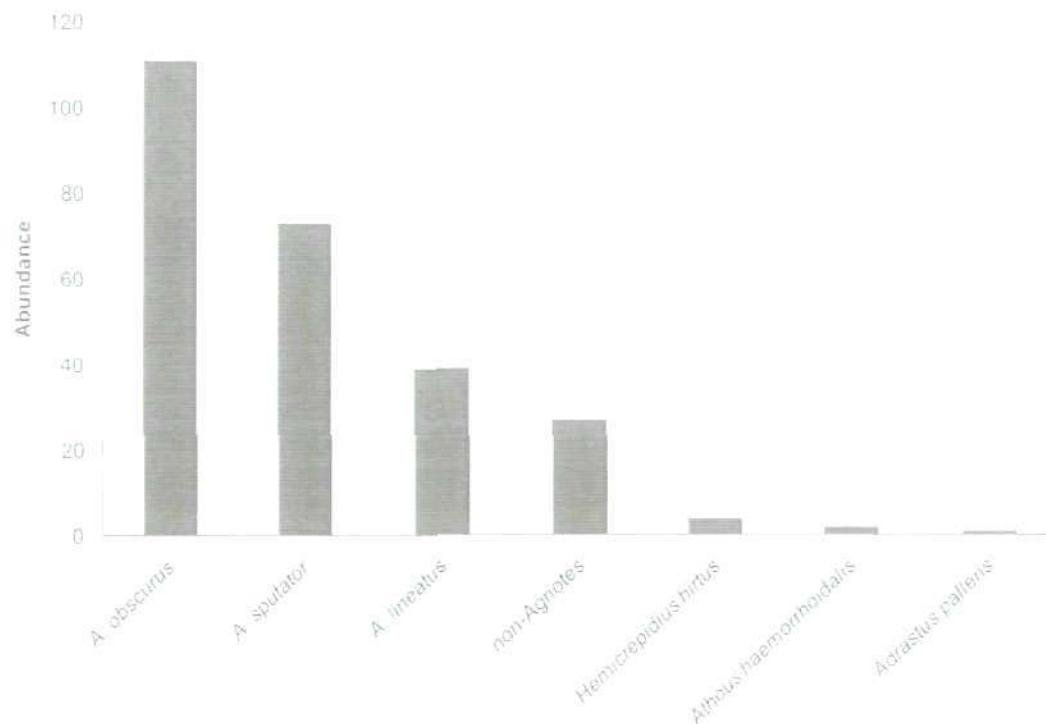
Figure 4.2 The abundance of each wireworm species from all successful identifications using T-RFLP and sequencing the 16S rRNA region.

The proportion of wireworm species differed between sampling location, even in sites relatively close to each other e.g. South Hams and Newton Abbot in Devon (Figure 4.3).
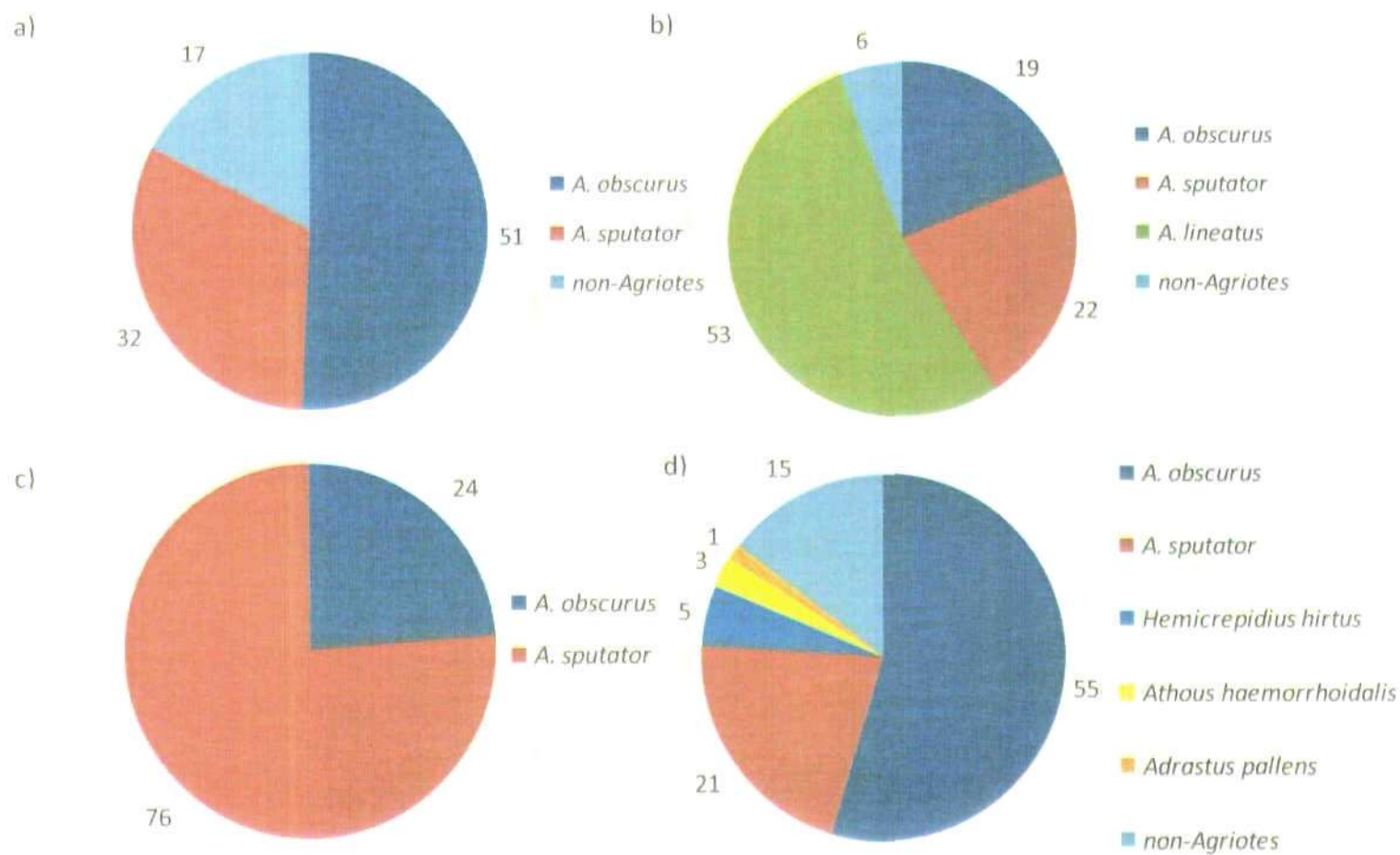
Figure 4.3 The proportion (%) of wireworm species trapped (the numbers next to pie sections) in a) South Hams, Devon, b) Newton Abbot, Devon, c) Somerset and d) Cambridge.

4.3.1.2 *Identification of non-*Agriotes *wireworms*

Three groups of non-*Agriotes* wireworms were identified. Group 1 consisted of 20 individuals belonging to three haplotypes (haplotype 1, 18 individuals; haplotype 2, 1 individual, haplotype 3, 1 individual). Group 2 contained 4 identical individuals (following the removal of sites containing gaps/missing data), and group 3 contained 3 identical individuals (following the removal of sites containing gaps/missing data; Appendix 2). Individuals in non-*Agriotes* group 3 were only found in Newton Abbot, Devon, but individuals in non-*Agriotes* groups 1 and 2 were found in the South Hams, Devon, and Cambridge.

The percentage difference between sequences in non-*Agriotes* groups 1, 2 and 3 ranged from 8.9 to 22.5% (Table 4.3) and alignments revealed several substitutions (Figure 4.4).

	1	2	3
Group 1		*2.6*	*1.8*
Group 2	22.5		*2.6*
Group 3	8.9	22	

Table 4.3 The percentage base differences per site from averaging over all sequence pairs (*p*-distance) between the three groups of non-*Agriotes* wireworms. Standard error estimates are shown above the diagonal in italics and were obtained by a bootstrap procedure (1000 replicates) in MEGA4. Gaps and missing data were eliminated from the dataset (complete deletion option).

a)

Group 1 Haplo 1	ATTTTTGAATTTTACTTCTGTAAAAGCTTAGATATTTTTTAAAGACGAGAAGACCCCAAGAGTTTTATGTTTACTAATAT
Group 1 Haplo 2	ATTTTTGAATTTTACTTCTGTAAAAGCTTAGATATTTTTTAAAGACGAGAAGACCCCAAGAGTTTTATGTTTACTAATAT
Group 1 Haplo 3	ATTTTTGAATTTTACTTTTGTAAAAGCTTAGATATTTTTTAAAGACGAGAAGACCCCAAGAGTTTTATGTTTACTAATAT
Group 2	TTTTTTGAATTTAACTTTTAGTAAAAGCTTTAATTTAAATTAAAGACGAGAAGACCCTAAGAGTTTAATAT-TATTTTTGT
Clustal Consensus	* *

Group 1 Haplo 1	AAAGTTTTTGGTTATTTGATTTTTTTATATTTAAATATTTGGTTGGGGGACTAAGATTAAATAAACTCTTAATTTTTAAT
Group 1 Haplo 2	AAAGTTTTTGGTTATTTGATTTTTTTATATTTAAATATTTGGATGGGGGACTAAGATTAAATAAACTCAAAATTTTTAAT
Group 1 Haplo 3	AAAGTTTTTGGTTATTTGATTTTTTTATATTTAAATATTTGGTTGGGGGACTAAGATTAAATAAACTCTTAATTTTTAAT
Group 2	AAGTTTATTTGGAATTTTTTATTTATTTTATTTAATATTTGGTTGGGGGACTAAGATTTAATAAACTCTTTTTTTTTAAAT
Clustal Consensus	* *

b)

Group 1 Haplo 1	ATTTTTGATTTTACTTCTGTAAAAGCTTAGATATTTTTTAAAGACGAGAAGACCCCAAGAGTTTTATGTTTACTAATATA
Group 1 Haplo 2	ATTTTTGATTTTACTTCTGTAAAAGCTTAGATATTTTTTAAAGACGAGAAGACCCCAAGAGTTTTATGTTTACTAATATA
Group 1 Haplo 3	ATTTTTGATTTTACTTTTGTAAAAGCTTAGATATTTTTTAAAGACGAGAAGACCCCAAGAGTTTTATGTTTACTAATATA
Group 3	ATTATTGATTTTACTTCTGTAAAAGCTTAGATTTTTTTTAAAGACGAGAAGACCCCAAGAGTTTTATATTTTTTTATATA
Clustal Consensus	* *

Group 1 Haplo 1	AAGTTTTTGGTTATTTGATTTTTTTATATTTAAATATTTGGTTGGGGGACTAAGATTAAATAAACTCTTAAAATTTTAAT
Group 1 Haplo 2	AAGTTTTTGGTTATTTGATTTTTTTATATTTAAATATTTGGATGGGGGACTAAGATTAAATAAACTCAAAAAATTTTAAT
Group 1 Haplo 3	AAGTTTTTGGTTATTTGATTTTTTTATATTTAAATATTTGGTTGGGGGACTAAGATTAAATAAACTCTTAAAATTTTAAT
Group 3	TAGTTTTTGGTTATTGGATTTTCTTATATTTAGATATTTAGTTGGGGGACTAAGATTGGATTAACTCTTAAAGTTTAAAT
Clustal Consensus	* *

c)

Group 2	TTTCTGTCTCTTTAATTTTTTTTTGATTTAACTTTAAAGTAAAAAGGCTTTAATTTAAATTAAAGACGAGAAGACCCTAT
Group 3	TTTCTGTCTCTTGAATTTATTATTGATTTTACTTCTAAGTTAAAAGGCTTAGATTTTTTTTAAAGACGAGAAGACCCCAT
Clustal Consensus	* *

Group 2	AGAGTTTAATATTATTTTTGTAATGATTTTTTGGAATTTTTTATTTATTTTATTTTTAATATTTGGTTGGGGTGACTAAA
Group 3	AGAGTTTTATATTTTTTTTATATTAAGTTTTTGGTTATTGGATTTTCTTATATATTTAATATTTAGTTGGGGTGACTTAA
Clustal Consensus	* *

Figure 4.4 Partial alignments of a) non-*Agriotes* group 1 (3 haplotypes) and group 2 b) group 1 and group 3 and c) group 2 and group 3, with clustal consensus (gaps and missing data have been removed).

4.3.1.3 Genetic distance and phylogeny of UK species

No exact matches were found when each of the three groups of non-*Agriotes* species was aligned with all other available UK click beetle sequences (26 in total), suggesting they are other species that have not yet been sequenced at the 16S rRNA region. Due to the short sequence length of some of the non-*Agriotes* species only 180 positions could be analysed in total for *p*-distance and constructing phylogenetic trees. However, even for the relatively short sequence lengths, pairwise divergence was up to 26% (excluding *Athous campyloides* which had an unusually high divergence of 56% and so was excluded from further analyses (see discussion in section 4.4.3; Table 4.4). The NJ (Figure 4.5) and MP (Figure 4.6) trees were similar, grouping the *Agriotes* sequences with each other, whereas in the ML tree (Figure 4.7) although *A. obscurus* and *A. sputator* haplotypes were grouped together other *Agriotes* species were not. Non-*Agriotes* groups 1 and 3 are grouped together in all trees, and in the same clade as *D. linearis* and *A. haemorrhoidalis* in the NJ and MP trees. There was lower support for the ML tree than the NJ and MP trees.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1 *Agriotes lineatus*		1	1	1	2	2	2	4	2	3	2	3	3	2	3	3	2	2	2	2	2	2	3	2	2	3
2 *Agriotes obscurus* haplo 1	2		0	0	2	2	2	4	2	3	3	3	3	3	3	3	3	2	2	2	3	2	2	2	2	3
3 *Agriotes obscurus* haplo 2	2	0		0	2	2	2	4	2	3	3	3	3	3	3	3	3	2	2	2	3	2	2	2	2	3
4 *Agriotes obscurus* haplo 3	2	0	0		2	2	2	4	2	3	3	3	3	3	3	3	3	2	2	2	3	2	2	2	2	3
5 *Agriotes sputator* haplo 1	6	6	6	6		1	1	4	2	3	3	3	3	3	3	3	2	2	2	2	3	2	3	2	2	3
6 *Agriotes sputator* haplo 2	7	6	6	6	1		1	4	2	3	3	3	3	3	3	3	2	2	2	2	3	2	3	2	2	3
7 *Agriotes sordidus*	7	6	6	6	2	2		4	2	3	3	3	3	2	3	3	2	2	2	3	3	2	2	2	2	2
8 *Agriotes pallidulus*	7	7	7	7	7	7	7		4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2
9 *Athous campyloides*	53	53	53	53	53	53	53	52		4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
10 *Athous haemorrhoidalis*	16	16	16	16	16	16	18	52	20		3	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3
11 *Stenagostus rhombeus*	15	15	15	15	14	14	13	54	17	19		3	3	3	2	3	3	3	3	3	3	3	3	2	3	3
12 *Agrypnus murinus*	15	15	15	15	17	17	16	52	17	19	20		3	3	3	3	3	3	3	3	3	3	3	3	3	3
13 *Ampedus balteatus*	16	16	16	16	17	16	17	52	15	24	21	20		3	3	3	3	3	3	3	3	3	3	3	3	3
14 *Aplotarsus incanus*	14	14	14	14	14	14	13	52	17	21	17	15	18		3	3	3	3	3	2	3	3	3	3	3	3
15 *Denticollis linearis*	15	16	16	16	15	15	15	54	18	11	14	21	19	20		3	3	3	2	3	3	2	2	2	3	3
16 *Panspaeus guttatus*	17	16	16	16	16	16	17	51	17	19	21	20	18	18	20		3	3	3	3	3	3	3	3	3	3
17 *Selatosomus cruciatus*	13	15	15	15	13	13	12	49	16	17	17	16	19	13	14	19		3	2	3	3	3	3	3	3	3
18 *Ctenicera cuprea*	12	12	12	12	11	11	12	52	13	16	17	13	17	13	18	16	15		2	2	3	3	3	3	3	3
19 *Hemicrepidius hirtus*	12	13	13	13	13	13	12	51	13	16	15	17	17	14	10	17	12	11		3	3	2	3	2	3	3
20 *Melanotus villosus*	12	12	12	12	12	12	13	53	15	19	17	15	16	7	19	17	13	11	14		3	3	3	3	3	3
21 *Selatosoma aenea*	13	15	15	15	15	15	14	53	16	17	20	16	19	16	15	22	15	15	13	14		3	3	3	3	3
22 Non-*Agriotes* gp 1 haplo 1	12	11	11	11	13	13	12	55	15	13	14	16	21	16	11	21	17	15	12	15	16		1	1	3	2
23 Non-*Agriotes* gp 1 haplo 2	14	13	13	13	15	15	13	55	17	15	16	17	22	17	12	23	19	17	14	16	17	2		1	3	2
24 Non-*Agriotes* gp 1 haplo 3	12	11	11	11	13	13	11	54	15	13	13	16	20	15	10	21	17	15	12	15	16	1	2		3	2
25 Non-*Agriotes* gp 2	10	11	11	11	11	12	12	54	11	20	17	18	17	15	19	19	17	13	16	13	15	16	18	16		3
26 Non-*Agriotes* gp 3	17	16	16	16	15	15	13	56	14	16	17	20	23	20	13	21	19	18	13	18	17	7	9	8	18	

Table 4.4 Pairwise *P*-distance (%) of all UK species used in the phylogenetic analysis
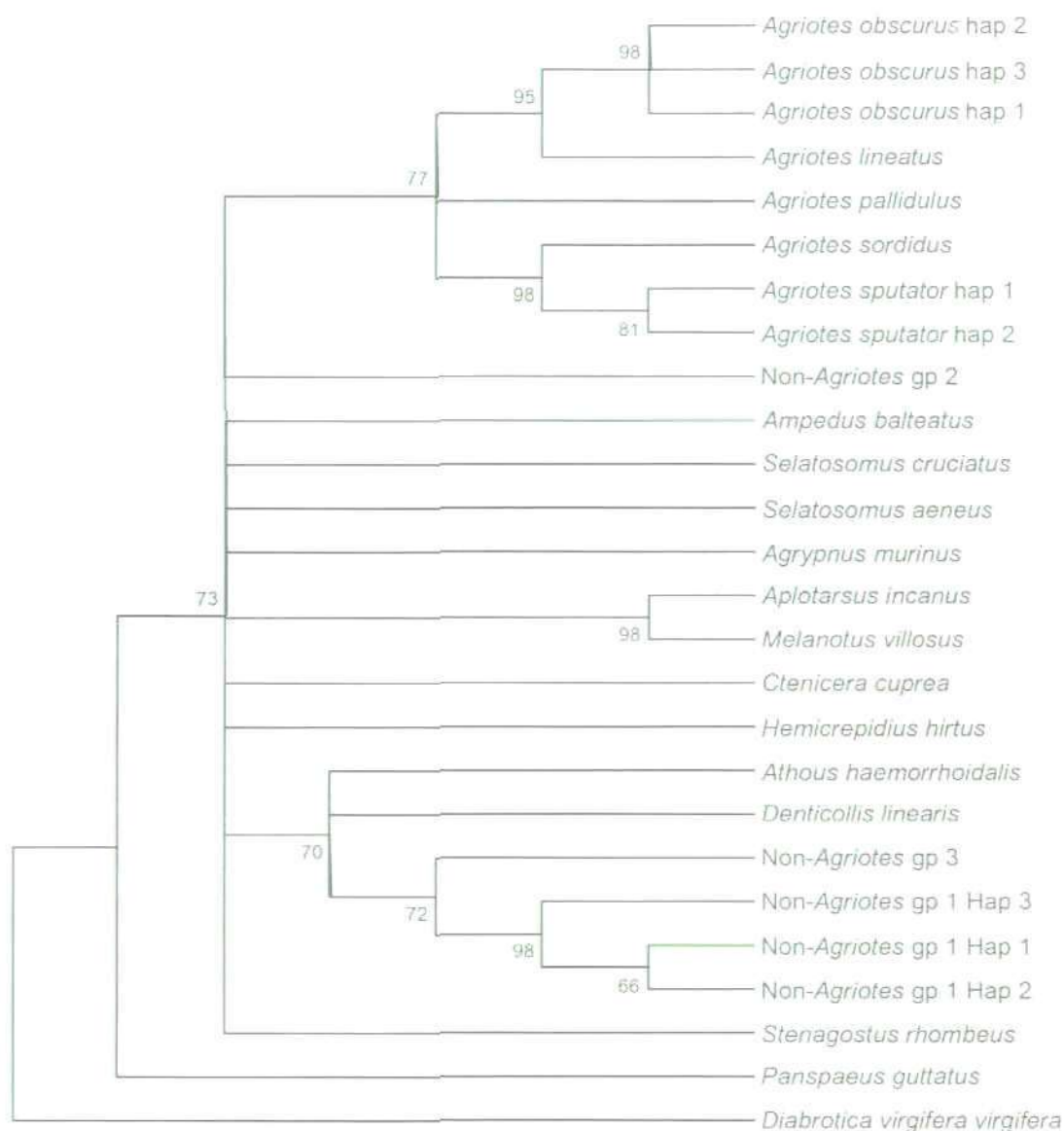
Figure 4.5 Neighbour joining tree, using *p*-distance, of 16S rRNA sequences of UK click beetle species and unidentified non-*Agriotes* wireworms. Bootstrap support values (1000 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. There were a total of 180 positions in the final dataset (all positions containing gaps and missing data were eliminated).
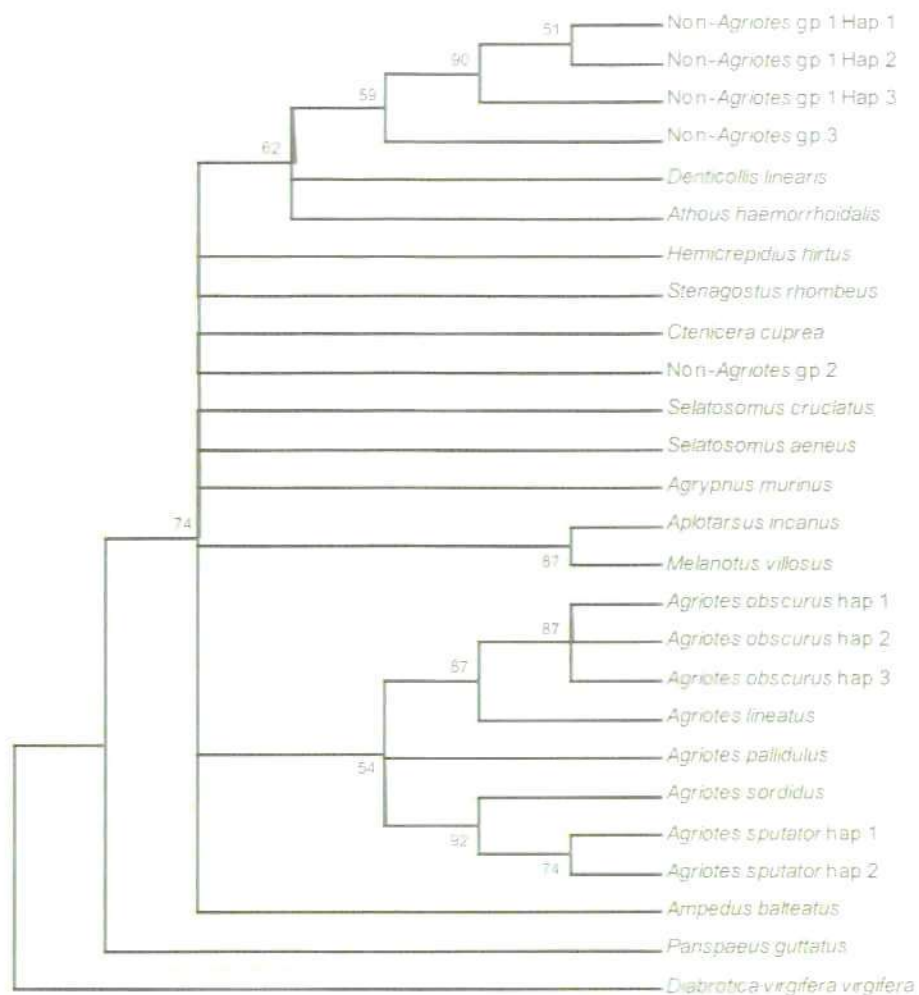
Figure 4.6 Maximum parsimony tree of 16S rRNA sequences of UK click beetle species and unidentified non-*Agriotes* wireworms. Bootstrap support values (1000 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree was obtained using Close-Neighbour-Interchange with search level 1 in which the initial trees were obtained with the random addition of sequences (100 replicates). All positions containing gaps and missing data were eliminated. There were a total of 180 positions in the final dataset, of which 62 were parsimony informative.

Figure 4.7 Maximum likelihood tree of 16S rRNA sequences of UK click beetle species and unidentified non-*Agriotes* wireworms using the TIM3+G model. Ln*L* = -320442.69. Bootstrap support values (500 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

4.3.2 Canadian species

4.3.2.1 Sequence data

The majority of the samples identified morphologically as the same species aligned well when sequences were compared, but there were some discrepancies. The *A. obscurus* individual did not align with the published *A. obscurus* sequences, and had high pairwise genetic distance when compared to *A. obscurus* (5%) and the other *Agriotes* species from the UK and Canada (6-10%; Table 4.5), suggesting there is an error with the sequence and/or identification. Similar issues were noted for the *Ctenicera* species in which there were low genetic distances between most *C. aeripennis aeripennis* and *C. destructor* samples, suggesting these are in fact one species, but one sample had a 12% distance to all others (Table 4.6). The *C. pruinina* sample from Oregon, US, was also highly different to those from Orangeville, Canada. When included in the phylogenetic analysis, sequences from these species were not grouped together as for other species, but were spread throughout the tree in different clades (Appendix 3). When these samples were removed the trees produced were more realistic (samples from the same species were grouped together). Therefore, these samples were excluded from further analyses. There were also high genetic distances between some *Limonius californicus* samples (up to 18%; Table 4.7) and *Hemicrepidius* spp. (13.7%), which were also removed from further analyses. Possible reasons for these results, including mis-identification and the presence of nuclear mitochondrial inserts (numts), are discussed in section 4.4.4.

	1	2	3	4	5	6	7	8	9	10	11	12
1. *A. obscurus* Canadian		1	1	1	1	2	2	2	2	2	1	2
2. *Agriotes lineatus*	5		1	1	1	2	2	2	2	2	2	2
3. *Agriotes obscurus* haplo 1	5	3		0	0	2	2	1	2	2	1	2
4. *Agriotes obscurus* haplo 2	5	3	0		0	2	2	1	2	2	1	2
5. *Agriotes obscurus* haplo 3	5	3	0	0		2	2	1	2	2	1	2
6. *Agriotes sputator* haplo 1	7	7	6	6	6		0	1	1	2	2	2
7. *Agriotes sputator* haplo 2	7	7	7	7	7	0		1	1	2	2	2
8. *Agriotes sordidus*	6	7	6	6	6	2	2		2	2	2	2
9. *Agriotes pallidulus*	7	9	7	7	7	6	6	7		2	1	2
10. *Agriotes criddlei*	10	11	12	12	12	9	9	9	12		2	1
11. *Agriotes mancus*	7	8	6	6	6	7	7	7	7	10		2
12. *Agriotes stabilis*	10	12	13	13	13	10	10	10	11	2	11	

Table 4.5 *P*-distance (%) between the Canadian '*Agriotes obscurus*' sequence and other UK and Canadian *Agriotes* sequences. Standard error estimates are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Gaps and missing data were eliminated.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *C. aeripennis aeripennis* Tangent		*0*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*1.9*	*0.5*	*0.5*	*0.5*	*0.5*	*1.8*	*1.8*	*1.6*
2. *C. aeripennis aeripennis* Tangent	0		*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	*1.9*	*0.5*	*0.5*	*0.5*	*0.5*	*1.8*	*1.8*	*1.6*
3. *C. aeripennis aeripennis* Fairview	0.7	0.7		*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
4. *C. destructor* Swift Current	0.7	0.7	0		*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
5. *C. destructor* Swift Current	0.7	0.7	0	0		*0*	*0*	*0*	*0*	*0*	*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
6. *C. destructor* Drumheller	0.7	0.7	0	0	0		*0*	*0*	*0*	*0*	*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
7. *C. destructor* Strasbourg	0.7	0.7	0	0	0	0		*0*	*0*	*0*	*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
8. *C. destructor* Strasbourg	0.7	0.7	0	0	0	0	0		*0*	*0*	*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
9. *C. destructor* Lethbridge	0.7	0.7	0	0	0	0	0	0		*0*	*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
10. *C. destructor* Fillmore	0.7	0.7	0	0	0	0	0	0	0		*0*	*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
11. *C. destructor* Fillmore	0.7	0.7	0	0	0	0	0	0	0	0		*1.9*	*0*	*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
12. *C. destructor* Fillmore	12	12	12	12	12	12	12	12	12	12	12		*1.9*	*1.9*	*1.9*	*1.9*	*1.2*	*1.2*	*2*
13. *C. destructor* Fort Sasktachewan	0.7	0.7	0	0	0	0	0	0	0	0	0	12		*0*	*0*	*0*	*1.8*	*1.8*	*1.5*
14. *C. destructor* Meota	0.7	0.7	0	0	0	0	0	0	0	0	0	12	0		*0*	*0*	*1.8*	*1.8*	*1.5*
15. *C. destructor* Meota	0.7	0.7	0	0	0	0	0	0	0	0	0	12	0	0		*0*	*1.8*	*1.8*	*1.5*
16. *C. destructor* Inglis	0.7	0.7	0	0	0	0	0	0	0	0	0	12	0	0	0		*1.8*	*1.8*	*1.5*
17. *C. pruinina* Orangeville	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	4.8	11.3	11.3	11.3	11.3		*0*	*1.9*
18. *C. pruinina* Orangeville	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	4.8	11.3	11.3	11.3	11.3	0		*1.9*
19. *C. pruinina* Oregon	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	13.1	8.6	8.6	8.6	8.6	11	11	

Table 4.6 *P*-distance (%) between the *Ctenicera* spp. sequences (with sample location). Standard error estimates are shown above the diagonal in italics and were obtained by a bootstrap procedure (1000 replicates). Gaps and missing data were eliminated.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1		0	0	0	0	0	0	0	0	1	1	2	0	1	0	0	2	2	2	0
2	0		0	0	0	0	0	0	0	1	1	2	0	1	0	0	2	2	2	0
3	0	0		0	0	0	0	0	0	1	1	2	0	1	0	0	2	2	2	0
4	0	0	0		0	0	0	0	0	1	1	2	0	1	0	0	2	2	2	0
5	0	0	0	0		0	0	0	0	1	1	2	0	1	0	0	2	2	2	0
6	0	0	0	0	0		1	0	0	1	1	2	0	1	0	0	2	2	2	0
7	0	0	0	0	0	1		0	0	1	1	2	0	1	0	0	2	2	2	0
8	0	0	0	0	0	0	0		0	1	1	2	0	1	0	0	2	2	2	0
9	0	0	0	0	0	0	0	0		1	1	2	0	1	0	0	2	2	2	0
10	2	2	2	2	2	2	2	2	2		0	2	1	1	1	1	2	2	2	1
11	2	2	2	2	2	2	2	2	2	0		2	1	1	1	1	2	2	2	1
12	18	18	18	18	18	18	18	18	18	18	18		2	2	2	2	2	2	2	2
13	0	0	0	0	0	0	0	0	0	2	2	18		1	0	0	2	2	2	0
14	1	1	1	1	1	1	1	1	1	3	3	18	1		1	1	2	2	2	1
15	0	0	0	0	0	0	0	0	0	2	2	18	0	1		0	2	2	2	0
16	0	0	0	0	0	0	0	0	0	2	2	18	0	1	0		2	2	2	0
17	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15		2	2	2
18	17	17	17	17	17	17	17	17	17	16	16	16	17	17	17	17	17		0	2
19	17	17	17	17	17	17	17	17	17	16	16	16	17	17	17	17	17	0		2
20	0	0	0	0	0	0	0	0	0	2	2	18	0	1	0	0	15	17	17	

Table 4.7 *P*-distance (%) between *Limonius californicus* sequences. Standard error estimates are shown above the diagonal in italics and were obtained by a bootstrap procedure (1000 replicates). Gaps and missing data were eliminated.

4.3.2.2 Intraspecific genetic variation

There was a high haplotype diversity for some species (*L. infuscatus, H. bicolor, A. mellilus* and *M. similis*) but a similar diversity for most where more than one individual was sequenced (with high standard deviation due to small sample size; Table 4.8). Nucleotide diversity was variable within species, but the small sample size limits the information that can be inferred from these results. Intraspecific *P*-distances were generally <1% between samples from the same species (excluding those mentioned in section 4.3.2.1) e.g. *L. infuscatus* (Table 4.9a), *L. agonus* (Table 4.9b), *A. mellilus* (Table 4.9c) and *A. stabilis* (Table 4.9d), and for two seperate species (*A. criddlei* and *A. stabilis*) there was no intraspecific difference between sequences.

Species	Location	N	H_N	H_d	π
Limonius californicus	Taber, AB	3	1	0	0
	Drake, SK	3	2	0.667 ± 0.314	0.0023
	Claresholm, AB	3	2	0.667 ± 0.314	0.0023
	Oliver, BC	3	2	0.667 ± 0.314	0.1216
	Swift Current, SK	1	-	-	-
	Fort Saskatchewan, AB	1	-	-	-
	Moose Jaw, SK	3	2	0.667 ± 0.314	0.1139
	Wakaw, SK	3	2	0.667 ± 0.314	0.0051
Limonius infuscatus	Fort Macleod, AB	3	3	1 ± 0.272	0.0067
Limonius agonus	Ridgetown, ON	4	1	0	0
	Parkhill, ON	3	2	0.667 ± 0.314	0.0044
Limonius canus	Kamloops, BC	1	-	-	-
Hypnoides bicolor	Brandon, MB	3	2	0.667 ± 0.314	0.0279
	Parkland, AB	3	2	0.667 ± 0.314	0.0988
	Gull Lake, SK	1	-	-	-
	Montmartre, SK	3	2	0.667 ± 0.314	0.0138
	Calgary, AB	3	1	0	0
	Elkhorn, MB	3	3	1 ± 0.272	0.0432
	Avonlea, SK	3	3	2 ± 0.272	0.0138
	Teulon, MB	3	2	0.667 ± 0.314	0.0138
	Boissevain, MB	3	2	0.667 ± 0.314	0.0138
	Orangeville, ON	2	1	0	0
	Domremy, SK	1	-	-	-
	Altona, MB	3	2	0.667 ± 0.314	0.0138
	Ste. Rose, MB	2	2	1 ± 0.5	0.0034
	Inglis, MB	1	-	-	-
	Manning, AB	2	1	0	0
	Fairview, AB	2	1	0	0
	Dawson Creek, BC	2	1	0	0
Ctenicera aeripennis aeripennis	Tangent, AB	2	1	0	0
	Fairview, AB	1	-	-	-
Ctenicera destructor	Swift Current, SK	2	1	0	0
	Drumheller, AB	1	-	-	-
	Strasbourg, SK	2	1	0	0
	Lethbridge, AB	1	-	-	-
	Fillmore, SK	3	2	0.667 ± 0.314	0.0828
	Fort Sasktachewan, AB	1	-	-	-
	Meota, SK	2	1	0	0
	Inglis, MB	1	-	-	-
Ctenicera pruinina	Orangeville, ON	2	1	0	0
	Oregon	1	-	-	-
Agriotes criddlei	Vegreville, AB	3	1	0	0
Agriotes stabilis	Boissevain, MB	4	1	0	0
	Wakaw, SK	3	1	0	0
Agriotes mancus	Le Gardeur, QC	1	-	-	-
Agriotes obscurus	Orangeville, ON	1	-	-	-
Aeolus mellillus	Milden, SK	2	2	1 ± 0.5	0.0029
	Drinkwater, SK	1	-	-	-
Melanotus similis	Le Gardeur, QC	2	2	1 ± 0.5	0.0031
Hemicrepidius spp.	Indian Head, SK	1	-	-	-
	Brussels, ON	1	-	-	-

Table 4.8 Genetic diversity indices for the sequence data for each species in each location. N = number of forward sequences, H_N = number of haplotypes, H_d = haplotype diversity ± standard error, π = nucleotide diversity.

a)

	1	2
1		*0.3*
2	0.3	

b)

	1	2	3
1		*0.5*	*0.6*
2	0.7		*0.3*
3	1	0.3	

c)

	1	2	3
1		*0.3*	*0*
2	0.3		*0.3*
3	0	0.3	

d)

	1	2	3	4	5	6	7
1		*0*	*0*	*0*	*0.3*	*0.3*	*0.3*
2	0		*0*	*0*	*0.3*	*0.3*	*0.3*
3	0	0		*0*	*0.3*	*0.3*	*0.3*
4	0	0	0		*0.3*	*0.3*	*0.3*
5	0.3	0.3	0.3	0.3		*0.5*	*0*
6	0.3	0.3	0.3	0.3	0.7		*0.5*
7	0.3	0.3	0.3	0.3	0	0.7	

Table 4.9 P-distance (%) between forward sequences for a) *Limonius infuscatus*, b) *Limonius agonus*, c) *Aeolus mellilus*, d) *Melanotus similis*. Standard error estimates are shown above the diagonal in italics and were obtained by a bootstrap procedure (1000 replicates). Gaps and missing data were eliminated.

The genetic distance between *H. bicolor* samples was relatively high compared to other species (but not to the extent of those in section 4.3.2.1; Appendix 4). The Mantel test was significant for these samples, suggesting there is a correlation between genetic and geographic distance ($Z = 56543.1740$, $p < 0.001$; Figure 4.8). The haplotype map suggests there is some differentiation of haplotypes from the east to the west of Canada (Figure 4.9). The MSN also suggests the difference between haplotypes is large - there are many steps (mutated positions) between most of the haplotypes (Figure 4.10).
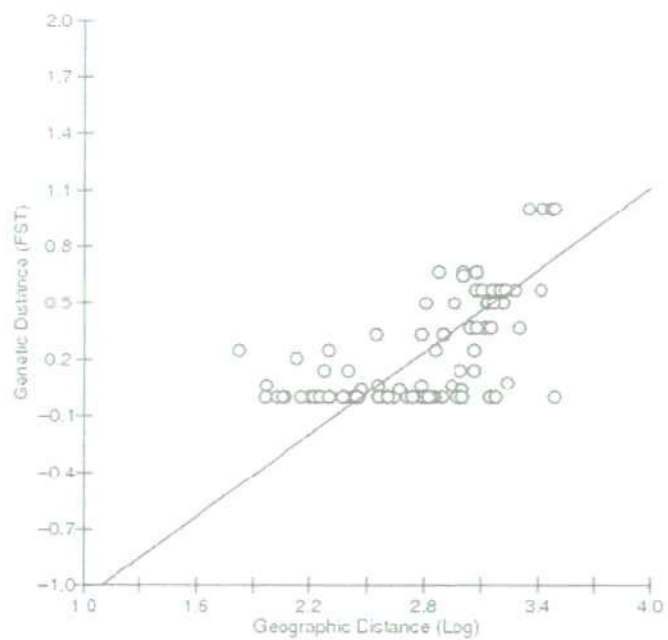
Figure 4.8 The relationship between geographic (log) distance and genetic distance (PhiST) for *Hypnoidus bicolor* samples ($R^2 = 0.54$).

Figure 4.9 Map of Canada showing *Hypnoidus bicolor* 16S rRNA haplotype frequencies in each sampling location.
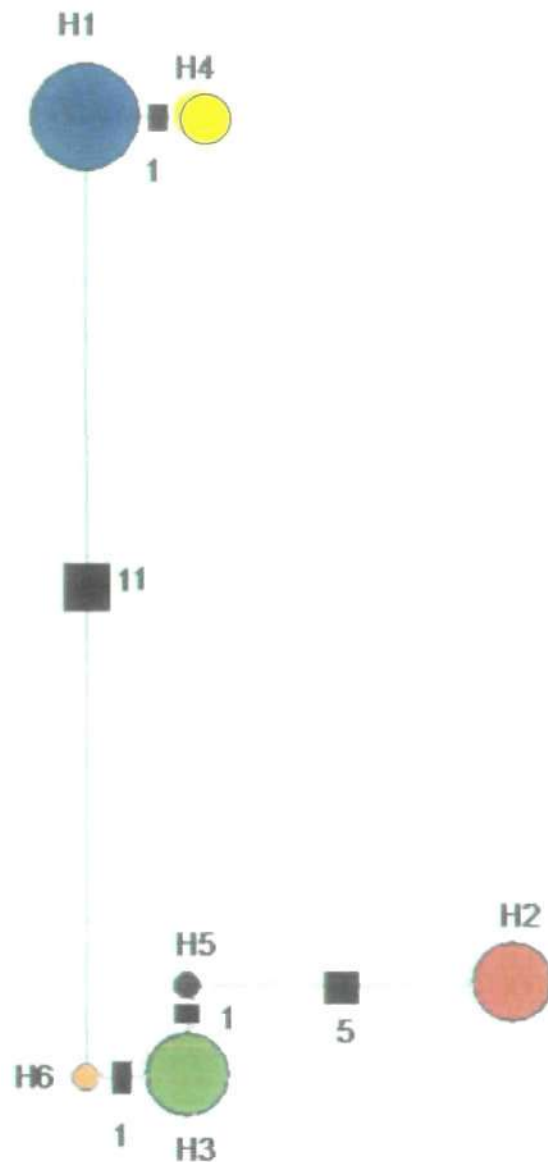
Figure 4.10 Minimum spanning network of *Hypnoidus bicolor* 16S rRNA haplotypes. Coloured circles represent different haplotypes; the sizes are proportional to the haplotype's frequency in the population, and the lengths of the joining lines are proportional to the number of nucleotide changes between haplotypes (the black boxes with numbers).

4.3.2.4 Phylogenetic relationships between Canadian species

The NJ (Figure 4.11), MP (Figure 4.12) and ML trees (Figure 4.13) trees are similar, and

the NJ and MP have high support for most of the branches. All species within the same

genus are placed within the same clade in the NJ and MP trees, but in the ML tree two

L. californicus samples are placed in a separate clade to the others. There does not

appear to be a geographic relationship between samples within the same clade.
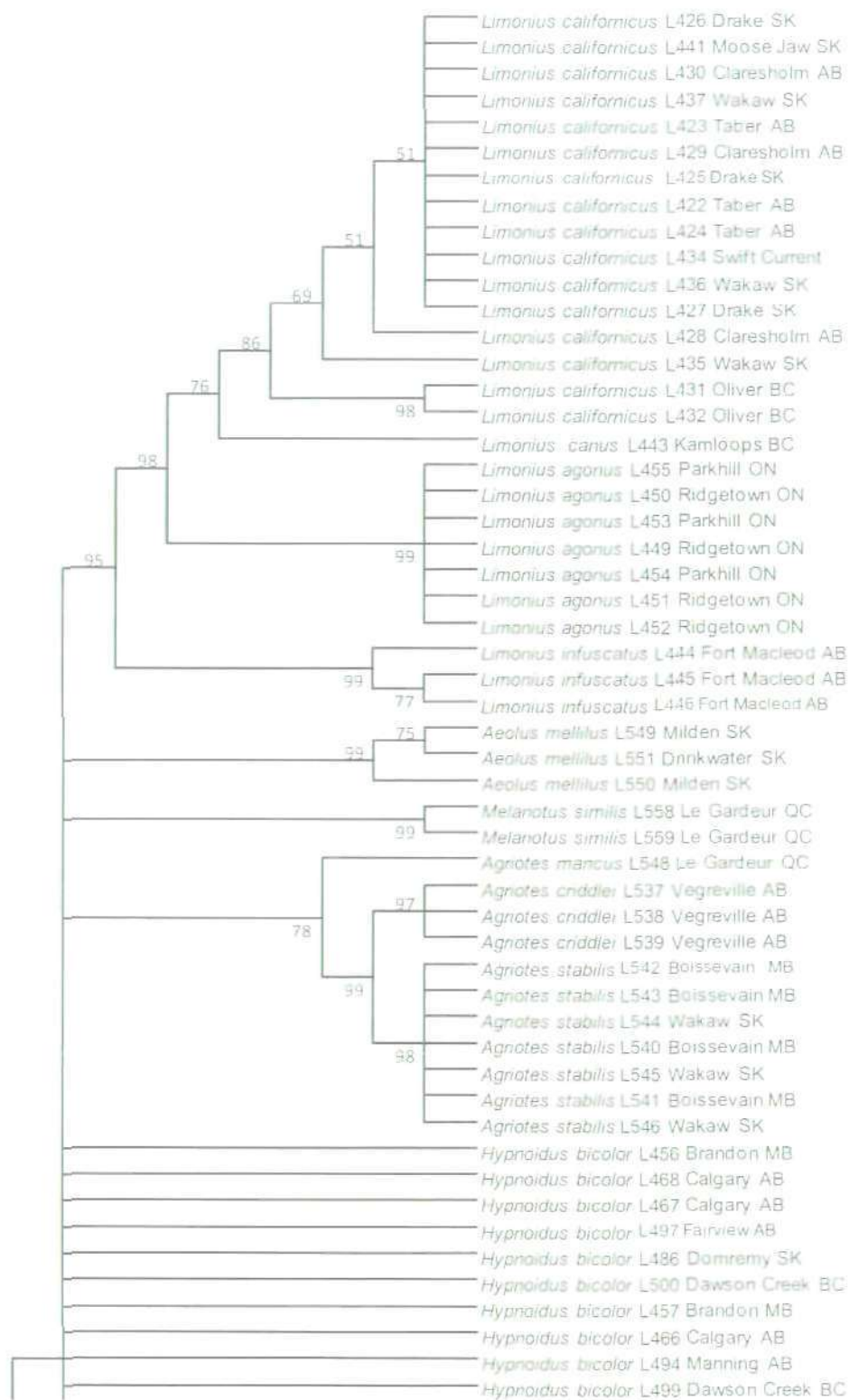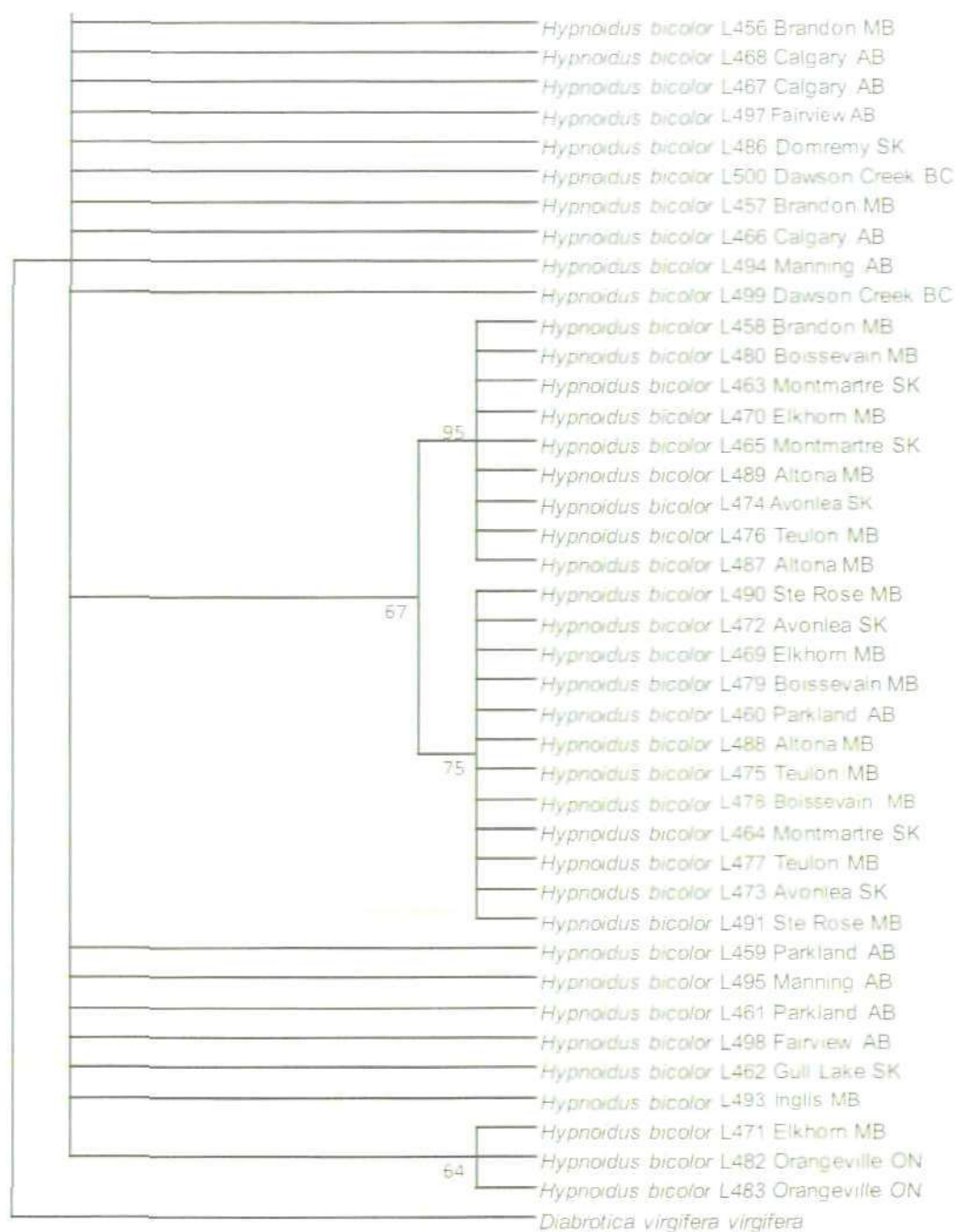
Figure 4.11 Neighbour joining tree, using *p*-distance, of 16S rRNA sequences of Canadian wireworm species. Bootstrap support values (1000 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. There were a total of 214 positions in the final dataset (all positions containing gaps and missing data were eliminated).

Limonius californicus L422 Taber AB
Limonius californicus L441 Moose Jaw SK
Limonius californicus L434 Swift Current
Limonius californicus L435 Wakaw SK
Limonius californicus L424 Taber AB
Limonius californicus L436 Wakaw SK
Limonius californicus L426 Drake SK
Limonius californicus L429 Claresholm AB
Limonius californicus L425 Drake SK
Limonius californicus L430 Claresholm AB
Limonius californicus L427 Drake SK
Limonius californicus L437 Wakaw SK
Limonius californicus L423 Taber AB
Limonius californicus L428 Claresholm AB
Limonius californicus L431 Oliver BC
Limonius californicus L432 Oliver BC
Limonius canus L443 Kamloops BC
Limonius agonus L452 Ridgetown ON
Limonius agonus L450 Ridgetown ON
Limonius agonus L455 Parkhill ON
Limonius agonus L449 Ridgetown ON
Limonius agonus L453 Parkhill ON
Limonius agonus L451 Ridgetown ON
Limonius agonus L454 Parkhill ON
Limonius infuscatus L446 Fort Macleod AB
Limonius infuscatus L444 Fort Macleod AB
Limonius infuscatus L445 Fort Macleod AB
Melanotus similis L558 Le Gardeur QC
Melanotus similis L559 Le Gardeur QC
Aeolus mellilus L550 Milden SK
Aeolus mellilus L549 Milden SK
Aeolus mellilus L551 Drinkwater SK
Agriotes mancus L548 Le Gardeur QC
Agriotes criddlei L537 Vegreville AB
Agriotes criddlei L539 Vegreville AB
Agriotes criddlei L538 Vegreville AB
Agriotes stabilis L545 Wakaw SK
Agriotes stabilis L540 Boissevain MB
Agriotes stabilis L542 Boissevain MB
Agriotes stabilis L541 Boissevain MB
Agriotes stabilis L546 Wakaw SK
Agriotes stabilis L543 Boissevain MB
Agriotes stabilis L544 Wakaw SK

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Figure 4.12 Maximum parsimony tree of 16S rRNA sequences of Canadian wireworm species. Bootstrap support values (1000 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree was obtained using Close-Neighbour-Interchange with search level 1 in which the initial trees were obtained with the random addition of sequences (100 replicates). All positions containing gaps and missing data were eliminated. There were a total of 214 positions in the final dataset, of which 76 were parsimony informative.
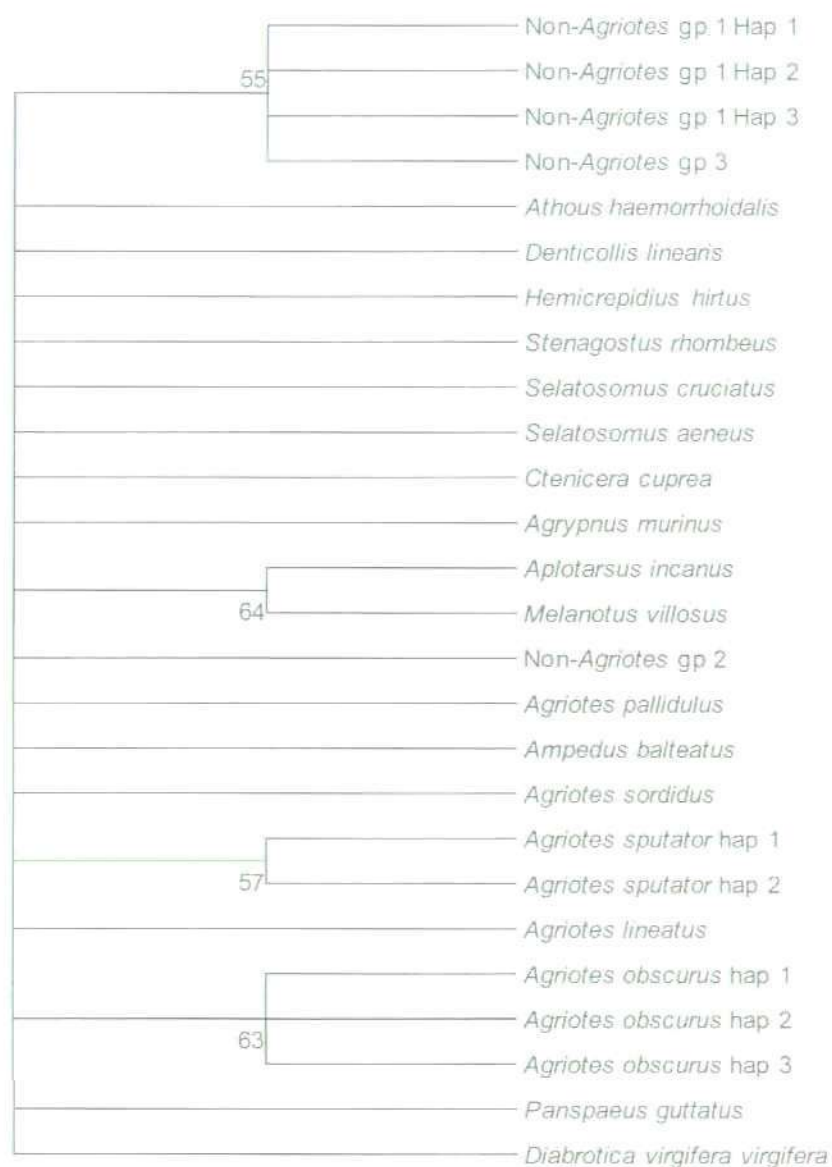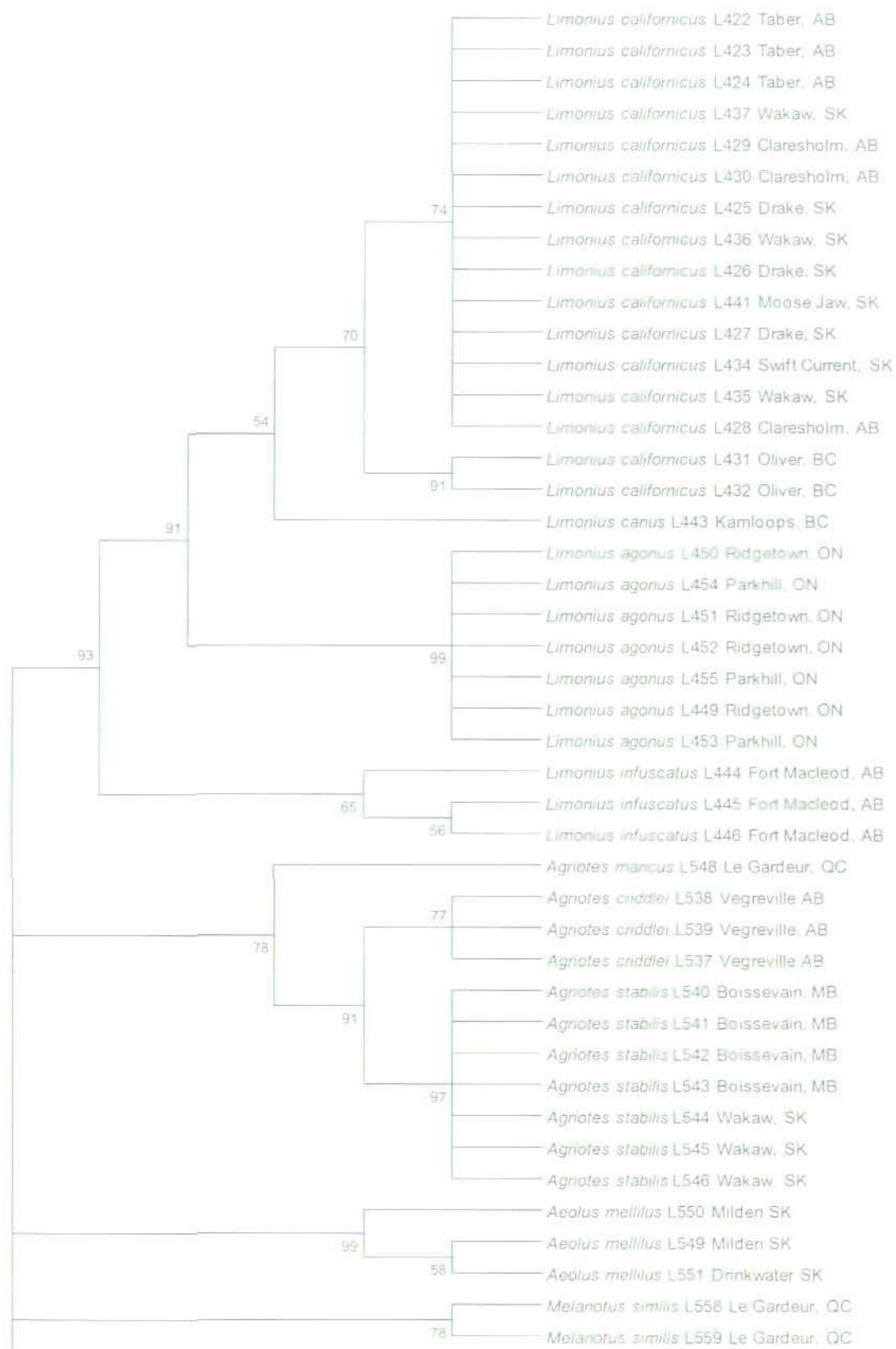
Figure 4.13 Maximum likelihood tree of 16S rRNA sequences of Canadian wireworm species using the GTR+G model. LnL = -1297.76. Bootstrap support values (500 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

4.3.3 Phylogenetic relations between all Elateridae species

The topography of the NJ (Figure 4.14), MP (Figure 4.15) and ML (Figure 4.16) trees for all Elateridae species are again very similar. Species in the same genus are grouped into the same clades and the relationships observed in the trees for the UK species and the Canadian species are again apparent, even with the addition of extra species and larger sequence lengths (238bp compared to 214bp for all Canadian forward sequences and 180bp for the UK species).
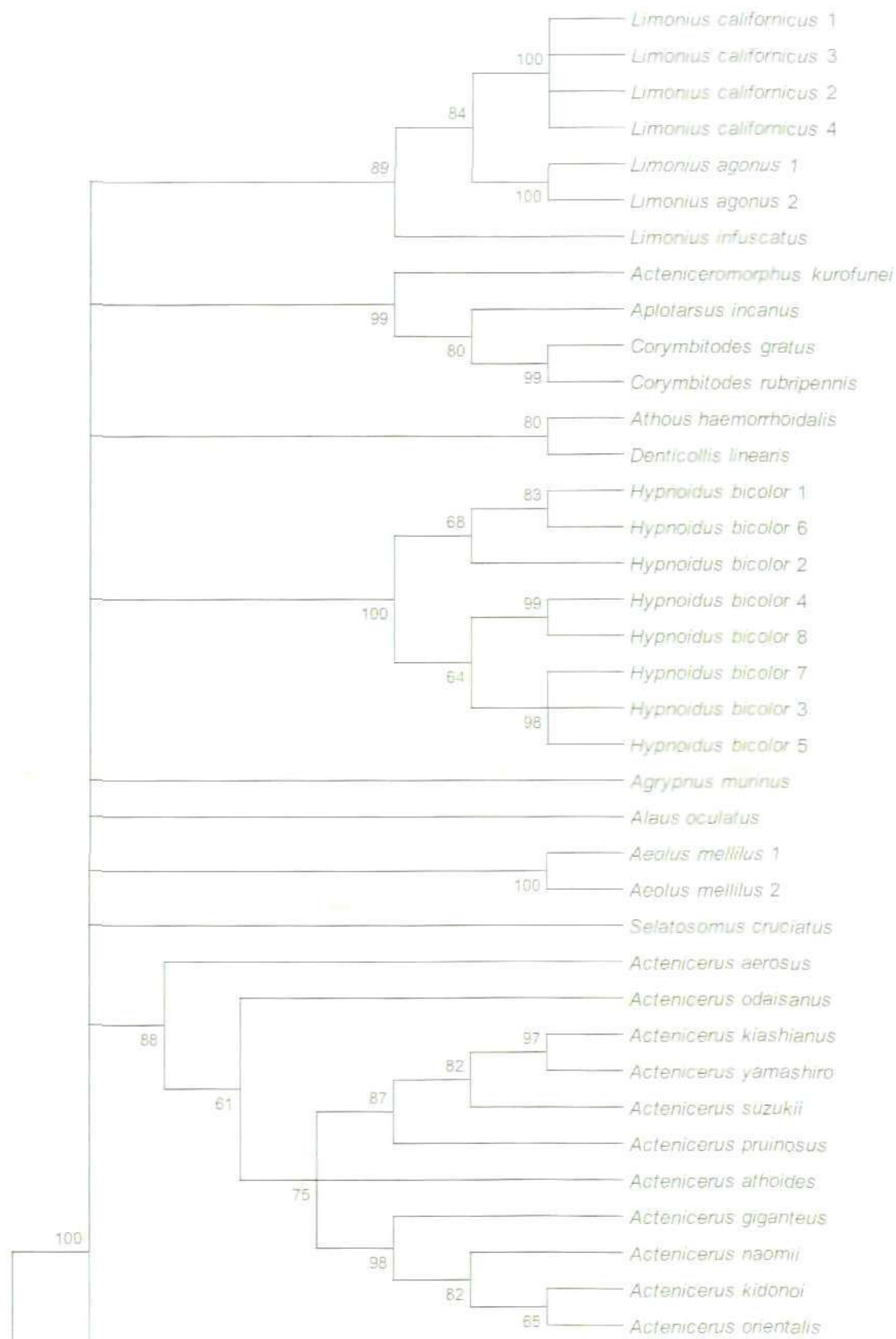
Figure 4.14 Neighbour joining tree, using *p*-distance, of 16S rRNA sequences of all Elateridae species. Multiple sequences from the same species are numbered and haplotypes are represented by 'hap'. Bootstrap support values (from 1000 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. There were a total of 238 positions in the final dataset (all positions containing gaps and missing data were eliminated).
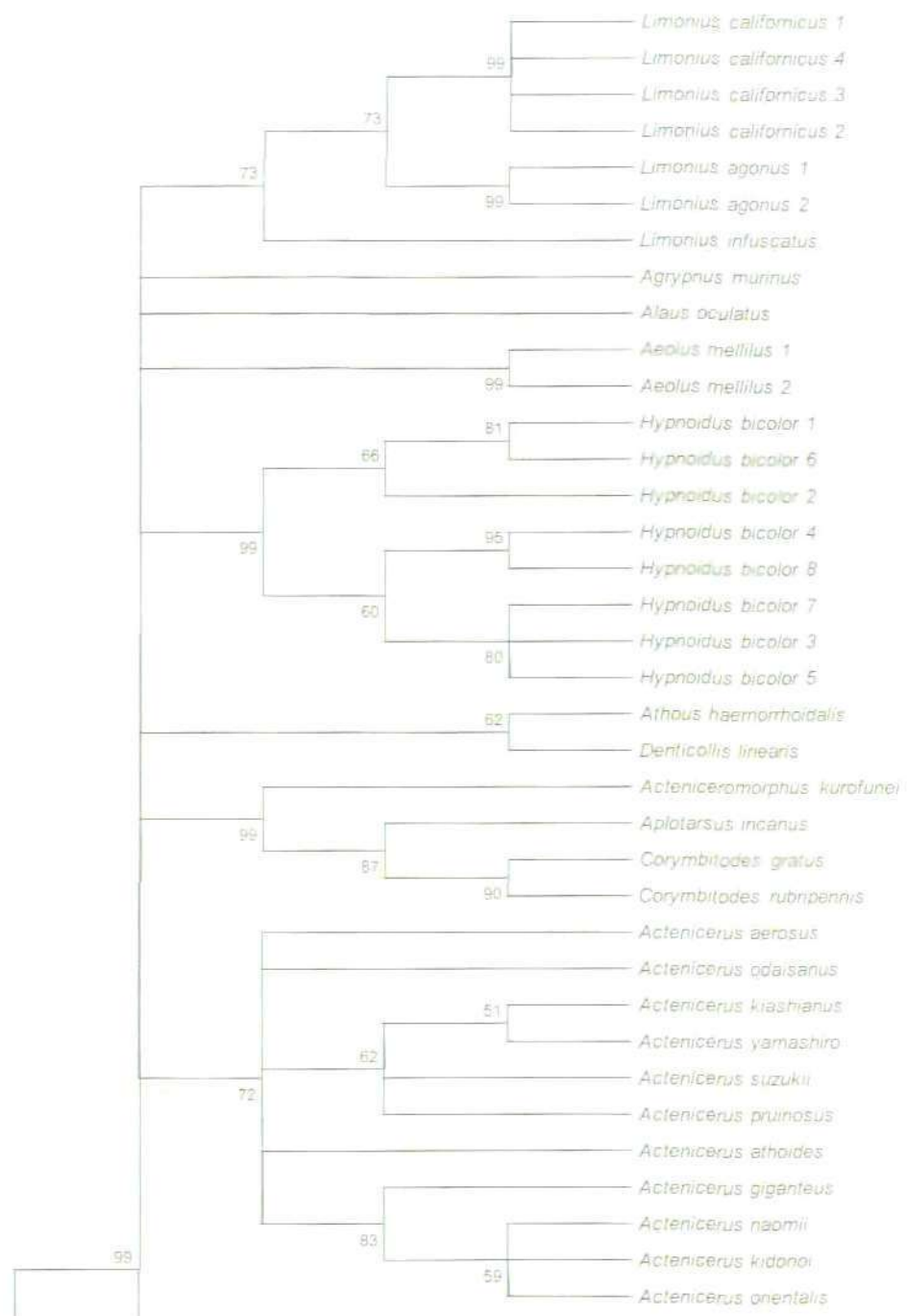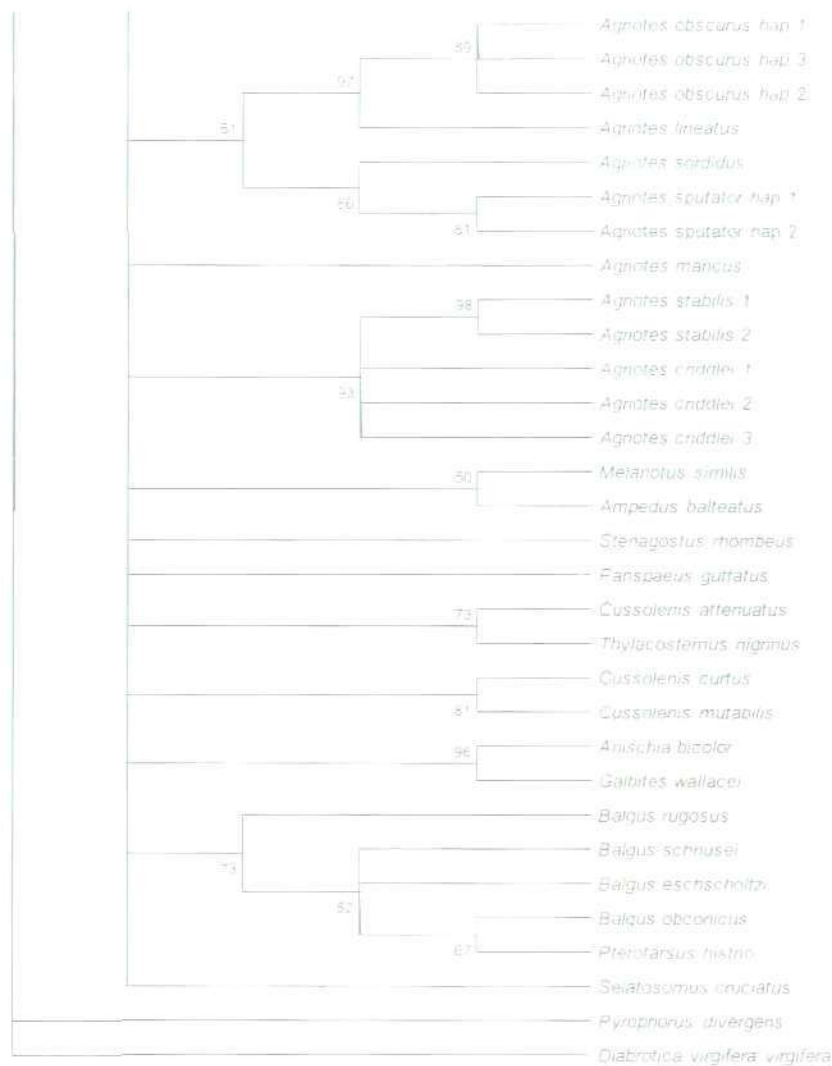
Figure 4.15 Maximum parsimony tree of 16S rRNA sequences of all Elateridae species. Multiple sequences from the same species are numbered and haplotypes are represented by 'hap'. Bootstrap support values (from 1000 replicates) are shown next to branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree was obtained using the Close-Neighbour-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (100 replicates). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 238 positions in the final dataset, of which 165 were parsimony informative.
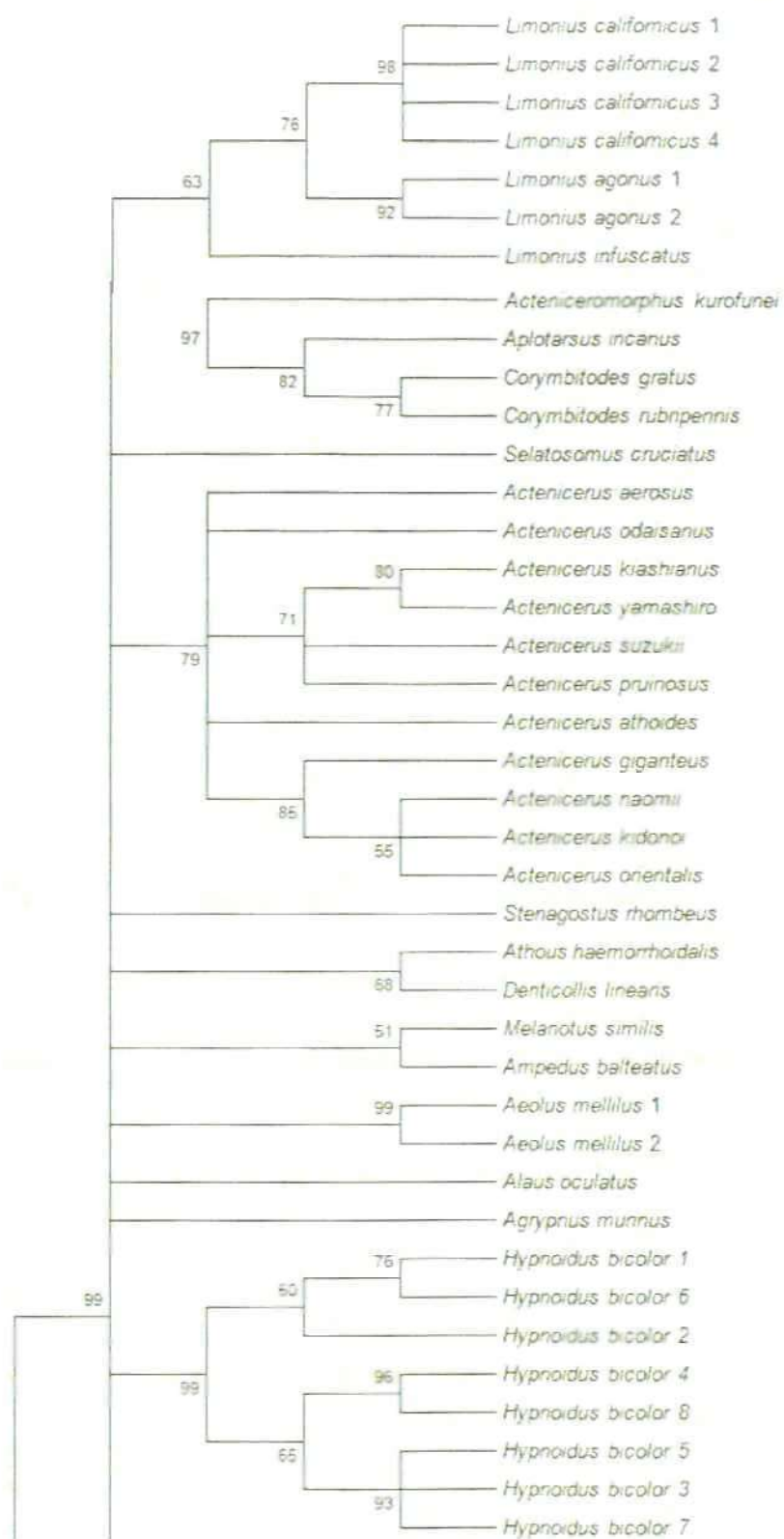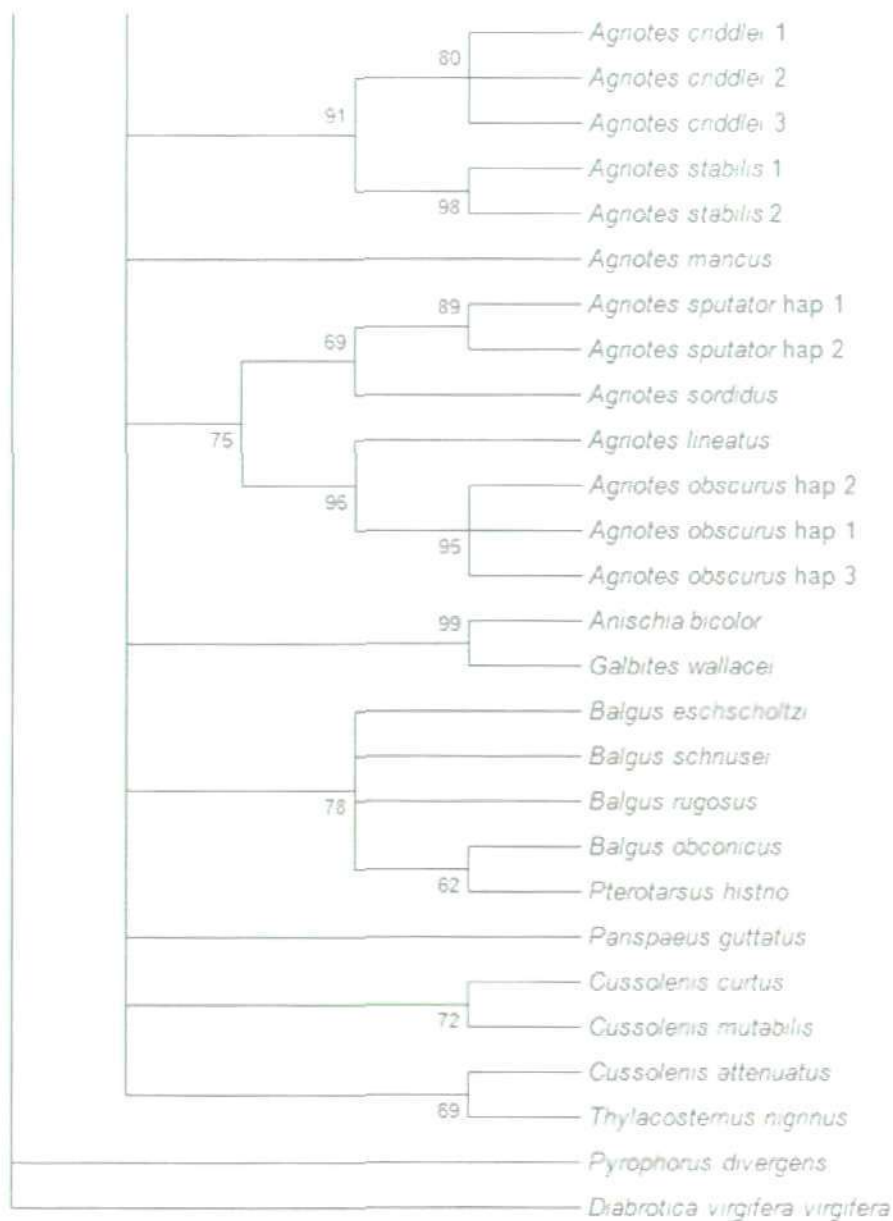
Figure 4.16 Maximum likelihood tree of 16S rRNA sequences of all Elateridae species using the TIM3+G model. −LnL = -4185.2. Multiple sequences from the same species are numbered and haplotypes are represented by 'hap'. Bootstrap support values (from 500 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

4.4 Discussion

4.4.1 Proportions and abundance of UK wireworm species

Agriotes obscurus were by far the most abundant wireworm species identified out of all samples processed by T-RFLP. Previous studies have reported the dominance of *A. obscurus* wireworms across the UK (Miles, 1942b; Roberts, 1919), with localised areas in which *A. sputator* (e.g. Salt & Hollick, 1944) and *A. lineatus* are more abundant (Anon, 1948). However, the majority of studies have not differentiated between wireworm species and so much of the information on species distributions available is based on that of adults. For example, in the large-scale survey from 1939-1942, *A. obscurus* was the most common adult species found in Northern fields, whilst in Midland areas numbers of *A. lineatus* and *A. sputator* increased, and in the South as a whole *A. sputator* was most common (Anon, 1948). As was reported in Chapter Two, the proportions and abundance of adults do not necessarily match that of wireworms of the same species and so it is difficult to compare these results with those found here. In addition, sampling was not carried out on as large a scale as previous surveys and only small sample sizes were available for most locations (these were not part of designed sampling plans but are the result of chance finds).

It is, however, interesting to note the difference in proportions and abundance of species identified in the South Hams and Newton Abbot in Devon, located approximately 16 miles apart. Whereas in these samples *A. obscurus* are the least abundant and *A. lineatus* the most abundant in Newton Abbot, in the South Hams the opposite situation is true (Table 4.2; Figure 4.2). More non-*Agriotes* wireworms are also present in the South Hams than Newton Abbot. There were differences in scale of sampling (90 fields vs. 6 fields), the number of cores taken (20 per field vs. 192-864 per

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field), and sampling method (soil core and bait trap vs. soil core only), as well as

management differences so, as was suggested in Chapter Three, sampling design (and

sample size) probably affects data obtained on wireworm species distributions and

abundance.

*4.4.2 Identification of non-*Agriotes *wireworms*

Most of the non-*Agriotes* wireworms found in the wireworm survey (Anon, 1948) were

from fields in hilly districts of the Midlands, the North and Wales and also on derelict

soils in the North Downs in Kent. The survey also found *Corymbites* spp. (now

Actenicerus sjaelandicus Muller, *Ctenicera cuprea*, and *Selatosomus aeneus* Linnaeus)

in the South West and *Athous* were widespread over most of the country. However

non-*Agriotes* sequences did not match those from *C. cuprea, S. aeneus, A.*

haemorrhoidalis and *A. campyloides* Newman. *Ctenicera cuprea* and *S. aeneus* were

found on Dartmoor, an area of high altitude relative to locations in which non-*Agriotes*

wireworms were found, but despite other wireworm species compared here being

found in grassland soil e.g. *A. campyloides, A. pallens, Agrypnus murinus* Linnaeus, no

successful identifications were made. Other species for which sequences were

obtained included wireworms that develop in trees and decaying wood or woodland

soil (*Stenagostus rhombeus* Olivier, *Ampedus balteatus* Linnaeus, *Denticollis linearis*

Linnaeus and *Selatosomus cruciatus* Linnaeus), sequences of which may have been

expected not to align with those of the non-*Agriotes* wireworms found in grassland

fields. However, for many click beetle species there is little information on larval

habitats and one such species, *H. hirtus* , thought to develop in decaying wood

(Wallace, 2009) was found in soil cores from agricultural land in Cambridge in this

study. Other possible species, for which sequence data were not available in this study but that could provide a match include *Hypnoidus riparius* Fabricius, *Prosternon tessellatum* Linnaeus, *Kibunea minuta* Linnaeus and *Sericus brunneus* Linnaeus, found in the wireworm survey (Anon, 1948), and *A. pallens* which was found in Cambridge but was not successfully sequenced.

4.4.3 Genetic distance and phylogeny of UK species

The low genetic divergence of sequences between group one and group three of non-*Agriotes* suggests they may be closely related, possibly in the same genus as each other. For example, the *Agriotes* species studied here differ by up to 7% (between *A. lineatus* and *A. pallidulus* Illiger; Table 4.4), similar to that between non-*Agriotes* group 1 and group 3 (8.9%; Table 4.3). They are also in the same clade in all trees (Figure 4.5, Figure 4.6, and Figure 4.7). *Agriotes acuminatus* was the only UK *Agriotes* species which was not included in phylogenetic analysis. Based on the genetic divergence between the other *Agriotes* species (0-7%), that non-*Agriotes* group 2 could be *A. acuminatus* may be unlikely, but *p*-distances differ widely among groups of insects (interspecific variation can be up to 30.7% in some insect families; Cognato, 2006). For example, sequence divergences at the 16S region in a single species of seed weevil (Coleoptera: Curculionidae) is in the range of 0.2-3.68% (Li et al., 2008), for two species of mayfly was 2.15% (Yeh, Yang & Kang, 1997), between two weevil species complexes (Coleoptera: Curculionidae) in the region of 11.9-13.8% (Langor & Sperling, 1997), for six species in the family Pieridae 5.5% to 21.7% (Sobti et al., 2007) and for species of true bugs (Insecta: Heteroptera) in the same family was on average 19.9% (Jung, Duwal & Lee, 2011). *Dalopius marginatus* Linnaeus, a species within the same tribe as the

Agriotes species (Agriotini; Mendel, 2008) is another possible candidate, but although widespread this species primarily inhabits woodland (or abandoned agricultural land) feeding on tree roots/seedlings (Jedlička & Frouz, 2007).

Athous campyloides had a relatively high level of sequence divergence (49-56%) from the other UK species, including *A. haemorrhoidalis*. The sequence was obtained from a morphologically identified adult sample and while it is possible it was misidentified, it may be that a numt (nuclear copy of mitochondrial DNA) has been amplified and sequenced (Richly & Leister, 2004, see also section 4.4.4). Numts are often characterised by double peaks in sequencing (Song et al., 2008), a problem experienced for *Agriotes accuminatus* for which no reliable sequence could be obtained. This would need to be investigated further in the laboratory, possibly by designing specific primers for these species (Moulton, Song & Whiting, 2010), but conclusively by entire mtDNA sequencing.

There are limitations in using only 26 relatively short sequences with varying amounts of missing data for phylogenetic analyses. Despite this, differences between sequences were found and associations between species in these trees are relatively consistent, corresponding to the most recent published taxonomic checklist for Elateridae (Mendel, 2008). For example, this checklist places *D. linearis* and *A. haemorrhoidalis* in the same tribe (Denticollini), and these species are also positioned in the same clade with over 50% support values in the NJ and MP trees.

4.4.4 Intra- and interspecific variation in Canadian wireworm species

There were ambiguous sequence results for some of the Canadian wireworm species (*Ctenicera* spp., *Limonius californicus*, *A. obscurus* and *Hemicrepidius* spp.). Some of these may represent misidentification, since as mentioned above, there are difficulties in distinguishing between the morphology of some of these species as there are for those in the UK and Europe. This may well be the case for some of the *Ctenicera destructor* and *C. aeripennins aeripennis* samples, and might explain the large divergence between the *Hemicrepidius* spp. and *Agriotes obscurus* with the published *Agriotes* sequences (Table 4.5); *Agriotes sparsus* is also present in agricultural land in Canada (Vernon et al., 2010) . However, for *L. californicus* and some of the *C. destructor* and *C. pruinina* species, as for *Athous campyloides* in the UK sequences, the presence of numts may be a potential issue. There are few reliable ways of checking for numts in sequence data which have already been obtained, particularly for ribosomal mtDNA genes which do not accumulate diagnostic frameshift or premature stop mutations like coding loci (Olson & Yoder, 2002). Producing a phylogenetic topology, as done here and aligning sequences to secondary structure models are suggested methods, but these rely on prior knowledge of phylogenetic relationships and the availability of secondary structure models for the taxa in question, which is often not the case. In this study when these sequences were removed the trees were more realistic: prior to removal some sequences from the same species were scattered throughout the tree. After removal of these anomalous sequences samples from the same species were grouped together in the NJ (Figure 4.11), MP (Figure 4.12) and ML (Figure 4.13) trees, indicating the reliability of this data for investigating species relationships.

For the Canadian samples, the raw sequence data did not obviously suggest more sequence ambiguities than is usual when sequencing click beetles, thus potential numts were initially overlooked and their presence became apparent only during analysis. Knowledge of the presence of numts is important, particularly for identification methods using barcoding, since the number of unique species based on the standard metric of 3% sequence divergence can be overestimated (Song et al., 2008). For future molecular research on the Elateridae it may be more appropriate to obtain sequence data from known adult samples, which would allow larval sequence data to be checked and so prevent the problem of separating species based on larval characters and produce more accurate phylogenetic topologies. This may also help in the detection of numts. PCR products and sequence data should be well scrutinised to detect possible co-amplification and sequencing of numts.

The small sample size from each location limits the interpretation of the number of haplotypes found in different species in this study. However, the phylogenetic trees did not suggest any structure consistent with geographic location (Figure 4.11, Figure 4.12 and Figure 4.13). For *H. bicolor* it was possible to carry out a preliminary assessment of the genetic structure of geographically separated samples, but the genetic distance was relatively high between some of the samples (up to 4.6%; Appendix 4), which if using the 3% threshold for species differentiation used in DNA barcoding (Song *et al.*, 2008) would place these as different species. This suggests some of the 'haplotypes' are in fact cryptic species. The results of the Mantel test (Figure 4.8), haplotype map (Figure 4.9) and MSN (Figure 4.10) show several haplotypes/cryptic species are present, that some are more common than others e.g. 'haplotype' 1, and that this may be related to geographic location. For example many of the 'haplotype' 1 sequences are

located in mid to west Canada, whilst in the far east only 'haplotype' 4 is found (and present in one other population in the middle states). In the MSN 'haplotype' 1 is connected to 'haplotype' 4 by 11 steps and these samples are found relatively close to each other, suggesting these may be mixed populations of cryptic species. Further sampling may reveal more mixing of these haplotypes/cryptic species. It would be useful to obtain adult *H. bicolor* and related species sequences from these locations to determine the identity of these cryptic species and whether this is related to geographic distribution. This information may give some idea as to whether what is thought of as one pest species is actually a complex of morphologically cryptic species. This species also has a parthenogenic mode of reproduction, with only females reported as being found in the North Western areas of the Prairie provinces (Alberta, Saskatchewan and Manitoba; Broatch, 2010), but there is little available information on this at present so a robust method of identification may aid studies on the biology and ecology of this/these pest species.

4.4.5 Phylogeny of all Elateridae

The phylogenies produced here using all available 16S rRNA sequence data are the largest of their kind (published at least) for economically important members of the Elateridae in the UK, Europe and Canada. In other studies, understanding the evolution of bioluminescence has been the main objective (Arnoldi et al., 2007; Sagegami-Oba, Oba & Ôhira, 2007), or studies have focused on specific sub-families (Vahtera, Muona & Lawrence, 2009). In general, the three tree building methods used in this study produced consistent results, lending support to the robustness of the data. Some differences were noted in the ML tree of UK species and in the ML tree of all Elateridae

e.g. *Panspaeus guttatus* was placed together with *A. sputator* whereas in the NJ and MP trees these species were more distantly related. There was relatively high support for many branches in all trees, even where sequence data were short and ambiguous (with missing data and gaps). Similar findings to those of Sagegami-Oba et al. (2007) were noted for the 'all Elateridae' data; although few of the same species were used (most were from Japan) similarities were noted in the positions of those in the same genera on the trees. For example, in the Sagegami-Oba et al. (2007) study, *Acteniceromorphus, Actenicerus, Corymbitodes, Hemicrepidius, Denticollis, Athous, Limonius* and *Corymbitodes* species are grouped within a large clade, and here these taxa are also more closely related (though the MP tree is more ambiguous). In addition, *Melanotus, Ampedus* and *Agriotes* species are contained within the same clade in this published study, and are closely related (within the same clade in the ML tree) here. This indicates that 16S rRNA may be useful in assessing species relationships and constructing phylogenies of elaterids. However, further adult and wireworm sampling or obtaining DNA from known museum specimens are needed to verify some of these results and obtain accurate identification, particularly for the Canadian wireworm species. Nevertheless, these data provide a starting point for assessing evolutionary relationships and classification of elaterid species. The addition of other loci, such as COI for which protocols have now been developed (Staudacher et al., 2011), together with other mitochondrial, nuclear and morphological data, may allow more accurate identification of species (and numts). Considering the pest status of many Elateridae worldwide, sequence data is lacking but as these methods develop to include more species (particularly using next generation sequencing) this should in turn improve knowledge of the relationships and distribution of damage causing species.

Chapter 5   Comparison of walking movements and response to sex pheromones

of male *Agriotes* click beetles

5.1 Introduction

The importance of *Agriotes* movement in agricultural land was realised in early studies

on click beetle biology and ecology, but only general descriptions of movement

behaviour and extent were provided (Cohen, 1942; Gough & Evans, 1942; Roebuck,

Broadbent & Redman, 1947). Recently, due to the implementation of sex pheromone

traps for *Agriotes* species in Europe (Furlan *et al.*, 2001a), more attention has been

given to click beetle movement in agricultural land, but published experimental studies

comparing the potential dispersal ability via investigating individual movement and

response to sex pheromones of *A. obscurus*, *A. lineatus* and *A. sputator* are lacking.

Adult trap catches were intended to be used to determine the wireworm population in

the soil instead of soil sampling, which is laborious and time-consuming. However,

subsequent studies using the traps found that although the distributions of adult male

A. obscurus and *A. lineatus* are similar at the landscape scale (Blackshaw & Vernon,

2006), when studied at the field scale their spatial distributions differ (Blackshaw &

Vernon, 2008), suggesting differences in the ability of sex pheromone traps to detect

adult spatial patterns. As was highlighted in Chapter 2 this has implications for the use

of adult trap counts in determining the wireworm population present in the soil.

Interspecific differences in the speed and recapture rate in sex pheromone traps have

also been noted in the field; Hicks and Blackshaw (2008) found that *A. lineatus*

travelled fastest, followed by *A. obscurus* and then *A. sputator*. However, contradictory

results have been reported for maximum dispersal distances; although Sufyan,

Neuhoff and Furlan (2007) found low recapture rates of *A. lineatus* and *A. obscurus* at release distances of 60m, the majority being trapped 10m from the release point, using carbon stable isotopes Schallhart *et al.* (2009) reported 'long-distance' migration (up to 80m) as being common in *A. obscurus*. Inherent problems with mark-release-recapture studies (e.g. marker application, failing to trap marked samples, estimation of maximum dispersal distance is limited by furthest distance at which traps are set) mean that comparisons between studies and species are difficult. In addition, if there are differences in species' responses to sex pheromones then this will affect the rate at which individuals are re-trapped, but the dispersal ability of the three species with and without pheromones has not been quantified. This information would enable assessment of the applicability of pheromone trapping for risk assessment and control. Relating this to wireworm distribution may improve our understanding of the relationship between adult and larval distributions, which in turn will allow more accurate risk assessment to be undertaken. Unlike the studies described above which have used field based approaches to assess click beetle dispersal and response to sex pheromones (e.g. Hicks & Blackshaw, 2008; Sufyan, Neuhoff & Furlan, 2007), in this study three types of controlled laboratory experiment were used, for the first time, to explore walking behaviour in male *Agriotes* click beetles: a locomotion compensator, Y-tube olfactometer and arena. An advantage of using these methods over field based studies is that underlying inter- and intra-specific variation in walking movements and response to sex pheromones can be quantified without the possibility of external site-specific factors affecting the behaviour of individuals. The aims of the study were to:

1. Compare walking movements between *A. obscurus*, *A. lineatus* and *A. sputator*

2. Determine the effect of sex pheromones on male *Agriotes* click beetle movement

3. Determine the suitability of a locomotion compensator, Y-tube olfactometer and arena for investigating the walking movements of click beetles.

The hypotheses were:

1. There are significant differences in speed and track length between species, in the order A. *lineatus* > A. *obscurus* > A. *sputator*

2. In the presence of sex pheromones the walking speed of all species increases, and movement is directional and less tortuous than when no sex pheromones are present

5.2 Methods

5.2.2 Sample collection

Yatlor sex pheromone traps (Furlan *et al.*, 2001b; Toth *et al.*, 2003) were used to trap adult male A. *obscurus*, A. *sputator* and A. *lineatus* click beetles. Trapping was carried out in grassland at Seale Hayne, University of Plymouth, Devon (OS Grid Ref SX 8287 7314), from 9th June to 25th July 2008 and 11th April to 6th June 2009, and in grass fields at North Wyke Research, Okehampton, Devon (OS Grid Ref SX 6591 9851) and Yelland Cross Farm, Rattery, Devon (OS Grid Ref SX 7397 6255) from 13th April to 26th July 2010. Traps were emptied weekly and replaced in the same position. Sex pheromones were changed every 6 weeks. Captured beetles were stored in a dark controlled temperature room at 15°C prior to testing.

5.3 Locomotion compensator

5.3.1 Methods

The locomotion compensator (Tracksphere LC 300, Syntech, Hilversum, The Netherlands; Figure 5.1) consists of a sphere (300mm diameter), onto which a beetle is placed, with a camera located directly above that measures the beetle's displacements. The sphere rotates opposite to these displacements by means of two electric motors, and two encoders contacting the sphere transmit the rotational movements to a computer as incremental X and Y coordinates (Syntech, 2004). An air stream can be directed over the sphere via a stimulus applicator. Walking movements of each individual were measured over 5 minutes, and the following parameters were measured to quantify the walking behaviour of each species: average walking speed, track length, vector length and straightness (Table 5.1). To determine the effect of sex pheromones on *A. sputator* walking behaviour, two further parameters were measured: upward straightness and upward length (Table 5.1).

Figure 5.1. Syntech LC-300 locomotion compensator with control unit and portable PC (Syntech, 2004)

Due to problems with calibration of the locomotion compensator, experiments started later in the adult click beetle season than anticipated, and therefore sample sizes were smaller than originally planned and differed between species depending on availability (N = 12 A. *obscurus*, N = 17 A. *lineatus* and N = 5 A. *sputator*). For the same reasons, it was only possible to test response to sex pheromones for a small number of A. *sputator* individuals (N = 4).

Experiments were carried out at 15°C and were illuminated by a fluorescent light located directly above the sphere. A white cardboard screen was placed around the sphere to prevent external influences affecting beetle behaviour. The rubber o-rings on the motors and encoders and the sphere itself was rinsed with distilled water and 70% ethanol after every five beetles and/or between species and treatments, as per other studies (e.g. van Tilborg et al., 2003). For the interspecific quantification of

walking movements, each beetle was tested once in still air. The response of *A. sputator* to sex pheromones was tested using three treatments: still air, air flow and pheromone. The former two treatments serve as controls to test the general walking behaviour of the beetles. Each individual ($N = 4$) was tested once in each treatment (three times in total). Air was passed from a pump at approximately 45ml/s into either a plastic pot containing no stimulus (for the 'air flow' trials) or one containing an *A. sputator* sex pheromone vial (Csalomon®, Plant Protection Institue, Hungary). On leaving the pot air was directed over the sphere via a stimulus applicator. Beetles were allowed to acclimatise on the sphere for 1 minute before recording started. Trials in which individuals did not move after 3 minutes, or where the sphere failed to track for the full 5 minutes, were removed from the dataset.

5.3.2 Statistical analysis

Average speed, track length and straightness were normally distributed and the homogeneity of variance assumption was met, therefore differences between species were tested using a one-way ANOVA, with Fishers Least Significant Difference (LSD) as a post-hoc test. Although data for *A. sputator* response to sex pheromones on the locomotion compensator were normally distributed with equal variance, due to the small sample size and relatively large number of explanatory variables (five variables each with three treatment levels), there were too few degrees of freedom to perform multivariate tests using a repeated measures MANOVA, or the Friedman Test (the non-parametric equivalent). The low power and associated risk of committing Type I or Type II errors with small sample sizes meant that testing the statistical significance of differences between treatments was deemed inappropriate. Following the

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recommendations of Cumming *et al.* (2007) data for each individual beetle was plotted for each parameter measured, rather than using the mean, as smaller *n* values result in wider inferential error bars and less precise estimates of true population values, which could lead to inaccurate interpretation of results.

Parameter	Description
Average speed	Average of walking speeds (including zero speeds) in mm/s.
Track length	Total length of the walking track (sum of track lengths per second), measured in mm
Vector length	Net displacement from the origin, measured in mm
Straightness	A quotient of the vector length and track length, ranging from 0 (tortuous) to 1 (linear)
Upward straightness	A quotient of upward length and track length, ranging from -1 (tortuous) to +1 (straight), measuring the straightness of insect movement towards or away from the stimulus source.
Upward length	Net upward displacement (movement toward stimulus), measured in mm. A negative sign indicates movement away from the stimulus source.

Table 5.1. The walking parameters measured during the locomotion compensator and arena experiments used to quantify the walking behaviour of adult male *A. obscurus*, *A. lineatus* and *A. sputator*.

5.3.3 Results

There were significant differences in average speed (F (2) = 10.225, p = <0.001; Figure 5.2a) and track length (F (2) = 10.800, p = <0.001; Figure 5.2b), but no significant difference in straightness, between species. These differences (determined by Fisher's LSD) were between *A. sputator* and *A. lineatus* (average speed, p = <0.001); track length, p = <0.001).
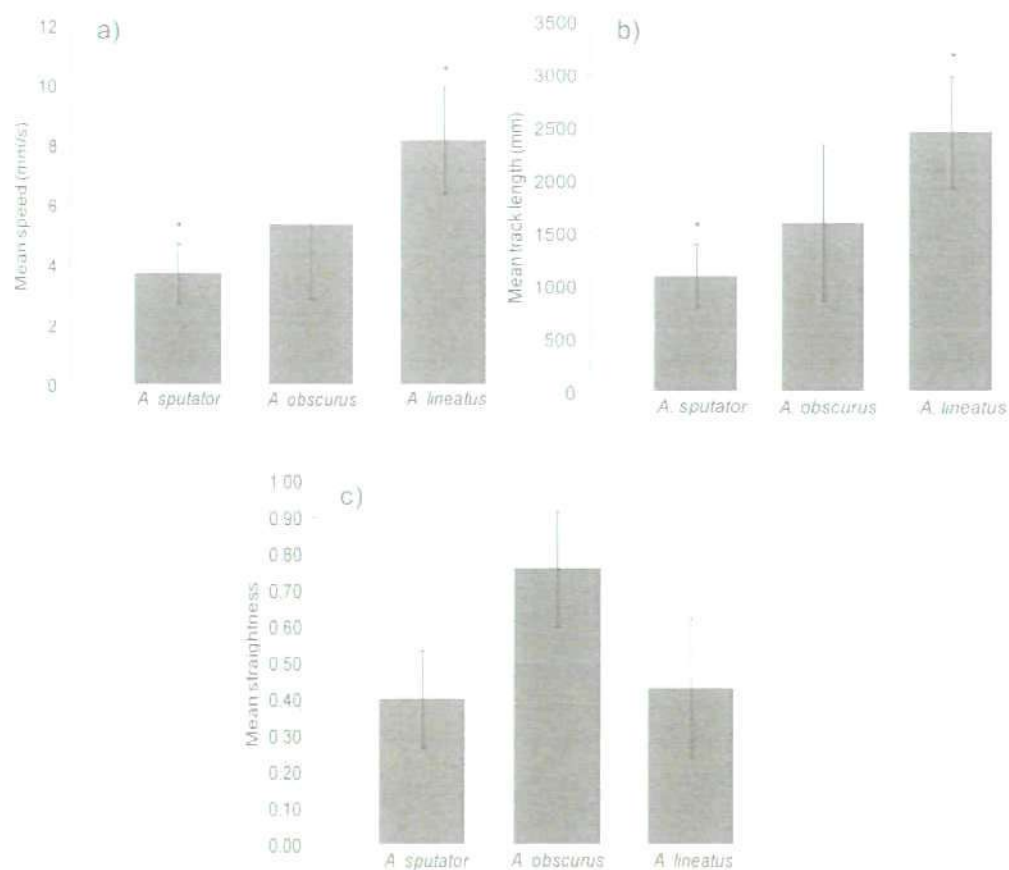
Figure 5.2. a) Mean walking speed (mm/s), b) track length (mm) and c) straightness of

A. obscurus (*N* = 5), *A. sputator* (*N* = 17) and *A. lineatus* (*N* = 12) on the locomotion

compensator. The bars represent the 95% confidence intervals, and asterisks indicate

significant differences between species (*p*<0.001).

A. sputator walking speed, track length and straightness tended to decrease between

still air, airflow and pheromone treatments (Figure 5.3a, b, c). However, upward

straightness and upward length was more variable between individuals and treatments

(Figure 5.3d, e).
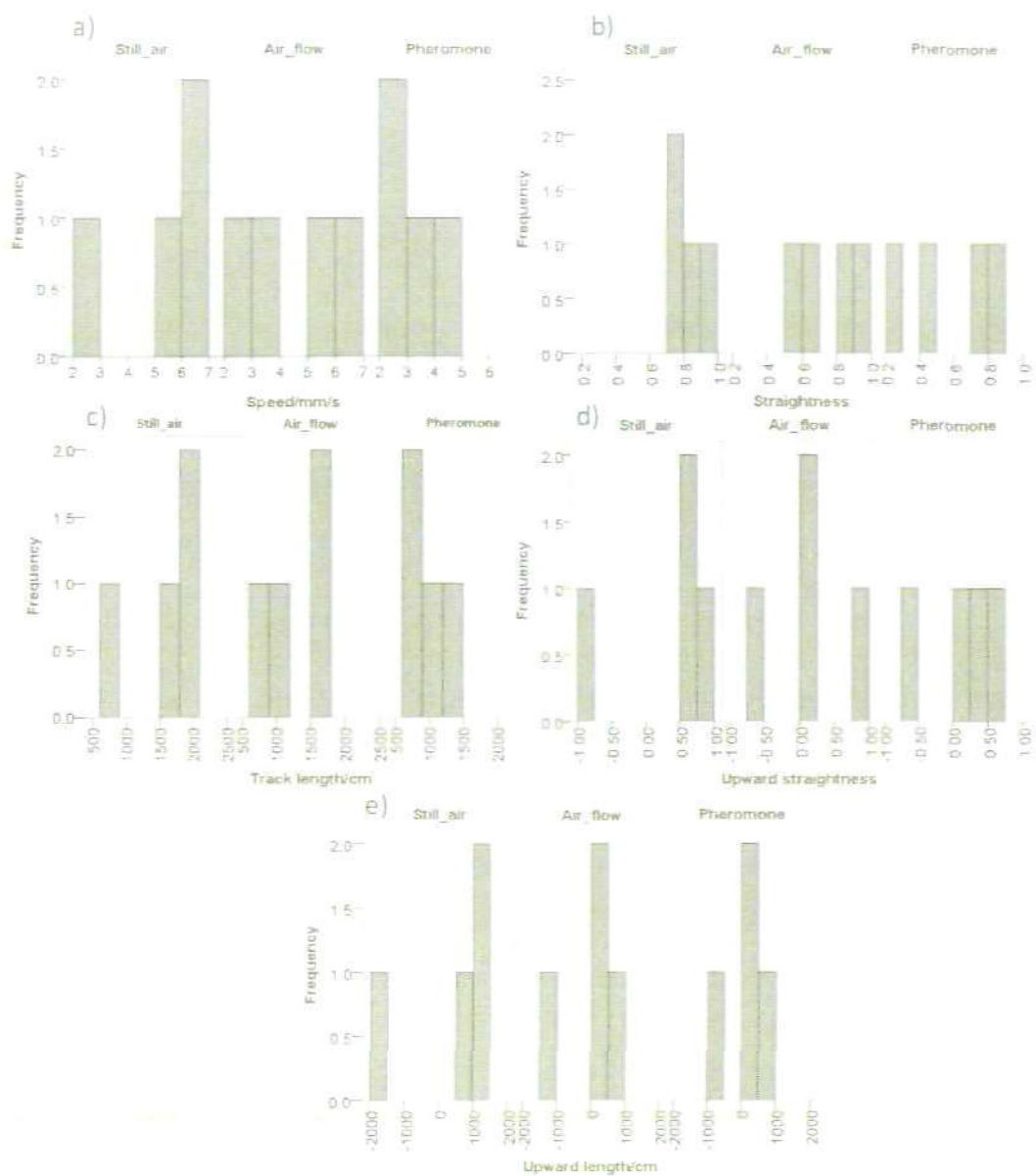
Figure 5.3. a) average speed (mm/s), b) straightness (quotient of the vector length and the track length, ranging from 0 to 1), c) track length (mm), d) upward straightness (quotient of upward length and the track length, ranging from +1 to -1), and e) upward length (net movement toward stimulus – in mm), of *A. sputator* individuals in still air, airflow and pheromones.

5.4 Y-tube olfactomer

Three experiments were carried out with the aim of finding an appropriate design to study intra- and inter-specific variation in click beetle walking movement and response to sex pheromones.

Experiments were carried out using a glass Y-tube olfactometer (1.4cm diameter, main tube length 7cm, arm length 6cm; Soham Scientific, Ely, Cambridgeshire). A 10cm long glass tube was connected to the main tube using a semi-transparent plastic connector in order to measure time taken to walk 100mm. Each arm was connected with plastic tubing to either a plastic pot (8cm diameter, 10cm height) containing a sex pheromone vial (Csalomon®, Plant Protection Institue, Hungary) or an empty pot as a control, and these were connected to an air pump via a pot of distilled water, charcoal and a flow meter (flow rate 100mm/s), which provided a clean, moisturised air flow. A further pump, connected to the long tube via plastic tubing and a rubber bung, pulled air through the system (Figure 5.4). Beetles were allowed to crawl into the tube by disconnecting the rubber bung and replacing once the beetle was inside to seal the system. The Y-tube was placed on a white sheet of cardboard, surrounded by a white cardboard screen to prevent external distractions. A fluorescent light directly above illuminated the experimental area, and the temperature was between 16-19°C. An extractor fan removed air which had passed through the Y-tube. The Y-tube was rinsed with 70% ethanol and distilled H_2O and the Y-tube arms swapped over between all individual trials. An extractor fan was left on for at least an hour between sex pheromone experiments to prevent cross-species sex pheromone contamination.

The time taken to walk 100mm was used to calculate average walking speed and arm choice (right or left in the control, and pheromone or non-pheromone in the

treatment) was recorded for each individual. Beetles were determined to have made a choice when they had walked 3cm into the Y-tube arm. If no choice was made after 3 minutes the trial was excluded from the decision dataset (but speed data retained).
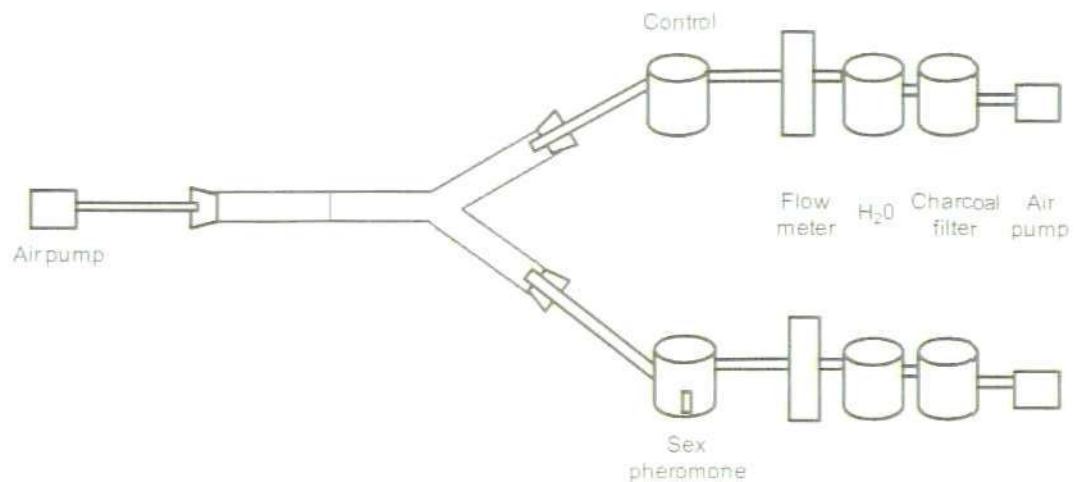
Figure 5.4. Y-tube olfactometer experimental arrangement for recording click beetle movement and response to sex pheromones. Air was pumped through a charcoal filter, distilled H_2O and a flow meter (flow rate 100ml/s) before entering the pot containing the odour source (sex pheromone vial or an empty pot as a control). Air was pulled through the system via a pump connected to the long arm of the Y-tube. This could be disconnected to allow a beetle to be placed at the start of the Y-tube.

5.4.1 Experiment 1

Four *A. lineatus* beetles were tested 10 times each, firstly in the control and secondly in the pheromone treatment, in order to determine the variation in individual walking behaviour between tests within the same individual and to test the equipment. A repeated-measures ANOVA was used to assess whether there was any significant variation in average speed between individuals, and in pheromone and control

treatments. The mean speed of the 10 tests for each beetle was used in order to avoid pseudoreplication (Lazic, 2010). Speed data were plotted for each individual and treatment. Since the sample size was small (N <10) significance testing for arm choice was not appropriate. Instead, in order to visually assess the variation in arm choice between individuals and treatments, the number of choices made for the left and right or pheromone and control (no pheromone) arm was plotted for each individual.

There was a significant difference between individuals (F(1) = 93.009, p = < 0.001), but not between treatments. These differences were between beetle 2 and beetles 1, 3 and 4 in the pheromone treatment (Figure 5.5).
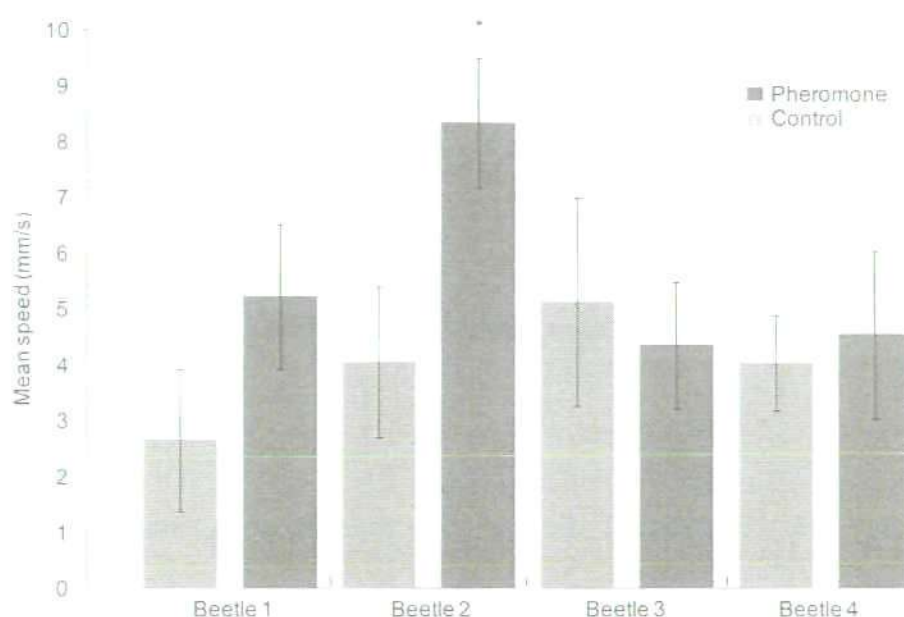
Figure 5.5. Mean speed (mm/s; of 10 replicates) of male *A. lineatus* click beetles (N = 4) in a Y-tube olfactometer with pheromone and control (no pheromone) treatments. Bars represent 95% confidence intervals, and asterisks indicate significant differences (P<0.05).

There was considerable variation in speed of individuals between tests in both the

pheromone and control treatments. For example, in the control treatment the speed

of beetle 1 increased from 3mm/s to 10mm/s between tests 9 and 10 (Figure 5.6),

while in the pheromone treatment speed from 6mm/s to 12 mm/s between tests 1

and 6 (Figure 5.7).
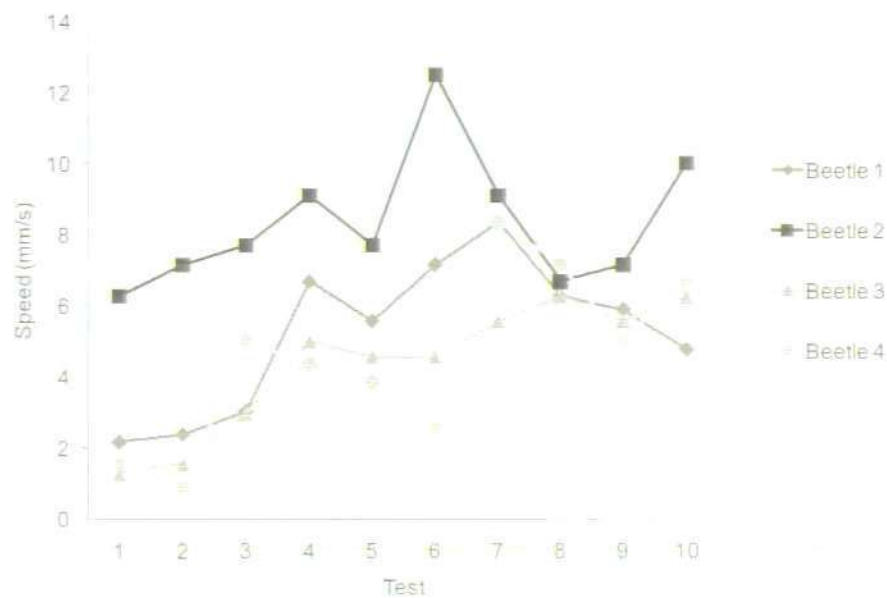
Figure 5.6. Average speed (mm/s) of male *A. lineatus* click beetles in each replicate (*N*

= 4, 10 replicates each) in a Y-tube olfactometer in the control treatment (no sex

pheromones).
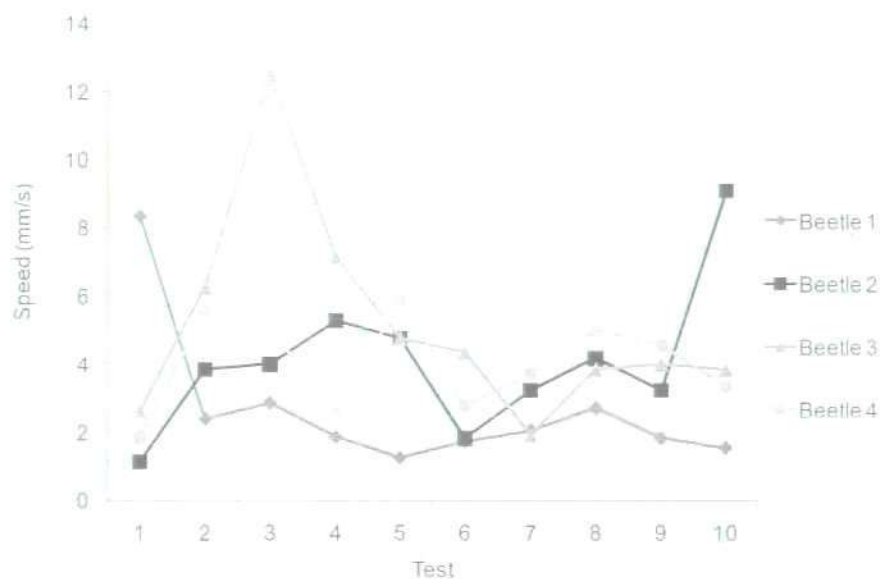
Figure 5.7 Average speed (mm/s) of male *A. lineatus* click beetles in each replicate (*N* = 4, 10 replicates each) in a Y-tube olfactometer with sex pheromones.

Arm choice was also variable between individuals and treatments. Beetle 2 chose only the left arm in all 10 replicates in the control treatment (Figure 5.8) and the pheromone treatment (Figure 5.9), while the pheromone arm was chosen as frequently as the non-pheromone arm (Figure 5.10). Similarly, beetle 3 chose the left arm more often in the control and pheromone treatment, but the pheromone arm more often in the treatment. The arm choice of beetle 1 and beetle 4 did not suggest a strong arm preference in pheromone or control treatments.

Figure 5.8. The proportion of choices made by male *A. lineatus* beetles (*N* = 4, 10 replicates) for the left and right arm of the Y-tube olfactometer in the control (no pheromone) treatment.

Figure 5.9. The proportion of choices made by male *A. lineatus* beetles (*N* = 4, 10 replicates each) for the left and right arm of the Y-tube olfactometer in the pheromone treatment.
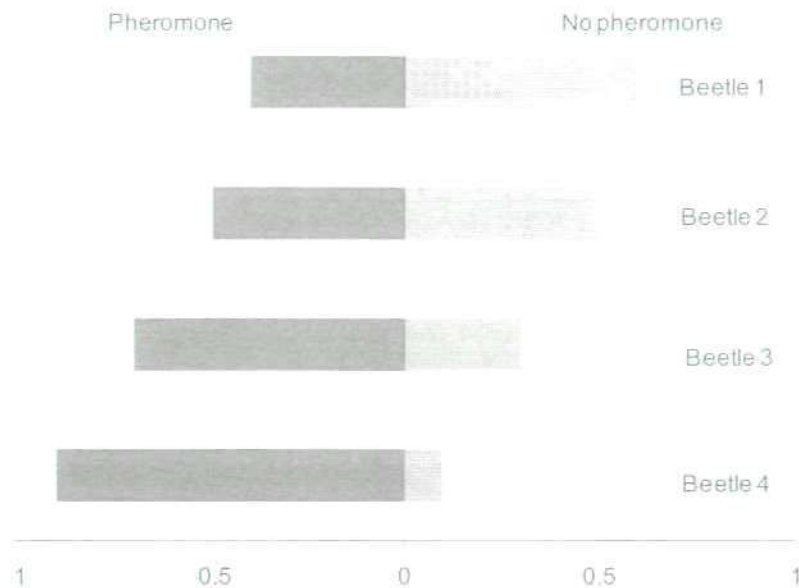
Figure 5.10 The proportion of choices made by male *A. lineatus* beetles (*N* = 4, 10 replicates each) for the pheromone and no pheromone (control) arm of the Y-tube olfactometer.

5.4.2 Experiment 2

In this experiment, interspecific differences in walking speed in sex pheromone and control treatments was assessed. Four individuals from all three species were tested in each treatment, and the number of replicates for each individual was reduced to three. In addition, each replicate was carried out in a different Y-tube, and the order of testing of individuals was varied randomly. Average walking speed was normally distributed and the homogeneity of variance assumption was met, therefore a one way ANOVA was used to statistically analyse the results. *Agriotes sputator* was not compared to the other two species in the pheromone treatment due to missing data (individuals turned around and tried to exit the tube); therefore a t-test was used to compare *A. obscurus* and *A. lineatus* walking speed. The average speed for the 3

replicates was used for each individual in each species to avoid pseudoreplication (Lazic, 2010). Again, due to the small sample size (N <10) the binomial test could not be used to determine the significance of arm choice. In order to visually assess choices made between *A. lineatus* and *A. obscurus* individuals, the number of choices made for the left and right or pheromone and control (no pheromone) arm was plotted for each species.

There was no significant difference in mean walking speed within or between (Figure 5.11) species in the control and pheromone treatments.
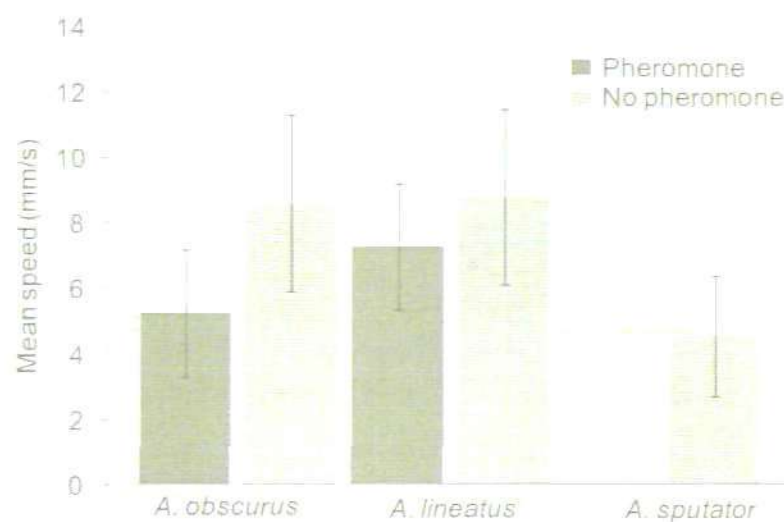
Figure 5.11 Mean walking speed (mm/s) of *A. obscurus* (N = 4), *A. lineatus* (N = 4) and *A. sputator* (N = 4) in a Y-tube olfactometer in pheromone and non-pheromone (control) treatments. Bars represent 95% confidence intervals.

Overall, there was little difference in the proportion of choices for the left or right arm (or the pheromone or control arm) of the Y-tube olfactometer for *A. lineatus* (Figure 5.12), but *A. obscurus* individuals preferred the left to the right arm in the control treatment (Figure 5.13).
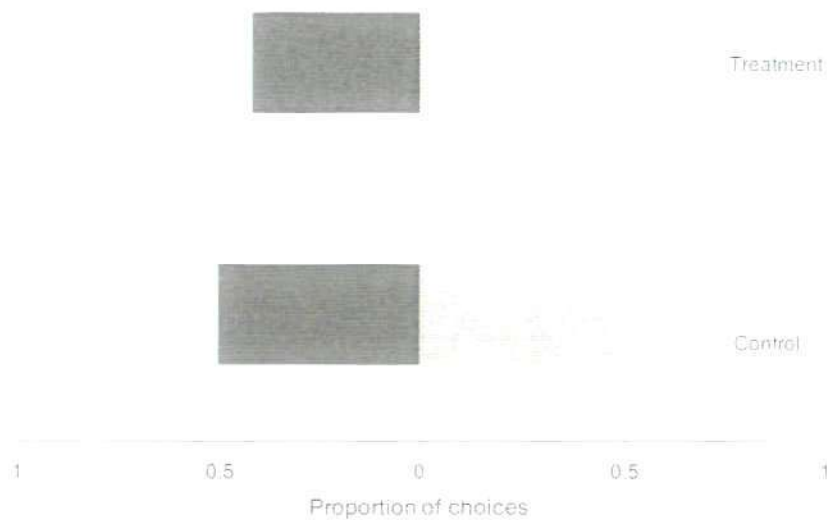
Figure 5.12 Proportion of choices made by male *A. lineatus* beetles (N = 4; 3 replicates) for each arm of the Y-tube olfactometer in the control (left arm= dark grey, right arm = light grey) and pheromone (pheromone arm = dark grey, non- pheromone (control) arm = light grey) treatments.
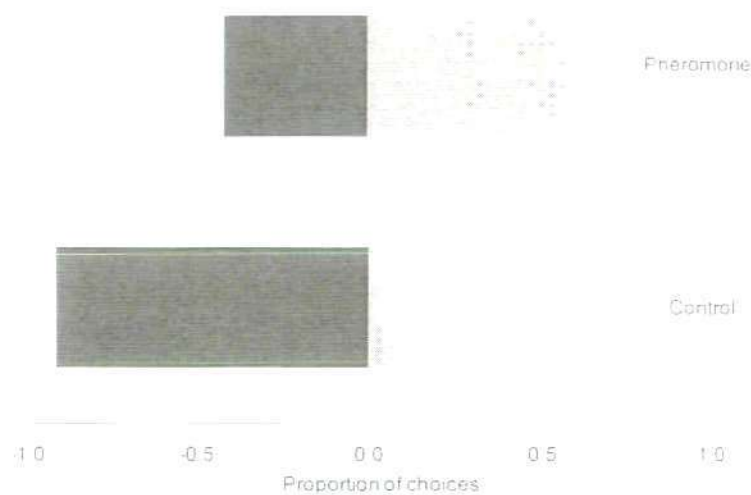
Figure 5.13 Proportion of choices made by male *A. obscurus* beetles (N = 4; 3 replicates) for each arm of the Y-tube olfactometer in the control (left arm= dark grey, right arm = light grey) and pheromone (pheromone arm = dark grey, non- pheromone (control) arm = light grey) treatments.
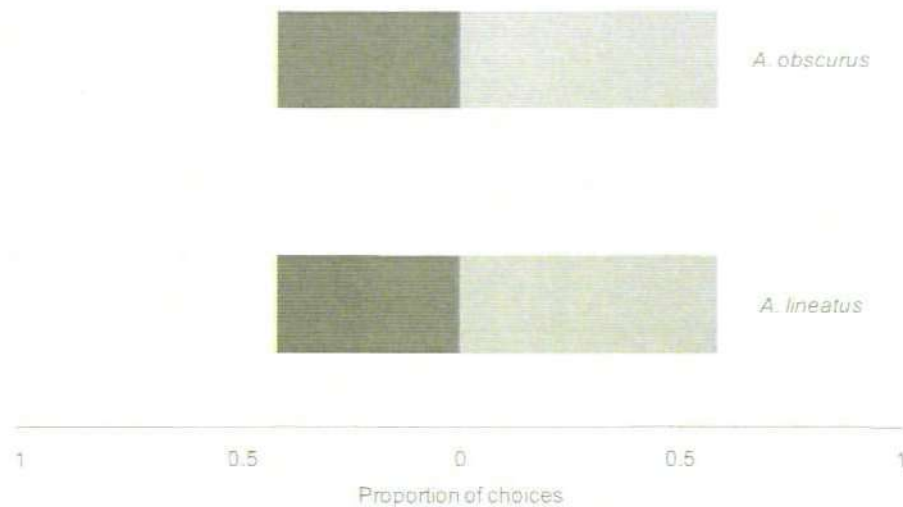
Figure 5.14 Proportion of choices made by male *A. obscurus* and *A. lineatus* beetles (*N* = 4; 3 replicates) for each arm of the Y-tube olfactometer in the pheromone treatment; left arm= dark grey, right arm = light grey.

5.4.3 Experiment 3

In this experiment speed and arm choice with no pheromones was tested using as many available individual beetles as possible. A larger sample size would enable a more robust assessment of the variation in speed between species, and the applicability of the equipment for measuring this locomotion variable. Unfortunately, sample sizes were variable and although normally distributed the homogeneity of variance assumption was not met. The non-parametric Kruskall Wallis test was therefore used to determine whether there were any significant differences in walking speed between the species. Arm choice was plotted as the proportion of choices for the left and right arm of the olfactometer.

There was no significant difference in walking speed between *A. lineatus*, *A. obscurus* and *A. sputator* (Figure 5.15). The proportion of choices for the left and right arm of the Y-tube was also very similar, within and between species (Figure 5.16).
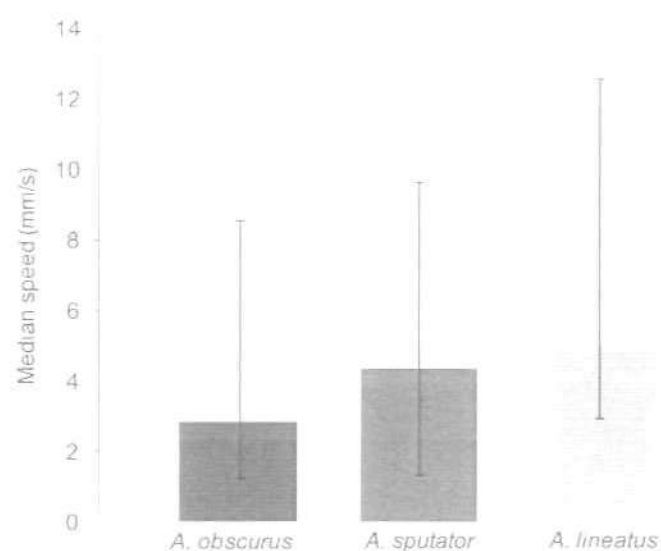
Figure 5.15 Median speed of male *A. obscurus* (N = 4), *A. sputator* (N = 32) and *A. lineatus* (N = 14) in a Y-tube olfactometer. Bars represent the 25% and 75% percentiles.

Figure 5.16 The proportion of choices made by male *A. obscurus* (N = 4), *A. sputator* (N = 32) and *A. lineatus* (N = 14) beetles for each arm of the Y-tube olfactometer.

5.5 Arena

Automated video tracking using an arena was set up as follows: a white plastic arena (40cm diameter, 10cm height) was placed on an A3 sheet of white paper, above which a video camera fixed onto a stand was placed in order that the field of view covered the whole set-up. Two lamps, one on either side of the arena, were used to illuminate the experimental area. The camera was attached to a computer containing a frame grabber which digitises the video output and stores it in the computer memory. Ethovision XT 7 (Noldus Information Technology, Wageningen, The Netherlands) was used to measure and analyse click beetle walking movements over 10 minutes (walking speed, track length, vector length and straightness; Table 5.1). Detection was based on grey scaling, and the position of the beetle was recorded 25 times/s and stored as X, Y- coordinates. Recording started as soon as the beetle was placed in the centre of the arena. Only *A. obscurus* ($N = 29$) beetles were available for trials, which were run over 24 days. The arena was rinsed with 70% ethanol and distilled H_2O and a new sheet of paper was used after every 5 trials and/or between days.

5.5.1 Comparison of measurements between experiments

In order to compare experiments, arena walking tracks were analysed over 5 minutes. Day of trial had no measurable effect on any of the observed behaviour (Kruskall-Wallis test, $p > 0.05$), and therefore results were combined. Since data for *A. lineatus* and *A. sputator* was not available for all three experiments, a Mann-Whitney U-test was used to determine whether there was any significant difference in measured walking speed, track length, vector length and straightness of *A. obscurus* in the locomotion compensator, Y-tube olfactometer and arena experiments.

There was no significant difference in the measured walking speed of *A. obscurus* between the three experiments, but a significant difference in track length ($U = -3.477$, $N = 34$, $p < 0.001$), vector length ($U = -3.477$, $N = 34$, $p < 0.001$) and straightness ($U = -3.428$, $N = 34$, $p < 0.01$; Table 5.2) between the individuals measured on the locomotion compensator and in the arena.

	Average speed (mm/s)	Track length (mm)	Vector length (mm)	Straightness	N
Locomotion compensator	5.34 (2.86 - 7.82)	1600.12 (857.07 - 2343.17)	1166.595 (517.89 - 2131.1)	0.76 (0.6 - 0.92)	5
Olfactometer	3.38 (1.1 - 5.7)	-	-	-	4
Arena	8.46 (6.86 - 10.06)	2536.71 (2058.60 - 3014.83)	159.22 (148.31 - 170.21)	0.11 (0.05 - 0.16)	29

Table 5.2 Walking parameters for *A. obscurus* in three different experiments (locomotion compensator, Y-tube olfactometer and arena). The mean and 95% confidence intervals (in brackets) for speed (mm/s), track length (mm), vector length (mm) and straightness are given.

Walking tracks in the arena were variable between individuals, but beetles mainly walked in loops around, or close to, the edge of the arena (Figure 5.17). In contrast, tracks on the locomotion compensator were generally more linear with fewer turns (Figure 5.18).
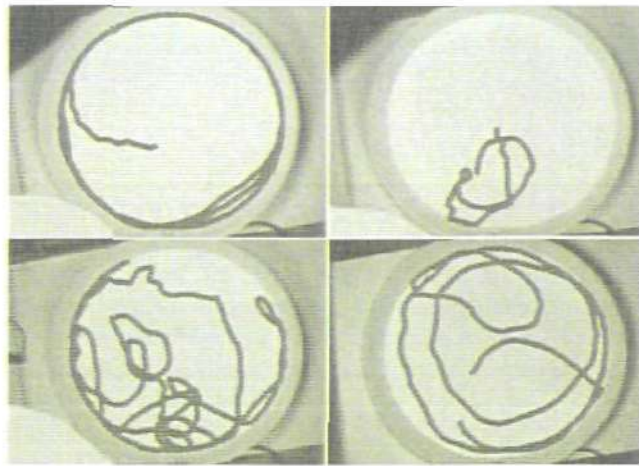
Figure 5.17 Examples of walking tracks produced by individual *A. obscurus* beetles in an arena over 5 minutes.

Figure 5.18 Example of tracks produced by an *A. sputator* beetle on the locomotion compensator with pheromones (left) and in the control (clean air flow) (right), and an *A. obscurus* beetle in still air (bottom). The beetle starts walking from the centre of the graph. Air flow and pheromone direction is indicated by the arrow.

5.6 Discussion

5.6.1 Interspecific walking movements

The difference in walking speed of the three species on the locomotion compensator (*A. lineatus* > *A. obscurus* > *A. sputator*; Figure 5.2a), although limited by small samples size, concur with the results of the mark-release-recapture study (Hicks & Blackshaw, 2008). These differences in movement rates between species may account for the observed difference in their distributions, as if *A. lineatus* are able to disperse more quickly and over larger distances, then they are likely to be trapped in higher numbers over a given time period, as was observed in Chapter 2 and by Hicks and Blackshaw (2008). Indeed, as might be expected, since beetles rarely stopped walking there was also a significant difference in track length between these species; Figure 5.2b). Hicks and Blackshaw (2008) estimated the average speed of *A. lineatus* to be 1.28m/day, *A. obscurus* 0.89m/day and *A. sputator* 0.46m/day, assuming movement is in a straight line towards the pheromone trap, and Kishita *et al.* (2003) determined the average dispersal distance of *Melanotus okinawensis* to be 130.1m/day. Extrapolation of measurements on the locomotion compensator would result in an average speed of 511.48m/day for *A. obscurus*, 326.59m/day for *A. sputator* and 790.9m/day for *A. lineatus*. These figures are unrealistic given that individuals do not move all day, and are likely to encounter changes in habitat and conspecifics/predators that alter their walking behaviour and speed. This suggests that in order to model dispersal at larger scales based on small scale experimental measurements, data on key behaviours affecting movement in the field are required (Firle et al., 1998; Morales & Ellner, 2002). The lack of any significant difference in average speed between species in the Y-tube olfactometer experiments (experiment 2; Figure 5.11 and experiment 3; Figure

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5.15), is possibly due to the experimental set-up and the unsuitability of this equipment for measuring click beetle walking movement (see section 5.3).

Although click beetles are thought to disperse mainly by walking, these species have been observed in flight over the three seasons of sex pheromone trapping for this study and by other researchers (Crozier, Tanaka & Vernon, 2003; Fryer, 1941; Roebuck, Broadbent & Redman, 1947), though no studies have considered the dispersal implications of this in detail. The Yatlor traps are designed to trap both flying and walking beetles; however, although modifications were made using a sticky trap lining in 2008, no flying beetles were captured. It is not known whether this was due to deficiencies in the sticky trap modification or lack of flying beetles at the time the traps were placed. A further study of 12.2m suction trap catches from the Rothamsted Insect Survey (Harrington & Woiwod, 2007) from 2005 – 2008 revealed no click beetles. Hicks (2008) suggests that *A. lineatus* may disperse by walking and occasional flight over long distances when weather conditions, particularly temperature, trigger flight. Kishita et al. (2003) also suggest that warm temperatures activate the response of male *M. okinawensis* to sex pheromones, stimulating long-distance flight. Quantitative investigation of click beetle flight may reveal further differences in dispersal ability between species.

5.6.2 Response to sex pheromones

There was a tendency for *A. sputator*'s average speed, track length and straightness to decrease between treatments (still air < air flow < pheromone) on the locomotion compensator, though responses were more variable in terms of upward straightness and upward length between individuals in each treatment. Previous studies have

found that for some species tested on the locomotion compensator upward straightness and speed increased when a stimulus was applied (e.g. Donze, McMahon & Guerin, 2004; Otalora-Luna, Perret & Guerin, 2004), but for others straightness and speed was reduced with increased stimulus concentration, behaviour that may indicate a searching phase (Hammock, Vinyard & Dickens, 2007). It may have been expected in the Y-tube olfactometer experiment that individuals would consistently choose the pheromone arm over the control arm of the Y-tube, but this was not the case for all *A. obscurus* and *A. lineatus* individuals (although significance was not tested). There was a tendency to choose the left arm for two of the *A. lineatus* beetles (Figure 5.8 and Figure 5.9), which may indicate problems with the experimental set-up, but specific reasons are difficult to hypothesise since sample sizes were small; with larger sample sizes these results may seem anomalous. The apparent upward trend in *A. lineatus* walking speed with repeated exposure to sex pheromones (Figure 5.5) compared to the control (Figure 5.6) in the Y-tube olfactometer experiment may suggest individuals are learning to respond, or that they need repeated exposure to the stimulus to respond. Alternatively, they may be looking for a way to escape; there were significant problems in the operation of the Y-tube olfactometer experiment for *A. sputator*, where although it was possible to measure speed of this species in the control treatment, as soon as the beetle entered the Y-tube in the pheromone treatment they would turn around and exhibit escape behaviour. Unlike *A. lineatus* and *A. obscurus*, they were able to change direction in the tube due to their small size. It is possible that *A. sputator* may have been more sensitive to sex pheromone exposure; practically it was very difficult to ascertain the amount of pheromones present in the equipment and the only way to control it was by changing the airflow. Another possibility is that this species responds differently to sex pheromones. The

beetles tested were not sexed and so it is not certain that they were all male, but previous research has shown that the number of females trapped in sex pheromone traps is only approximately 1% of the total and so this is unlikely to be a factor (Hicks, 2008). *Agriotes sputator* was the only species tested with pheromones on the locomotion compensator, so testing of *A. obscurus* and *A. lineatus* may reveal differences, as they have in general walking behaviour on the locomotion compensator.

Since all the beetles used in these experiments had been previously exposed to sex pheromones this may have affected results. It was attempted to capture adult beetles using pitfall traps over a 2 week period in agricultural fields (traps were emptied every 2-3 days), but none were obtained. A more efficient method of capturing live adult beetles would be required to test the response of non-sex pheromone trapped beetles to sex pheromones, but this may also allow capture of female beetles for analysis of movement behaviour.

5.6.3 Comparison of measurements between experiments

Although *A. obscurus* walking speed was not influenced by experimental design, there were significant differences in track length, vector length, straightness, and behaviour of beetles, between the locomotion compensator and arena experiments. This suggests that beetles respond differently depending on the experimental set-up. That no significant difference in average speed was found between the species in the Y-tube olfactometer may be due to the experimental design. Speed was measured in a straight tube with a relatively narrow diameter over 100mm, which may be too short a distance and too restrictive to movement for any apparent differences to emerge. This

design may also cause concentration of the pheromone which in a small area may disorientate the beetles. In sex pheromone traps in the field, pheromone vials are 25cm above the ground, and so the pheromone is more likely to disperse and become less concentrated depending on the wind direction. However, in these experiments the pheromone was probably present in a concentrated plume, which could have overwhelmed the beetle. Rather than applying pheromone with a narrow stimulus tube on the locomotion compensator, as in this experiment, an alternative might be the use of a wind tunnel (Thiery & Visser, 1986; Tinzaara et al., 2003). This consists of a large tube (100cm length, 80cm height; Visser, 1976) containing the stimulus at one end, with a contracted opening at the other from which air flows over the sphere, which may allow more natural dispersal over the beetle. Another method could be to carry out experiments with a locomotion compensator *inside* a larger wind tunnel, increasing the area of pheromone dispersion which will re-create conditions closer to those in the field. As limited walking parameters can be measured in the Y-tube olfactometer, the locomotion compensator and arena are more suitable for assessing click beetle movement behaviour.

Whereas the locomotion compensator has no boundaries and beetles are able to move a limitless distance (beetles rarely stopped walking on the locomotion compensator), movement in the arena is restricted by the arena walls, meaning once beetles encounter an edge they tend to continuously walk around it trying to find an escape route (in the majority of tracks the beetle spent at least some time walking around the edge e.g. Figure 5.17). This also had an effect on straightness; whereas it was close to 1 (linear movement) for beetles on the locomotion compensator, in the arena tracks it was closer to 0 (tortuous movement), which can be seen visually in the

tracks produced in each experiment (Figure 5.17 and Figure 5.18). The experimental

environment of both the arena and the locomotion compensator are likely to seem

hostile to the click beetles, since there was no shelter, food or conspecifics, and it is

therefore possible that their behaviour represented that of escape; whereas tortuous

paths allow greater exploration of an area, linear paths will effectively locate resources

spaced further apart, allowing escape from an unsuitable area (Morales & Ellner,

2002). This behaviour has also been observed in German cockroaches in stressful

situations relating to unfamiliar environments in an arena (Durier & Rivault, 2003).

Firle et al. (1998) and Morales and Ellner (2002) suggest that to describe movement at

larger scales based on measurements of small scale movements, the complexities of

individual behaviour need to be taken into account. In this study, sample sizes for

some species and/or experiments were relatively small (N = 4 or 5), but individual

variation was noted. Measurement of a large number of individuals for each species, in

as wide a range of habitats and situations likely to be encountered in the field as

possible, would allow generation of an accurate random walk model, meaning easily

studied small scale walking behaviours could be scaled up to determine dispersal

ability at the scale at which it occurs.

Despite the limitations noted here, significant differences in walking movements

between A. lineatus, A. obscurus and A. sputator were found and there was some

success, for A. sputator at least, in measuring response to sex pheromones. These

results corroborate already existing data on click beetle walking speed and

distribution. It would not be recommended to use the Y-tube olfactometer protocol

tested in this study for measurement of click beetle walking movement, but with

modification this could have some use in testing adult female crop preference, which

may provide information on whether olfactory cues are used determining oviposition sites. Modification of the experimental designs used for the locomotion compensator and arena, discussed above, could include the use of non-sex pheromone trapped adults, a wind tunnel instead of a stimulus tube for application of pheromones, the testing of each beetle only once to prevent learning/escape behaviour, modification of the (arena) environment to include habitat structures, and testing a larger sample size. In addition, experimental studies on flight to test observations noted in the field would provide further, more accurate information on click beetle dispersal dynamics. Indirect methods of measuring dispersal via molecular analyses (gene flow between populations; Chapter 6) combined with this information would provide estimates of maximum dispersal distances.

Chapter 6   The development of amplified fragment length polymorphism (AFLP)

markers for assessing click beetle dispersal in agricultural land

6.1 Introduction

The small scale, within field movement of adult male *Agriotes obscurus*, *A. lineatus* and

A. sputator has been assessed using mark-release-recapture (Hicks & Blackshaw,

2008), carbon stable isotopes (Schallhart *et al.*, 2009), and behavioural studies (see

Chapter 5), revealing differences in walking speed between species. However, larger

scale studies i.e. at the farm, regional or national scales, are lacking. Knowledge of a

pests ability to invade and reproduce in new habitats is important, since this

information can be used to determine the correct spatial scale at which to implement

management practices (Kazachkova, Meijer & Ekbom, 2008). In the case of *Agriotes*

obscurus, *A. sputator* and *A. lineatus* differences in dispersal range may also partly

explain the interspecific differences in adult and wireworm distribution observed in

previous studies (see Chapters 2 and 3). The limitations of directly observing

movement in the field and the laboratory were discussed in Chapter 5.

Since dispersal and subsequent successful reproduction leads to gene flow, a range of

molecular markers and methods have been developed and applied to estimate

dispersal range on the basis of genetic similarity among pairs of populations in relation

to the geographic distance between them (Bohonak, 2002; Broquet & Petit, 2009;

Manel et al., 2003; Rousset, 1997). For example, Darvill et al. (2006) used

microsatellites to estimate the dispersal range of a rare bumblebee species, and Van

Der Wurff et al. (2003) used microsatellites and three enzyme–amplified fragment

length polymorphism (TE- AFLP) to determine the dispersal ability of the soil invertebrate *Orchesella cincta* Linnaeus.

For this study it was originally intended to use microsatellites to assess adult male dispersal at the farm, regional and national scales. A small panel of microsatellite loci were available for *Agriotes*; sequences were previously isolated, but not further tested by J. Ellis (at University of Plymouth). However none were found to be polymorphic on initial screening in the early stages of research for this thesis. Therefore, amplified fragment length polymorphisms (AFLP) were chosen as an alternative. The original AFLP method, developed by Vos et al. (1995), has been applied mainly to research on plants, bacteria and fungi, the majority being crop species or economically important organisms (Bensch & Åkesson, 2005), but recently the approach has been used for studies on insects e.g. the pollen beetle *Meligethes aeneus* Fabricius (Kazachkova, Fahleson & Meijer, 2004; Kazachkova, Meijer & Ekbom, 2008), the moth *Ephestia kuehniella* Zeller (Ryne & Bensch, 2008), Spruce Gall Adelgids (Ahern, Hawthorne & Raupp, 2009) and Colorado potato beetle *Leptinotarsa decemlineata* Say (Hawthorne, 2001).

Although AFLPs are largely dominant in nature and so can be less informative than co-dominant markers, such as microsatellites, they are commonly cited as having several advantages over other molecular markers e.g. microsatellites, mitochondrial DNA (MtDNA), random amplified polymorphic DNA (RAPD) and expressed sequence tags (EST). These include time and cost efficiency, that no prior genomic knowledge of the target organism is needed, that they can be analysed using manual polyacrylamide/agarose gel electrophoresis or automated genotypers, they are reproducible, and only minimal (albeit high quality) amounts of DNA are needed

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(Mueller & Wolfenbarger, 1999). However, due to considerable problems in the development process, AFLP markers could not be optimised for *A. obscurus*, *A. lineatus* or *A. sputator* despite considerable investment in overcoming the technical difficulties encountered. The process and results from each step will be described and possible reasons for the failure to generate markers discussed, with suggestions for future optimisation of the protocol.

6.2 Development of a specific AFLP technique for click beetles

The AFLP technique, originally described by Vos et al. (1995), involves 4 steps (Meudt & Clarke, 2007). Step 1: genomic DNA is digested with two restriction enzymes (a rare cutter and a frequent cutter, usually *Eco*RI and *Mse*I), producing three types of DNA fragments: *Eco*RI-*Eco*RI, *Mse*I-*Eco*RI, *Mse*I-*Mse*I. Step 2: Double stranded *Eco*RI and *Mse*I adapters with complementary 'sticky ends' (unpaired nucleotides on the terminal end of the adapters) are ligated to the restriction fragments. Step 1 and 2 can be performed in the same reaction. Step 3: Using primers that are complementary to the adapter sequences, with the addition of one nucleotide (A, C, T or G) at the 3' end (*Eco*+1 and *Mse*+1), a pre-selective PCR amplifies a subset of these fragments. Since only a specific subset of the many available fragments will be amplified (only those with bases flanking the restriction sites that are complementary to the selective nucleotides of the primers), the number of fragments is reduced. Step 4: A second, selective PCR reduces the number of fragments further (to a suitable number for analysis), using the same primers with an additional 2 selective bases (*Eco*+3 and *Mse*+3). Primers with different combinations of selective bases will amplify different sub-sets of loci, and each individual, selective *Eco*+3 primer can be labelled with a

different fluorescent dye (meaning *EcoRI-EcoRI* and *MseI-EcoRI* fragments will be visualised), enabling PCR products to be pooled for capillary electrophoresis. Labelled fragments are then detected by electrophoresis, and polymorphisms are visible on a Genetic Analyser (in this case an AB3130) as peaks present in some samples but not others, caused by the gain or loss of a restriction site, change in the selective primer binding site, or a length polymorphism between restriction sites (Meudt & Clarke 2007). A binary data matrix is produced by aligning multiple individual profiles and scoring based on the presence (1) or absence (0) of a peak.

Modifications to the original protocol for different study organisms have been made, including type of restriction enzymes, starting amounts of template DNA, type of *Taq* and the number of bases added to selective primers (Kazachkova, Fahleson & Meijer, 2004). Similarly, here a specific protocol was developed for the AFLP analysis of click beetles.

6.2.1 Samples

Adult male *A. obscurus*, *A. lineatus* and *A. sputator* click beetles, trapped using species-specific sex pheromone traps from grass fields, were available from 10 sites in the UK; Figure 6.1). In Devon, 6 sites were located 2-19km apart in the South Hams, sampled in 2004, and 1 was located in Okehampton approximately 33km away, sampled in 2010 (Figure 6.1a). Two sites were located in Cambridge approximately 21km apart, sampled in 2008 and 2010. One site was located in Scotland, approximately 740km from the Cambridge sites and 900km from the Devon sites, sampled in 2010. Samples were either transferred to 70-100% ethanol or frozen following collection in the field. Development was primarily carried out using *A. obscurus* beetles only, under the

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assumption that the protocol would be suitable for all three species, possibly with minor optimisation.

Figure 6.1 Location of sample sites for *A. obscurus*, *A. lineatus* and *A. sputator* adult male click beetles for AFLP analysis a) in Devon (6 sites in the South Hams; Tidwell, Riverford, Borough, Yelland Cross, Home Park and Higher Ludbrook, and 1 site in Okehampton and b) across the UK.

6.2.2 DNA extraction

Complete digestion of DNA in the restriction-ligation step is critical since partial digestion may result in false bands being interpreted as polymorphisms. Contamination of DNA with inhibitors can prevent complete digestion, and so clean DNA is an important pre-requisite for the AFLP assay (Reineke, Karlovsky & Zebitz, 1998). To ascertain the most appropriate protocol for DNA extraction from click beetles (in terms of DNA concentration and purity), DNA was extracted from the legs, head and thorax (avoiding the abdomen, which may contain contaminants) of *A.*

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obscurus, A. sputator and *A. lineatus* adult click beetles using salt/chloroform (Rico, Kuhnlein & Fitzgerald, 1992), spin column (DNeasy Blood and Tissue Kit; Qiagen, Crawley, UK) or ammonium acetate (Original protocol described by Nicholls *et al.*, 2000) extraction methods, and re-suspended in 50μl DNA grade H_2O. Further purification steps included gel extraction of 5μl DNA from 2% agarose gel using spin columns (QIAquick Gel Extraction Kit; Qiagen); spermine precipitation (Reineke et al., 1998) and digestion of the extract with 1 unit RNaseI at 37°C for 1 hour to remove excess RNA. The concentration and purity of DNA was analysed using a Nano-Drop 1000 spectrophotometer (LabTech, Ringmer, UK) and gel electrophoresis (2% agarose gel made with 10% TBE buffer, 110V, 1.5 hours).

The variability in DNA concentration between samples was large, even within the same species using the same extraction methods (Table 6.1). For both ammonium acetate and salt/chloroform extraction protocols, more concentrated DNA was obtained from frozen beetles, suggesting this is the best method for preservation. Although salt/chloroform DNA extraction was suitable for molecular analyses of click beetle DNA used previously (T-RFLP and sequencing), for AFLPs, methods which use phenol or chloroform may result in partial digestion of DNA if these are not removed efficiently (Kazachkova, Fahleson & Meijer, 2004). Therefore, although relatively concentrated and pure DNA was obtained, it was decided not to adopt this method. The most appropriate method was found to be an ammonium acetate protocol with several modifications (Table 6.1; Appendix 5): 1. Samples were left in the DigSol digestion buffer with Proteinase K at 55°C overnight, without shaking. 2. Following the addition of 100% ethanol during the pellet washing stage, samples were placed at -20°C for 20 minutes before centrifugation for 20 minutes 3. After the final wash with 500μl 70%

ethanol the tubes were centrifuged for an extra 5 minutes at 11337g. 4. The DNA pellets were dried on a heat block at 50°C for 1-2 minutes until completely dry and resuspended in 50µl DNA grade H₂O.

Subsequent purification of extractions with spermine precipitation and gel extraction resulted in very low concentrations of DNA, and although the addition of the RNAseI step reduced the smearing produced by DNA extracts (via the removal of RNA), it did not visibly affect the success of the restriction-ligation step, and so no further purification methods were carried out for subsequent extractions.

Species	Preservative method	Ammonium acetate	Salt/chloroform	Spin column	Spermine precipitation after ammonium acetate	Spermine precipitation after salt/chloroform	Spermine precipitation after spin column
A. lineatus	Frozen	4.4	60.6	-	0.5	7.2	-
A. lineatus	Frozen	69.7	47.7	10.1	6.4	7.3	-
A. lineatus	100% ethanol	-	38.9	48.7	-	3	15.5
A. obscurus	Frozen	37.9	8.4	-	6.4	0.7	-
A. obscurus	100% ethanol	-	13.2	16.2	-	0.7	6.4
A. obscurus	100% ethanol	-	6.9	1.8	-	1.3	1.3
A. obscurus	Frozen	76.5	-	8	-	-	-
A. obscurus	Frozen	27.1	-	10.4	-	-	-
A. sputator	Frozen	22.7	-	17.1	-	-	-
A. sputator	Frozen	56.3	43.5	-	4.1	1.8	-
A. sputator	100% ethanol	-	41.7	29.1	-	5	8.8

Table 6.1 The DNA concentration (ng/μl) of samples extracted using ammonium acetate, salt/chloroform and spin column methods, and purified using spermine precipitation for A. obscurus, A. lineatus and A. sputator adult click beetle samples.

6.2.3 Restriction enzyme digest

Although *Mse*I and *Eco*RI are the most commonly used restriction enzymes in AFLP analyses, other studies have used combinations with *Taq* (frequent four-base cutter, often used for mammalian and vertebrate genomes) and *Pst*I (rare six-base cutter, often used for large genomes of > 5000MB (Vos & Kuiper, 1997). Here, all four of these restriction enzymes were tested in combination to determine which would produce the best size range of fragments: an even distribution of fragments up to around 700bp (Kazachkova, Fahleson & Meijer, 2004). Different enzyme (10 units, 50 units or 60 units) and DNA concentrations (100ng, 150ng or 200ng) were tested.

Reactions consisted of ammonium acetate extracted *A. obscurus* DNA, restriction enzymes and 100ng/µl BSA, made up to 30µl total volume with DNA grade H_2O. Digestions were carried out at 37°C for 3 hours, with inactivation for 15 minutes at 65°C. The whole reaction was then run out on a 2% agarose gel for 1.5 hours at 110V. The most suitable combination of restriction enzymes was determined to be *Eco*RI/*Mse*I, which produced a smear from around 200-800bp (Figure 6.2). Therefore this combination was used in subsequent reactions. The concentration of DNA did not significantly improve restriction enzyme digestion, although it did improve the visibility of the reaction on the gel. DNA concentration is not thought to be a critical factor in AFLP assays since a number of protocols have been successfully developed using a wide range of DNA concentrations (Trybush *et al.*, 2006). Restriction enzyme concentrations of 50 and 60 units were not successful, but 10 units were sufficient for the reaction to work.
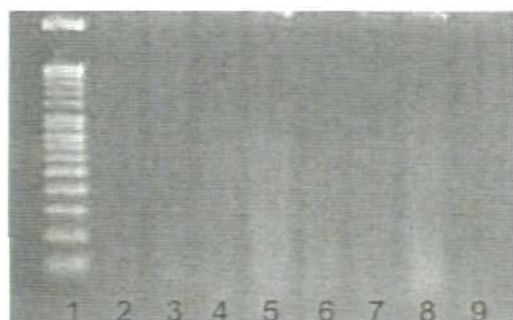
Figure 6.2 Restriction enzyme digest run out on a 2% agarose gel for 1.5 hours. Lane 1, 50bp ladder *Mse*I; lane 2, *Eco*RI; lane 3, *Pst*I; lane 4, *Taq*I; lane 5, *Mse*I/*Eco*RI; lane 6, *Mse*I/*Taq*I; lane 7, *Mse*I/*Pst*I; lane 8, *Eco*RI/*Taq*; lane 9, *Eco*RI/*Pst*I.

6.2.4 Restriction/ligation

The restriction-ligation can be carried out in one step, and so this was done based on the protocol of Kazachkova, Fahleson and Meijer (2004) with some modifications. *Eco*RI and *Mse*I adapters were as described by (Vos *et al.*, 1995, ; Table 6.2). Restriction-ligation was carried out in a total reaction volume of 30µl in individual 0.5ml PCR tubes, combining 200ng template DNA, 10X ligation buffer, 2mM ATP, 100ng/ul BSA, 0.05M NaCl, 10 units each of *Eco*RI and *Mse*I, 10 units T4 DNA ligase, and 5pm of each double stranded adapter (1nmol of each strand was mixed with 200µl DNA grade H_2O, heated to 98°C for 5 minutes then cooled slowly to room temperature (1nmol of each strand was mixed with 200µl DNA grade H_2O, heated to 98°C for 5 minutes then cooled slowly to room temperature; Weising et al., 2005). These were incubated at 37°C for 3 hours, then 65°C for 10 minutes to inactivate the enzymes. To check the reaction had worked, 25µl was run out on a gel, as per the restriction enzyme digest.

MseI adpaters	5' GAC GAT GAG TCC TGA G 3'
	3' TAC TCA GGA CTC AT 5'
EcoRI adapters	5' CTC GTA GAC TGC GTA CC 3'
	3' CAT CTG ACG CAT GGT TAA 5'
EcoRI pre-sel	GAC TGC GTA CCA ATT CC
MseI pre-sel	GAT GAG TCC TGA GTA AC
EcoRI sel 1	6FAM GAC TGC GTA CCA ATT CCC G
EcoRI sel 2	NED GAC TGC GTA CCA ATT CCT A
EcoRI sel 3	PET GAC TGC GTA CCA ATT CCA T
EcoRI sel 4	6FAM GAC TGC GTA CCA ATT CCG A
EcoRI sel 5	NED GAC TGC GTA CCA ATT CCT G
EcoRI sel 6	PET GAC TGC GTA CCA ATT CCA C
MseI sel 1	GAT GAG TCC TGA GTA ACT T
MseI sel 2	GAT GAG TCC TGA GTA ACA G
MseI sel 3	GAT GAG TCC TGA GTA ACA A
MseI sel 4	GAT GAG TCC TGA GTA ACG G
MseI sel 5	GAT GAG TCC TGA GTA ACC C
MseI sel 6	GAT GAG TCC TGA GTA ACT C

Table 6.2 Sequences of AFLP adapters and pre-selective ('pre-sel') and selective ('sel')

primers (5' to 3') used in the development process including fluorescent label where

appropriate (6FAM, PET or NED).

6.2.5 Pre-selective amplification

The restriction-ligation reaction was diluted 1:20 with low TE buffer (10mM Tris,

0.1mM EDTA; as per Kazachkova, Fahleson & Meijer, 2004) and 4µl amplified with a

Taq PCR Core Kit using 0.2mM dNTP's, 1 unit *Taq* DNA polymerase, 10X PCR buffer and

5pM of each core primer (*Eco*RI pre-sel and *Mse*I pre-sel; Table 6.2), made up to a total

volume of 20µl with DNA grade H_2O. Reaction conditions were initial denaturation at

94°C for 2 minutes, followed by 32 cycles of 94°C for 30seconds, 56°C for 1 minute,

and 72°C for 1 minute, and a final elongation step at 72°C for 5 minutes, as per

Kazachkova et al. (2004). 5µl of the PCR products were run out on a gel to check the

reaction had worked (indicated by a smear centered around 200bp; Trybush et al.,

2006).

Figure 6.3 Pre-selective amplification of two *A. obscurus* samples. Lane 1, 50 bp ladder;
lane 2, sample 1; lane 3, blank; lane 4, sample 2.

6.2.6 Selective amplification

Pre-selective PCR products were diluted 1:20 with low TE buffer, and 2.5µl were then
mixed together with the same reagents and quantities as the pre-selective
amplification (in a total volume of 10µl). Six fluorescently labelled selective *Eco*RI
primers were tested with each *Mse*I primer systematically, to give 36 combinations in
total (Table 6.2), in order to choose the most appropriate primer pair (in terms of
number of peaks). Initial denaturation was for 2 minutes at 94°C. The annealing
temperature was 65°C for 30s in the first cycle, then the temperature was reduced by
0.7°C for the next 12 cycles, and finally 23 cycles were carried out at 56°C for 30s.
Denaturation was 94°C for 30s, and extension was 72°C during each of these cycles.
When run out on a gel as per previous steps, individual bands can be seen in each
sample (Figure 6.4).
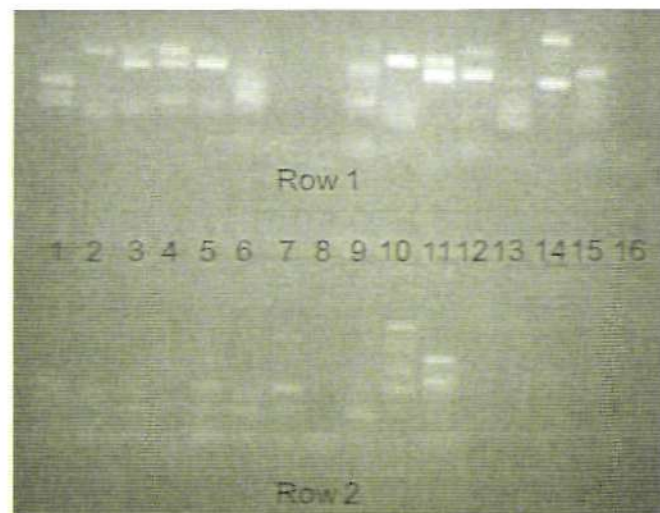
Figure 6.4 Selective amplification of samples from Cambridge (Cam) and the South Hams (SH). **Row 1:** Lane 1: Cam 1 sel 1; lane 2: Cam 1 sel 5; lane 3: Cam 1 sel 21; lane 4: Cam 1 sel 22; lane 5: Cam 1 sel 17; lane 6: Cam 1 sel 29; lane 7: Cam 2 sel 1+5 (failed); lane 8: Cam 2 sel 21+22 (failed); lane 9: Cam 2 sel 17+29; lane 10: Cam 3 sel 1+5; lane 11: Cam 3 sel 21+22; lane 12: Cam 3 sel 21+22; lane 13: SH 1 sel 1+5; lane 14: SH 1 sel 21+22; lane 15: SH 1 sel 17+29; lane 16: SH 2 sel 1+5 (failed). **Row 2:** Lane 1: SH 2 sel 21+22; lane 2: SH 2 sel 17+29; lane 3: SH 3 sel 1+5, lane 4: SH 3 sel 21+22; lane 5 SH 3 sel 17+29; lane 6: SH 4 sel 1+5; lane 7: SH 4 sel 6 21+22; lane 8: SH 4 sel 17+29; lane 9: Cam 4 sel 1+5; lane 10: Cam 4 sel 21+22; lane 11: Cam 4 sel 17+29.

6.2.7 Fragment analysis

Diluted selective PCR products (1μl) were added to 0.3μl of LIZ-500 size standard (Applied Biosystems) and 15μl of Hi-Di Formamide and denatured for 2 minutes at 95°C before being analysed on an Applied Biosystems 3130 Genetic Analyzer. GeneMapper v. 4 was used to visualise fluorescently labelled fragments using the default settings for AFLP analysis.

6.3 Results

Initial results for tests of one selective primer combination per lane (for one sample) were promising, with large numbers of peaks present (Figure 6.5), suggesting all previous steps were successful. From these tests, four selective primer combinations were chosen to test between 6-8 samples from Cambridge and the South Hams (sel 1: *Eco*RI 1 and *Mse*I 1, sel 5: *Eco*RI 1 and *Mse*I 5, sel 21: *Eco*RI 4 and *Mse*I 4, and sel 22: *Eco*RI 4 and *Mse*I 4), producing approximately 120 polymorphic peaks (e.g. Figure 6.6). The number of peaks analysed in AFLP studies varies, but the aim was for 100-200 polymorphic peaks in total (for all primer combinations), and so these primers appeared suitable for further testing on a larger number of samples from other regions.

In these tests selective PCR products were not diluted before fragment analysis. However, this resulted in many 'off-scale' peaks (the darker peaks in Figure 6.5), making the profile difficult to analyse. Therefore, dilutions of 1:20, 1:50, 1:100, 1:200 and 1:1000 were tested. Subsequent testing found that a dilution of 1:100 reduced the off-scale peak size without the loss of too many of the smaller fragments (a minimum fluorescent threshold of 100 was decided upon – any peaks below this would not be considered in analyses; Kazachkova, Fahleson & Meijer, 2004). Peaks below 80bp were not considered when counting polymorphic peaks, Double peaks, or "plus A" peaks, caused by the addition of an extra base by *Taq* polymerase, also obscured peak profiles and T4 DNA polymerase was used to remove the extra base (Ginot *et al.*, 1996), but was unsuccessful. Other attempts to correct this included promoting the addition of the extra base to ensure all peaks have the same profile, thereby increasing the efficiency of peak calling; the elongation period at 72°C was increased to 30

minutes in the selective amplification and the amount of MgCl₂ to 2mM (Applied

Biosystems, 2000), but no significant improvement was noted.

It was noticed that some samples from the same location varied somewhat in the

number of peaks produced by the same primer pair (e.g. Figure 6.6), and so reducing

this variability and improving reproducibility was the main concern in the next stages

of development.
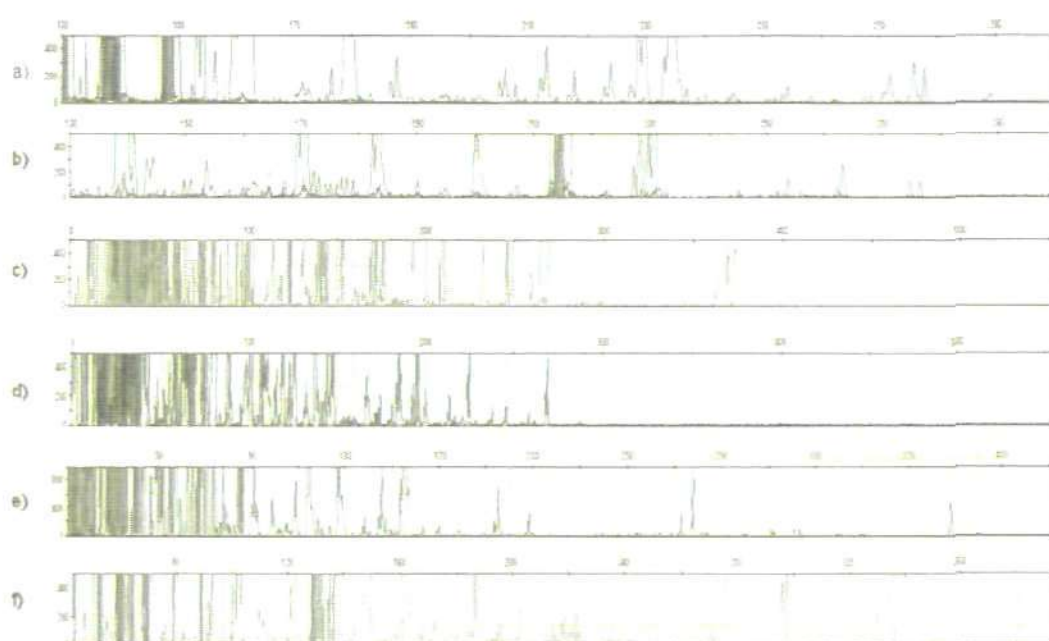
Figure 6.5 GeneMapper profiles for a single *A. obscurus* sample amplified with

selective primer combination a) *Eco*RI sel 1 and *Mse*I sel 2; b) *Eco*RI sel 1 and *Mse*I sel

6; c) *Eco*RI sel 2 and *Mse*I sel 1; d) *Eco*RI sel 3 and *Mse*I sel 1; e) *Eco*RI sel 4 and *Mse*I sel

6; f) *Eco*RI sel 5 and *Mse*I sel 2.
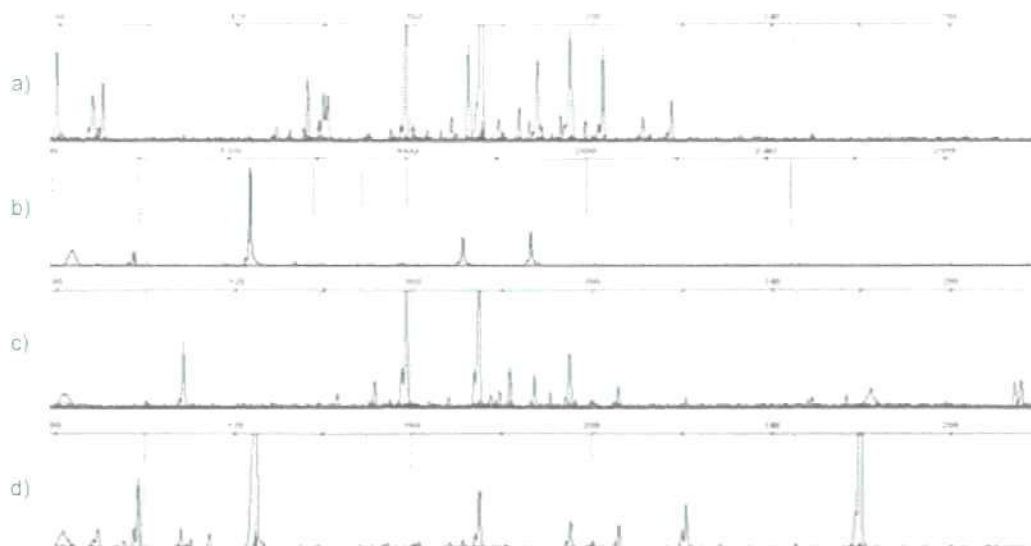
Figure 6.6 GeneMapper profiles for four *A. obscurus* samples (South Hams; profiles 1 and 2, Cambridge; profiles 3 and 4) amplified with selective primer combination *Eco*RI 1 and *Mse*I 1.

6.4 Optimisation problems

Problems were encountered with high variability in AFLP profiles between samples. Despite the same protocol being used, the number of peaks produced in the initial tests with the same primers above could not be reproduced in other samples. This lack of reproducibility was a major concern, since it is possible that resulting peak profiles were not related to location of the sample but to underlying problems with the method and/or samples. A number of tests were carried out to determine the possible cause of these problems.

There was some indication from previous work (J. Ellis, pers.comm) that adult *A. obscurus* DNA was more difficult to amplify than that of *A. lineatus* or *A. sputator*. However, when new DNA extractions from each species using spin columns and ammonium acetate were tested together with samples that had worked previously,

there were very few peaks in any of the profiles. Newly labelled primers were used in

primer combinations sel 5 (*EcoRI* 1 was labelled with NED), sel 21 (*EcoRI* 4 was labelled

with PET) and sel 22 (*EcoRI* 4 was labelled with VIC), in order that peaks could be

traced to the primer pair that amplified them (they were previously all labelled with

6FAM). This alone did not explain the failure of the analysis, since the original 6FAM

labelled *EcoRI* primer in sel 1 was used in these tests and this also failed to produce the

peak patterns seen previously. All original and newly labelled primers were checked for

the correct sequence, annealing temperature and self-complementarity using

OligoCalc (Kibbe, 2007) prior to use and no discrepancies were found. However, Meudt

and Clarke (2007) state that there will be differential amplitude of emission between

fluorophores which can affect reproducibility. This can be overcome by empirically

determining the optimum pooling ratio, but pooling PCR products or separating into

different wells did not affect the number of peaks in subsequent AFLP profiles.

Attempts were also made to increase the number of fragments amplified by removing

a base from each of the selective primers, but even for samples which worked

previously results were disappointing, and very few peaks were produced.

During development some variation in the intensity of the smears produced when pre-

selective PCR products were run out on agarose gels was noticed, suggesting variation

in the success of the reaction for different samples, but Trybush *et al.* (2006) state that

the quality of the pre-selective amplification is acceptable if the DNA smear is centred

around 200bp. To determine whether this had a knock-on effect in terms of number of

peaks produced, samples which were 'good' (a large, bright smear compared to other

samples) and 'bad' (small, less intense smear) were tested in fragment analysis, but

this did not appear to affect results. Trybush *et al.*, 2006 also noted that the original

adapters described by Vos et al. (1995) should not be used for pre-amplification with hot-start PCR (PCR in which the *Taq* is activated by an initial denaturation step at high temperature), as fragments are not amplified if template DNA is first denatured (Lan & Reeves, 2000). Therefore, pre-selective PCR was carried out for four samples with the addition of a 72°C hold for 2 minutes to allow the *Taq* polymerase to ligate both adapter strands (Wolf, 2000). Gel results indicated a clear smear centred around 200bp (Figure 6.7a), and three out of the four samples were uniform, but results were not significantly better than those originally obtained (Figure 6.3). The selective amplification also produced bright, uniform smears (the same sample was again slightly different to the others; Figure 6.7b), but AFLP profiles were not improved, and in fact separate bands were no longer visible as they were previously (Figure 6.4). The original adapters were used previously with no problems in this protocol, and by Kazachkova, Fahleson and Meijer (2004) with hot-start PCR, so again this is unlikely to be the primary factor involved in the failure of the protocol.
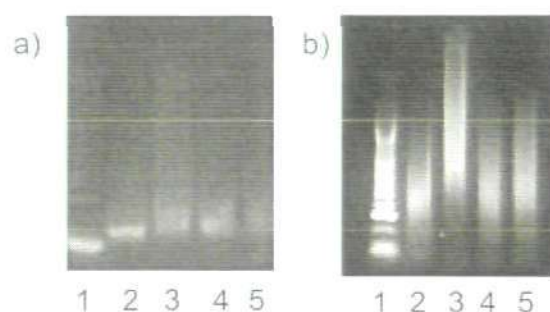
Figure 6.7 a) pre-selective amplification of four *A. obscurus* samples: lane 1: 50bp ladder; lane 2: South Hams 1; lane 3: South Hams 2; lane 4: Cambridge 1; lane 5: Cambridge 2, b) selective amplification (sel 1) of the same four samples (in the same lane positions) following the addition of an extra hold for 2 minutes at 72°C before the pre-selective PCR cycle.

New reagents in all steps of the protocol were used, including restriction enzymes, adapters, primers and PCR reagents. The addition of Q solution, an additive that amplifies difficult templates by modifying the melting behaviour of nucleic acids (Qiagen, Crawley, UK) did not significantly affect pre-selective or selective amplification gel results or AFLP profiles in GeneMapper, nor did the addition of 2mM $MgCl_2$ or increasing the amount of *Taq* polymerase. The optimised Trybush et al. (2006) protocol which worked for a range of other genomes was also attempted (using the restriction enzymes, adapters and primers described here) but no improvement was observed.

6.5 Discussion

Despite the modifications described above, within the given time-frame it was not possible to apply the protocol to the samples collected in the UK since further development was required. The reasons for failure of this protocol are still unclear and as unsuccessful attempts at producing AFLPs, or indeed any other molecular markers, are seldom published, it is difficult to ascertain whether this is unusual or not. There is, however, some indication as to possible explanations and further opportunities for optimisation.

As discussed, DNA quality is important as this has an ultimate effect on the number of bands produced. The extractions used in this protocol were relatively pure (according to A260/280 readings on the spectrophotometer), but some smearing was present when extracts were run out on gels. Throughout the molecular work with *Agriotes* adults and wireworms there have been similar issues; sample degradation was reported as an issue when samples were sent to be tested for use in microsatellite

development (G. Horsburgh, pers. comm.), and most of the other samples run out on gels had this profile, irrespective of the preservative method, the freshness of samples and the extraction method. This could indicate there are inhibitors present in the extracts which prevent subsequent reactions from working. Some species have been reported as having *Eco*RI specific inhibitors (Bensch & Åkesson, 2005), which also might explain why T-RFLP was not significantly affected, but AFLP reactions using *Eco*RI may be. Despite this, smears were present when restriction/ligation reactions were run out on gels and many fragments were produced in the initial tests. If click beetle sample quality is a factor a method which removes inhibitors whilst retaining a high DNA concentration may be required. For click beetles, degraded/impure DNA is an issue and as of yet a suitable extraction technique has not been found. Although a number of difficulties have been discussed, if quality of DNA extracts is the main issue then this could be relatively easy to resolve by testing a wider range of DNA extraction methods, such as CTAB (Kazachkova, Fahleson & Meijer, 2004; Murray & Thompson, 1980; Reineke, Karlovsky & Zebitz, 1998) and commercially available kits. The use of a kit would increase costs (especially since 30 individuals were intended to be studied from each site) but may prevent further expense in the long-term.

It has been reported that the presence of fragments itself is not necessarily an indication that previous steps in the protocol have worked. Vos et al. (1995) found that some bands were present following selective amplification even if the sample contained no DNA, likely caused by primer–dimers and interactions between adaptors (Bensch & Åkesson, 2005). Other researchers have also reported, via the BioTechniques molecular biology online forum (http://molecularbiology.forums.biotechniques.com/), a visible pre-selective smear on

a gel despite either only the forward or the reverse primer being used, suggesting this is not necessarily an indication that a reaction has worked. In addition, it was reported that the visibility of bands when selective amplifications are analysed on a gel could be indicative of the success of the protocol, and in this study this was also found to be the case. Running out restriction/ligation and pre-selective amplification reactions by gel electrophoresis is recommended in many AFLP protocols and published studies, including those followed for this study (Kazachkova, Fahleson & Meijer, 2004; Trybush et al., 2006), but if this does not provide accurate information there are implications for the rest of the development process, since efforts will be targeted at optimising the wrong stage of the protocol.

Several researchers developing AFLPs commented on the BioTechniques forum that they had experienced the same issues described in this chapter, including failure of AFLP reactions that had previously worked, despite the same samples and protocol being followed and the recommended modifications to the method being implemented. This suggests that the development of AFLPs is not always a quick alternative to other molecular markers, and that it may depend on the organism under study. However, there is potential that with additional time a suitable protocol based on the work carried out in this study could be developed and the extent of dispersal assessed using AFLPs for the three Agriotes pest species in the UK.

With the advent of metagenomics and the accessibility of associated technologies for studying not only model organisms but also those of ecological and evolutionary interest for which no reference genome exists, the development of molecular markers is likely to become much easier than it has been historically. Instead of probing randomly for unknown sequence regions, high-throughput methods for SNP (single

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nucleotide polymorphism) genotyping on a whole-genome scale are now able to genotype thousands of individuals from thousands of loci (Hudson, 2008). For example, next generation sequencing technologies were recently used to develop genomic resources for the non-model aphid *Aphis glycines* Matsumura, identifying 635 SNPs and 1,382 microsatellite markers from 278 million bp (Bai et al., 2010), and for the rapid identification of microsatellite loci in the water strider *Gerris incognitus* Drake and Hottes (Perry & Rowe, 2010). Such analyses were beyond the scope of this study, but are likely to become increasingly utilised for research of this kind in the future.

Chapter 7   Discussion

Extensive studies of *Agriotes* species in the UK since the 1940's led to much knowledge

being gathered about wireworms as pests and their biology. Despite this, they

continued to cause damage, since knowledge of important aspects of their ecology

was lacking (a fundamental requirement for the development of sustainable risk

assessment and pest management strategies). The relatively recent introduction of

new tools, such as sex pheromones and molecular methods of identification, have

allowed this to be more easily investigated, and the studies described here revealed

that the assumed understanding of *Agriotes* click beetle and wireworm ecology is not

complete, and furthermore, that it may be inaccurate.

7.1 Spatial distribution

A clear understanding of the distribution of pest species is an essential pre-requisite

for their control, and as such this was investigated in Chapter 2, Chapter 3 and Chapter

4 for UK and Canadian species, with the focus on the three main *Agriotes* pest species

in the UK (*Agriotes obscurus, A. lineatus* and *A. sputator*). The above-belowground life

cycle of click beetles, with belowground wireworms as the pest phase, makes it

difficult to assess their distribution. The lack of an efficient method to trap adults of

the three main pest species (*Agriotes obscurus, A. lineatus* and *A. sputator*) in the UK

and to identify *Agriotes* species (and to some extent other potential pest species) of

wireworms has prevented accurate information being obtained. The advent of sex

pheromone traps (Furlan et al., 2001a; Furlan et al., 2001b) and the T-RFLP method of

identification of these species (Ellis et al., 2009) have enabled this to be studied in

more detail here. The data in Chapter 2 suggested that the spatial relationship

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between *Agriotes* adult males and wireworms was not always straightforward; whereas large numbers of each *Agriotes* species were trapped in sex pheromone traps in agricultural fields, the proportion of wireworms of the same species (from the same fields) was not necessarily equivalent. No *A. lineatus* larvae were found at all, which is surprising considering the number of adults trapped and this species' inclusion in the *Agriotes* pest complex, which assumes no differences between these species. It also questions the location of *A. lineatus* larvae, which are assumed to be present based on the presence of adults. Possibilities discussed in Chapter 2 are that eggs are deposited elsewhere in fields i.e. in field margins which were not sampled in this study, or adults are better able to disperse and/or attracted to sex pheromones from a wider area than other species (see section 7.4), meaning they are moving in from other non-sampled areas. These could be their preferred habitats, possibly areas of undisturbed land not currently in cultivation e.g. set-aside. To a large extent these results may be affected by sampling method (of both adults and wireworms) and scale of sampling (see section 7.2).

The data presented in Chapter 2 showed that separating wireworms to species provides more information on their distribution than grouping them as a pest complex; the three species are not always found in the same place, suggesting there may be fundamental differences in their ecology. The non-*Agriotes* species trapped were not always found in large numbers, e.g. in Chapter 3 low numbers relative to *Agriotes* wireworms were found, but this may suggest there are certain site characteristics, or other factors, which affect the presence, abundance and distribution of these species. Previous surveys in the UK (Anon, 1948) suggested that in general non-*Agriotes* species are more abundant in areas of high altitude (i.e. northern moorland) or marsh areas,

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neither of which were characteristic of the locations sampled in these studies, though altitude was found to be related to non-*Agriotes* distribution in some (but not all) redundancy analyses plots in Chapter 2. These results give some clues as to other environmental factors that may play a role in influencing species distributions, such as the amount of weeding and grass duration, which lends support to an existing body of evidence which suggest these factors have an impact on the presence and/or abundance of species (Miles, 1942a; Parker & Seeny, 1997; Seal, McSorley & Chalfant, 1992).

Other soil dwelling insects might also be expected to influence wireworm species distributions through predation and/or competition for space and resources. Data presented in Chapter 3 suggest that, for the population densities and species in the population sizes and location sampled at least, there was no such relationship. However, the effect of scale on intra– and interspecific distributions was noted and as will be discussed (section 7.2) this needs to be taken into account when analysing ecological data, especially where the results will be used to inform management practices. These results on the spatial distribution of species with above-belowground life cycles have wider implications for ecological studies in general, since there are other species of both economic, conservation and biological pest management importance for which similar issues are likely to be of importance.

7.2 Sampling methods

If pest management is to be targeted at the areas in which wireworm pest species are found, then the lack of congruence between aboveground sex pheromone trapped adults, and belowground soil and bait trapped wireworm species needs to be taken

into account. Sex pheromone traps have been proven to be effective at trapping *Agriotes* species in agricultural land in studies described here (Chapter 2 and Chapter 3) and other published studies (Arakaki *et al.*, 2008; Ester, van Rozen & Griepink, 2001; Furlan *et al.*, 2001a; 2001b; Sufyan, Neuhoff & Furlan, 2007), but relating adult distribution to that of wireworms of the same species, as discussed, is problematic. It was discussed in Chapter 2 that the method of sampling wireworms (bait trap or soil core; two of the main methods used for sampling wireworms) can affect the species that are found and their apparent distribution and abundance. This in turn has implications for how the abundance and distribution of wireworms might relate to the abundance and distribution of adults trapped with sex pheromone traps, raising important issues with using these methods for risk assessment before planting of crops. The attraction of using sex pheromone traps for assessing wireworm presence and distribution is in the low cost and labour expense needed for monitoring compared to direct soil core or bait sampling. However, this study has highlighted that this may give inaccurate information on these pest species which could lead to poor decisions being taken in terms of which crops to grow in which fields. Until further information is gathered on the response of adult males to sex pheromone traps (comparisons in terms of attractiveness between species) and movement/dispersal ability (see section 7.4), it would not be recommended to use sex pheromone trapping as a surrogate for wireworm sampling to inform pest management application (Hicks & Blackshaw, 2008).

Scale of sampling affected the relationships found between soil core trapped wireworm species and other soil dwelling insect larvae, and their individual spatial distributions (Chapter 3). Scale is a well discussed factor in ecological studies since it

can have a major and important impact on the interpretation of patterns such as species diversity, distribution, relationship to environmental factors and species interactions. These in turn are important to understand when considering the effects of climate and land use change particularly on species of economic and conservation importance (Levin, 1992). Here, spatial distribution changed from a more aggregated to random distribution for most species as the sampling scale decreased from the field to the soil core scale. This is in line with the patchy wireworm distribution (and crop damage) often observed in the field (Blackshaw & Vernon, 2006; Salt & Hollick, 1946). The most appropriate scale at which to sample is likely related to the scale at which management will be carried out, so using information regarding species distributions at the field scale in risk assessment may be more appropriate for many crop growers.

Alternative, more efficient sampling methods would be useful, but at present are not available. Pitfall traps did not capture any click beetles from fields in which known populations were present in this study (though have been used successfully for trapping click beetles in Canada; Vernon & Pats, 1997). Forage traps, which consist of grass or hay spread onto a plastic sheet, can be used in cropped fields but again when used to try to capture other species (as reported in Chapter 4) no click beetles were captured. Non-attractive methods to capture adults moving within fields might give a more accurate interpretation of wireworm distribution in the soil in the same fields, but this relies again on having knowledge of click beetle movement and dispersal abilities. Practically, a non-attractive trap for adults would also be useful for capturing live males and females for laboratory studies on movement, response to sex pheromones and olfactory studies on female response to crop odours, and for capturing other click beetle species which would be of use for identifying non-*Agriotes*

wireworms. Trialling other traps for capturing flying click beetles, or further modifying

the traps used in these studies in planned experimental designs might also be useful to

assess this mode of dispersal in *Agriotes* species.

7.3 Species identification

The relatively large abundance and potential number of species of non-*Agriotes*

wireworms trapped in UK agricultural land were somewhat unexpected since the

majority of the literature states that *Agriotes* are the main species found in this

habitat, which raises issues concerning the *Agriotes* pest complex. Molecular

identification of these species was attempted using 16S rRNA sequence data in

Chapter 4, but failed to provide any alignments with UK species for which sequence

data were available. The phylogenetic analyses gives clues as to which species they are

similar to, but Elaterid sequence data, for 16S rRNA at least, are somewhat lacking, and

so further work is needed to provide correct alignments. This would involve obtaining

sequences from adult samples of known provenance and species identification.

The T-RFLP method (Ellis *et al.*, 2009) proved invaluable in assessing *Agriotes* species

relationships (including those with other insect pest larvae), spatial distribution, above-

belowground relationships with adults, and the effect of scale and sampling method

on these patterns, none of which had previously been assessed due to the difficulty

associated with morphological identification. The 16S rRNA sequence data also

provided useful information in terms of unravelling the phylogenetic and

phylogeographic relationships within this group, which may in the future provide a

method of determining potential pest species based on species relationships as more

sequence data become available. Comparing the ecology of pest and non-pest species

may indicate factors responsible for a species attaining pest status, as has been carried out for other taxa e.g. arionid slugs (Noble & Jones, 1996). It also provided a method of identifying (or potentially identifying were more sequence data available) other elaterid species. Using just one locus limits the scope of molecular ecology studies, and it would be useful to combine several nuclear and mitochondrial loci in future studies. One way in which this might aid these studies is in the identification of numts (nuclear mitochondrial DNA inserts). Since many of the Canadian wireworm species sequenced at 16S rRNA (see Chapter 4) had not been sequenced previously, and there were ambiguities in morphological species identification, it was difficult to ascertain whether samples were misidentified or contained numts. Using more than one locus would overcome these issues, as two or more sets of sequence data could be cross-referenced. Since this study was one of the largest of its kind in constructing phylogenetic relationships between click beetle species using 16S rRNA data, these issues have only just come to light. However, this could present potential problems for studies on click beetles using mitochondrial genes, since these data suggest numts may be prevalent in some click beetle species; potential numts were identified for UK species (*A. campyloides* and *A. accuminatus*), and in the Canadian species for *L. californicus*, *A. obscurus* and *Ctenicera* spp. Where possible, using known adult samples, rather than possibly cryptic wireworm samples, for assessment of population structure and phylogenetic relationships is suggested.

Another issue encountered, particularly in the development of AFLPs in Chapter 6, was DNA quality. This is a particular problem for adult click beetle DNA, so the determination of a DNA extraction method that provides large amounts of clean,

inhibitor free DNA that can be used in sensitive protocols such as AFLPs would improve the reliability of click beetle molecular ecology studies.

The presence of potential cryptic species in the Canadian wireworm samples is interesting in itself. Limited information is available for pest species in Canada at present, so accurate identification is essential for assessing the ecology and biology of damage causing species. Results outlined in Chapter Two show how this has been important for studies in UK agricultural land, and it should be a standard pre-requisite for any research on potential pests. In particular for *Hypnoidus bicolor* there was evidence for several cryptic species, and possibly some geographic structuring, but more samples would be needed to verify this. Since this species has other interesting attributes (it is emerging as a destructive pest species, and has a parthenogenic as well as sexual forms) it may be informative to investigate this further. With molecular and morphological data combined, it may be possible to produce more accurate identification keys. Increasing numbers of researchers are choosing molecular methods to answer key questions or problems in insect pest species ecology (including Elaterids; Lindroth & Clark, 2009; Sagegami-Oba, Oba & Ōhira, 2007; Staudacher *et al.*, 2011), as they become increasingly accessible and affordable to all.

7.4 Adult movement

Results from behavioural studies of *Agriotes* adult male click beetle movement corroborate already existing data on the movement rates (measured directly) of the three UK pest species. *Agriotes lineatus* displayed the highest average walking speed (which was significantly different from that of *A. sputator*), a finding supported by data measured indirectly in the field (Hicks & Blackshaw, 2008). This may provide some

insight into results reported in Chapter Two, since overall *A. lineatus* adult males had the highest abundance of the three species, but *A. lineatus* larvae were not found. If this species is more active and/or able to move faster then it may be more likely be trapped as an adult and have a more widespread distribution. This might also depend on the response to sex pheromones and conditions in the field. Chapter Five reported no apparent difference in response of the species to sex pheromones in terms of average walking speed, but significant individual variation was found between *A. lineatus* walking speeds in control and pheromone treatments. In the locomotion compensator experiment, *A. sputator's* walking speed decreased from still air to pheromone treatment, but this species behaved differently to *A. obscurus* and *A. lineatus* in the Y-tube olfactometer experiment. It is difficult to judge whether these results suggest there are differences in the underlying behaviour of the three species, or whether the experimental set-up had more influence on the observed responses. Sample sizes were small and only *A. sputator* was tested with pheromones on the locomotion compensator. The Y-tube olfactometer equipment used here was deemed inappropriate for studying click beetle movement behaviour, since limited walking parameters can be measured and behaviour may be affected by the enclosed experimental set-up. Therefore, the locomotion compensator and arena are likely to give better information since a range of parameters can be measured and beetle movement is not as restricted, though there are still issues with possible edge affects (where beetles walk around the edge and try to find an escape route) in an arena. A possible alternative to directly using data obtained in laboratory or field studies to infer information about dispersal at larger scales may be in the use of mathematical models. With larger samples sizes individual variation can be taken into account and information on parameters affecting insect movement in the field can be incorporated

(Firle et al., 1998; Morales & Ellner, 2002), a limitation in this study which had small sample sizes meaning were results extrapolated they may have been inaccurate. Using beetles which have not been trapped using sex pheromones would be more appropriate in both assessing male response to sex pheromones, and in extrapolating data obtained in the laboratory to all individuals, but again this relies on an efficient method of capturing large numbers of live beetles.

Development of the AFLP protocol was intended to be used to infer dispersal distances of the three *Agriotes* species and could not be finalised in the given time frame, but potential avenues for future optimisation include selection/development of an efficient method of DNA extraction, which may solve many of the issues encountered in later stages of the protocol. Alternatively, a different marker could be trialled (though a limited set of microsatellites were already tested without success), or high-throughput sequencing technology could be used to identify hundreds of potential marker loci e.g. Single Nucleotide Polymorphisms (SNPs) or microsatellites (Hudson, 2008), for further testing. Data from molecular markers would provide an estimate of large scale dispersal which is still lacking for the *Agriotes* species in the UK but may aid interpretation of the distribution data discussed here, and the development of effective pest management strategies. With a successful protocol developed, it might be possible to optimise this for other click beetle pest species, for example in Europe, the USA and Canada.

7.5 Conclusions

The use of novel techniques for studying the Elateridae has led to the acquisition of new information on economically important species, opening new avenues of

investigation and indicating priority areas for future research. The identification of wireworm species in UK agricultural land, where this has previously been very difficult or not attempted at all, led to a number of important findings: that the *Agriotes* pest complex may not be present or as widespread as previous studies have assumed/suggested; linking adult and wireworm species distributions depends upon sampling method; and that the distribution of wireworm species changes over different spatial scales. Similarly, identification of economically important Canadian species revealed possible cryptic species that would otherwise have been overlooked. Therefore, species identification is an important part of the risk assessment process and should be taken into account when methods are developed and put into practice. Studies on *Agriotes* movement suggest differences between the three UK pest species, and an accurate method of measuring the dispersal ability over larger scales, would provide further information on the species distributions observed here, and in the case of both UK and Canadian species, may aid the development and implementation of pest management strategies.

Appendices

Appendix 1 Abiotic variables used in the Redundancy Analysis and forward selection

Appendix 2 Samples in a) non-*Agriotes* group 2 and b) non-*Agriotes* group 3, with sampling location, sequence length in base pairs (bp) and total number if gaps/missing data (bp)

Appendix 3 Neighbour joining tree, using *p*-distance, of 16S rRNA forward sequences of Canadian wireworm species (including potential numts). Bootstrap support values (1000 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. There were a total of 214 positions in the final dataset (all positions containing gaps and missing data were eliminated)

Appendix 4 P-distance (%) between *Hypnoidus bicolor* forward sequences from different sampling locations in Canada. P-distance (%) between *Hypnoidus bicolor* forward sequences from different sampling locations in Canada. Colours and numbers (e.g. 'H1') correspond to haplotype. Standard error estimates are shown above the diagonal in italics and were obtained by a bootstrap procedure (1000 replicates). Gaps and missing data were eliminated

Appendix 5 DNA extraction of tissue using ammonium acetate precipitation

Appendix 1

Variable	Description
Environmental	
Altitude	Metres above sea level (range: 20-180m)
Distwc	Distance from watercourse (mm on a 1:25,000 map)
Aspect N	Degrees from grid North to an accuracy of 45°
Aspect E	Degrees from grid East to an accuracy of 45°
Slope	The percentage change in altitude of the field as a fraction of the distance of the change
Chemical	
pH	Measured on air-dried soil in the laboratory
Potassium	mg/l
Phosphorus	mg/l
Magnesium	mg/l
Salinity	EC1:5 Air-dried, ground soil was mixed with water in a 1:5 w/v ratio The mixture was then allowed to settle before being measured for electrical conductivity
Physical	
var5cmShear	Soil shear strength (a measure of compaction) at 5cm depth using a shear vane. Taken *in situ* on all fields on the same day in August, at 5 positions in each field
var10cmShear	Soil shear strength (a measure of compaction) at 10cm depth using a shear vane. Measurements taken *in situ* on all fields on the same day in August, at 5 positions in each field
Moisture	Conductance in mV as a surrogate for soil moisture measured using a theta probe (Delta-T devices), taken *in situ* on all fields on the same day in August, at 5 positions in each field
Sand %	Sand, silt and clay percentages were measured from soil samples using a particle size analyser (Malvern Instruments)
Silt %	Sand, silt and clay percentages were measured from soil

	samples using a particle size analyser (Malvern Instruments). Note: percentage clay was excluded from the analysis as it is redundant when percentages of sand and silt are included as variables
Bulk density	Bulk density was calculated using the method for disturbed soil (Tan 1996).
Stones %	The stone (>2mm diameter & <30mm) content of the soil was calculated as a % by weight of air-dried soil
OM %	Organic matter measured as percentage loss on combustion
Cultural	
GrassDura	Grass duration is the number of years the field has been in continuous grass. Permanent pasture was given an 'age' of 75 years (Parker and Seeney 1997)
Silage	The number of silage cuts was taken as a total number of cuts for that field over a three-year period
YrsinGrass	The number of years between 1999 and 2004 in which the field had been in grass. This allows for any effect of grass in an organic vegetable rotation
Yrsconvert	Years since conversion, a count of the years since the conversion period of the field was completed
Org Fert	Organic fertiliser is the number of years of the last five in which either slurry or farmyard manure (FYM) was applied in any quantity
Manure	Tonnes Hectare^{-1} of FYM or slurry applied to the field in 2003
Lime	Tonnes Hectare^{-1} of lime applied to field in 2003 (Mean: 0.45)
Cultivations	Number of cultivations (not including ploughing, rotavating or rolling) in 2003
Additive index	Number of additive applications to the field in 2003.
Roll	Number of passes by a roller in 2003
Rotavate	Number of passes by a rotavator in 2003
Bedform	Number of times a bed (for potatoes/carrots) was formed with a mechanical bed former in 2003 (Note: Maximum is one pass)

Destone	Number of times the field underwent mechanical destoning in 2003
Weeding	Number of times the field was weeded (flame, hand or mechanical) in 2003
AvgMan	Average tonnes Hectare of FYM or slurry applied to field per year (1999-2004).
AvgCultiv	Average number of cultivations (not including ploughing, rotavating or rolling) per year (1999-2004)
AvgAdditv	Average number of additive applications per year (1999-2004)
AvgLime	Average tonnes Hectare^{-1} of lime applied to field per year (1999-2004)
AvgRotav	Average number of passes by a rotavator per year (1999-2004)
AvgRoll	Average number of passes by a roller per year (1999-2004)
AvgBedForm	Average number of times a bed (for potatoes/carrots) has been formed mechanically per year (1999-2004)
AvgDestone	Average number of times the field has undergone mechanical destoning per year (1999-2004)
AvgofWeeding	Average number of times a field has been weeded (flame, hand or mechanical) per year (1999-2004)

Abiotic variables used in the Redundancy Analysis and forward selection

Appendix 2

a)

Haplotype	Sample	Location	Sequence length/bp	Gaps/missing data
1	6 10/18	Newton Abbot, Devon	259	26
1	L4	South Hams, Devon	250	4
1	L5	South Hams, Devon	250	6
1	L61	South Hams, Devon	250	4
1	L64	South Hams, Devon	250	4
1	L65	South Hams, Devon	250	6
1	L69	South Hams, Devon	250	8
1	L91	South Hams, Devon	250	5
1	L94	South Hams, Devon	250	4
1	L97	South Hams, Devon	250	5
1	L236	Cambridge	299	5
1	L277	Cambridge	287	16
1	L283	Cambridge	281	12
1	L292	Cambridge	281	14
1	L295	Cambridge	312	11
1	L297	Cambridge	292	10
1	L298	Cambridge	286	11
1	L108	Cambridge	334	5
2	L294	Cambridge	314	20
3	L58	South Hams, Devon	250	5

b)

Sample	Location	Sequence length/bp	Gaps/missing data
L52	South Hams, Devon	254	5
L56	South Hams, Devon	254	2
L103	Cambridge	265	6
L114	Cambridge	267	4

Samples in a) non-*Agriotes* group 2 and b) non-*Agriotes* group 3, with sampling

location, sequence length in base pairs (bp) and total number of gaps/ missing data

(bp)

Limonius californicus L423 Taber
Limonius californicus L429 Claresholm
Limonius californicus L430 Claresholm
Limonius californicus L441 Moose Jaw
Limonius californicus L425 Drake
Limonius californicus L434 Swift Current
Limonius californicus L436 Wakaw
Limonius californicus L437 Wakaw
Limonius californicus L472 Taber
Limonius californicus L424 Taber
Limonius californicus L426 Drake
Limonius californicus L427 Drake
Limonius californicus L428 Claresholm
Limonius californicus L435 Wakaw
Limonius californicus L431 Oliver
Limonius californicus L432 Oliver
Limonius canus L443
Limonius agonus L451 Ridgetown
Limonius agonus L454 Parkhill
Limonius agonus L455 Parkhill
Limonius agonus L449 Ridgetown
Limonius agonus L452 Ridgetown
Limonius agonus L450 Ridgetown
Limonius agonus L453 Parkhill
Limonius infuscatus L444 Fort Macleod
Limonius infuscatus L445 Fort Macleod
Limonius infuscatus L446 Fort Macleod
Limonius californicus L438 Fort Saskatchewan
Aeolus mellilus L549 Milden
Aeolus mellilus L551 Drinkwater
Aeolus mellilus L550 Milden
Melanotus similis L558
Melanotus similis L559
Hemicrepidius sp. L554 Indian Head
Agriotes mancus L548
Agriotes criddlei L538 Vegreville
Agriotes criddlei L539 Vegreville
Agriotes criddlei L537 Vegreville
Limonius californicus L433 Oliver
Agriotes stabilis L546 Wakaw
Agriotes stabilis L543 Boissevain
Agriotes stabilis L545 Wakaw
Agriotes stabilis L540 Boissevain
Agriotes stabilis L542 Boissevain
Agriotes stabilis L541 Boissevain
Agriotes stabilis L544 Wakaw
Ctenicera pruinina L536 Oregon
C. aeripennis aeripennis L503 Tangent
C. aeripennis aeripennis L504 Tangent
C. aeripennis aeripennis L505 Fairview
Ctenicera destructor L514 Swift Current
Ctenicera destructor L512 Swift Current
Ctenicera destructor L531 Meota
Ctenicera destructor L528 Fillmore
Limonius californicus L439 Moose Jaw
Ctenicera destructor L518 Strasbourg
Ctenicera destructor L523 Lethbridge
Ctenicera destructor L517 Drumheller
Limonius californicus L440 Moose Jaw
Ctenicera destructor L530 Fort Saskatchewan
Ctenicera destructor L520 Strasbourg
Ctenicera destructor L532 Meota
Ctenicera destructor L527 Fillmore
Ctenicera destructor L533 Inglis

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                                                 Ctenicera pruinina L534 Orangeville
                                    100          Hemicrepidius sp. L555 Brussels
                                                 Ctenicera pruinina L535 Orangeville
                                                 Hypnoidus bicolor L463
                                                 Hypnoidus bicolor L487
                                                 Hypnoidus bicolor L465
                                                 Hypnoidus bicolor L474
                                    87           Ctenicera destructor L529 Fillmore
                                                 Hypnoidus bicolor L480
                                                 Hypnoidus bicolor L470
                                                 Hypnoidus bicolor L489
                                                 Hypnoidus bicolor L458
                                                 Hypnoidus bicolor L476
                                                 Hypnoidus bicolor L490
                                                 Hypnoidus bicolor L472
                                                 Hypnoidus bicolor L478
                                                 Hypnoidus bicolor L479
                                                 Hypnoidus bicolor L464
                                                 Hypnoidus bicolor L477
                                    56           Hypnoidus bicolor L469
                                                 Hypnoidus bicolor L488
                                                 Hypnoidus bicolor L460
                                                 Hypnoidus bicolor L475
                                                 Hypnoidus bicolor L473
                                                 Hypnoidus bicolor L491
                                                 Hypnoidus bicolor L468
                                                 Hypnoidus bicolor L493
                                                 Hypnoidus bicolor L456
                                                 Hypnoidus bicolor L497
                                                 Hypnoidus bicolor L467
                                                 Hypnoidus bicolor L499
                                                 Hypnoidus bicolor L457
                                                 Hypnoidus bicolor L461
                                                 Hypnoidus bicolor L459
                                                 Hypnoidus bicolor L466
                                                 Hypnoidus bicolor L462
                                                 Hypnoidus bicolor L486
                                                 Hypnoidus bicolor L495
                                                 Hypnoidus bicolor L498
                                                 Hypnoidus bicolor L471
                                    64           Hypnoidus bicolor L482
                                                 Hypnoidus bicolor L483
                                                 Hypnoidus bicolor L494
                                                 Hypnoidus bicolor L500
                                                 Diabrotica virgifera virgifera
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Neighbour joining tree, using p-distance, of 16S rRNA forward sequences of Canadian wireworm species (including potential numts). Bootstrap support values (1000 replicates) are shown next to the branches; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. There were a total of 214 positions in the final dataset (all positions containing gaps and missing data were eliminated)

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Appendix 4

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. Brandon, MB H1		0	1.2	0	1.2	0	0	1.2	1.2	1.2	0	0	0	1.2	1.2	0.3	1.2	1.2	1.2	1.2
2. Brandon, MB H1	0	0	1.2	0	1.2	0	0	1.2	1.2	1.2	0	0	0	1.2	1.2	0.3	1.2	1.2	1.2	1.2
3. Brandon, MB H2	4.3	4.3	0	1.2	0.8	1.2	1.2	0	0.8	0	1.2	1.2	1.2	0.8	0	1.2	0.7	0.8	0	0.8
4. Parkland, AB H1	0	0	4.3		1.2	0	0	1.2	1.2	1.2	0	0	0	1.2	1.2	0.3	1.2	1.2	1.2	1.2
5. Parkland, AB H3	4.3	4.3	2.1	4.3		1.2	1.2	0.8	0	0.8	1.2	1.2	1.2	0	0.8	1.2	0.3	0	0.8	0
6. Parkland, AB H1	0	0	4.3	0	4.3		0	1.2	1.2	1.2	0	0	0	1.2	1.2	0.3	1.2	1.2	1.2	1.2
7. Gull Lake, SK H1	0	0	4.3	0	4.3	0		1.2	1.2	1.2	0	0	0	1.2	1.2	0.3	1.2	1.2	1.2	1.2
8. Montmartre, SK H2	4.3	4.3	0	4.3	2.1	4.3	4.3		0.8	0	1.2	1.2	1.2	0.8	0	1.2	0.7	0.8	0	0.8
9. Montmartre, SK H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1		0.8	1.2	1.2	1.2	0	0.8	1.2	0.3	0	0.8	0
10. Montmartre, SK H2	4.3	4.3	0	4.3	2.1	4.3	4.3	0	2.1		1.2	1.2	1.2	0.8	0	1.2	0.7	0.8	0	0.8
11. Calgary, AB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3		0	0	1.2	1.2	0.3	1.2	1.2	1.2	1.2
12. Calgary, AB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0		0	1.2	1.2	0.3	1.2	1.2	1.2	1.2
13. Calgary, AB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0		1.2	1.2	0.3	1.2	1.2	1.2	1.2
14. Elkhorn, MB H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3		0.8	1.2	0.3	0	0.8	0
15. Elkhorn, MB H2	4.3	4.3	0	4.3	2.1	4.3	4.3	0	2.1	0	4.3	4.3	4.3	2.1		1.2	0.7	0.8	0	0.8
16. Elkhorn, MB H4	0.4	0.4	4.6	0.4	4.6	0.4	0.4	4.6	4.6	4.6	0.4	0.4	0.4	4.6	4.6		1.2	1.2	1.2	1.2
17. Avonlea, SK H5	4.6	4.6	1.8	4.6	0.4	4.6	4.6	1.8	0.4	1.8	4.6	4.6	4.6	0.4	1.8	5		0.3	0.7	0.3
18. Avonlea, SK H3	4.3	4.3	0	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3	0	2.1	4.6	0.4		0.8	0
19. Avonlea, SK H2	4.3	4.3	0	4.3	2.1	4.3	4.3	0	2.1	0	4.3	4.3	4.3	2.1	0	4.6	1.8	2.1		0.8
20. Teulon, MB H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3	0	2.1	4.6	0.4	0	2.1	
21. Teulon, MB H2	4.3	4.3	0	4.3	2.1	4.3	4.3	0	2.1	0	4.3	4.3	4.3	2.1	0	4.6	1.8	2.1	0	2.1
22. Teulon, MB H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3	0	2.1	4.6	0.4	0	2.1	0
23. Boissevain, MB H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3	0	2.1	4.6	0.4	0	2.1	0
24. Boissevain, MB H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3	0	2.1	4.6	0.4	0	2.1	0
25. Boissevain, MB H2	4.3	4.3	0	4.3	2.1	4.3	4.3	0	2.1	0	4.3	4.3	4.3	2.1	0	4.6	1.8	2.1	0	2.1
26. Orangeville, ON H4	0.4	0.4	4.6	0.4	4.6	0.4	0.4	4.6	4.6	4.6	0.4	0.4	0.4	4.6	4.6	0	5	4.6	4.6	4.6
27. Orangeville, ON H4	0.4	0.4	4.6	0.4	4.6	0.4	0.4	4.6	4.6	4.6	0.4	0.4	0.4	4.6	4.6	0	5	4.6	4.6	4.6
28. Donnelly, SK H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3
29. Altona, MB H2	4.3	4.3	0	4.3	2.1	4.3	4.3	0	2.1	0	4.3	4.3	4.3	2.1	0	4.6	1.8	2.1	0	2.1
30. Altona, MB H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3	0	2.1	4.6	0.4	0	2.1	0
31. Altona, MB H2	4.3	4.3	0	4.3	2.1	4.3	4.3	0	2.1	0	4.3	4.3	4.3	2.1	0	4.6	1.8	2.1	0	2.1
32. Ste. Rose, MB H6	3.9	3.9	2.5	3.9	0.4	3.9	3.9	2.5	0.4	2.5	3.9	3.9	3.9	0.4	2.5	4.3	0.7	0.4	2.5	0.4
33. Ste. Rose, MB H3	4.3	4.3	2.1	4.3	0	4.3	4.3	2.1	0	2.1	4.3	4.3	4.3	0	2.1	4.6	0.4	0	2.1	0
34. Inglis, MB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3
35. Manning, AB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3
36. Manning, AB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3
37. Fairview, AB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3
38. Fairview, AB H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3
39. Dawson Creek, BC H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3
40. Dawson Creek, BC H1	0	0	4.3	0	4.3	0	0	4.3	4.3	4.3	0	0	0	4.3	4.3	0.4	4.6	4.3	4.3	4.3

P-distance (%) between *Hypnoidus bicolor* forward sequences from different sampling locations in Canada. *P*-distance (%) between *Hypnoidus bicolor* forward

sequences from different sampling locations in Canada. Colours and numbers (e.g. 'H1') correspond to haplotype. Standard error estimates are shown above the

diagonal in italics and were obtained by a bootstrap procedure (1000 replicates). Gaps and missing data were eliminated.

Appendix 4 cont.

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1. Brandon, MB H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
2. Brandon, MB H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
3. Brandon, MB H2	*0*	*0.8*	*0.8*	*0.8*	*0*	*1.2*	*1.2*	*1.2*	*0*	*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
4. Parkland, AB H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
5. Parkland, AB H3	*0.8*	*0*	*0*	*0*	*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
6. Parkland, AB H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
7. Gull Lake, SK H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
8. Montmartre, SK H2	*0*	*0.8*	*0.8*	*0.8*	*0*	*1.2*	*1.2*	*1.2*	*0*	*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
9. Montmartre, SK H3	*0.8*	*0*	*0*	*0*	*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
10. Montmartre, SK H2	*0*	*0.8*	*0.8*	*0.8*	*0*	*1.2*	*1.2*	*1.2*	*0*	*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
11. Calgary, AB H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
12. Calgary, AB H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
13. Calgary, AB H1	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
14. Elkhom, MB H3	*0.8*	*0*	*0*	*0*	*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
15. Elkhom, MB H2	*0*	*0.8*	*0.8*	*0.8*	*0*	*1.2*	*1.2*	*1.2*	*0*	*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
16. Elkhom, MB H4	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0*	*0*	*0.3*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*
17. Avonlea, SK H5	*0.7*	*0.3*	*0.3*	*0.3*	*0.7*	*1.2*	*1.2*	*1.2*	*0.7*	*0.3*	*0.7*	*0.5*	*0.3*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
18. Avonlea, SK H3	*0.8*	*0*	*0*	*0*	*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
19. Avonlea, SK H2	*0*	*0.8*	*0.8*	*0.8*	*0*	*1.2*	*1.2*	*1.2*	*0*	*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
20. Teulon, MB H3	*0.8*	*0*	*0*	*0*	*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
21. Teulon, MB H2		*0.8*	*0.8*	*0.8*	*0*	*1.2*	*1.2*	*1.2*	*0*	*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
22. Teulon, MB H3	2.1		*0*	*0*	*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
23. Boissevain, MB H3	2.1	0		*0*	*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
24. Boissevain, MB H3	2.1	0	0		*0.8*	*1.2*	*1.2*	*1.2*	*0.8*	*0*	*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
25. Boissevain, MB H2	0	2.1	2.1	2.1		*1.2*	*1.2*	*1.2*	*0*	*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
26. Orangeville, ON H4	4.6	4.6	4.6	4.6	4.6		*0*	*0.3*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*
27. Orangeville, ON H4	4.6	4.6	4.6	4.6	4.6	0		*0.3*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*
28. Donnemy, SK H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4		*1.2*	*1.2*	*1.2*	*1.1*	*1.2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
29. Altona, MB H2	0	2.1	2.1	2.1	0	4.6	4.6	4.3		*0.8*	*0*	*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
30. Altona, MB H3	2.1	0	0	0	2.1	4.6	4.6	4.3	2.1		*0.8*	*0.3*	*0*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
31. Altona, MB H2	0	2.1	2.1	2.1	0	4.6	4.6	4.3	0	2.1		*0.9*	*0.8*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
32. Ste. Rose, MB H6	2.5	0.4	0.4	0.4	2.5	3.3	4.3	3.9	2.5	0.4	2.5		*0.3*	*1.1*	*1.1*	*1.1*	*1.1*	*1.1*	*1.1*	*1.1*
33. Ste. Rose, MB H3	2.1	0	0	0	2.1	4.6	4.6	4.3	2.1	0	2.1	0.4		*2.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*	*1.2*
34. Inglis, MB H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4	0	4.3	4.3	4.3	3.9	4.3		*0*	*0*	*0*	*0*	*0*	*0*
35. Manning, AB H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4	0	4.3	4.3	4.3	3.9	4.3	0		*0*	*0*	*0*	*0*	*0*
36. Manning, AB H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4	0	4.3	4.3	4.3	3.9	4.3	0	0		*0*	*0*	*0*	*0*
37. Fairview, AB H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4	0	4.3	4.3	4.3	3.9	4.3	0	0	0		*0*	*0*	*0*
38. Fairview, AB H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4	0	4.3	4.3	4.3	3.9	4.3	0	0	0	0		*0*	*0*
39. Dawson Creek, BC H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4	0	4.3	4.3	4.3	3.9	4.3	0	0	0	0	0		*0*
40. Dawson Creek, BC H1	4.3	4.3	4.3	4.3	4.3	0.4	0.4	0	4.3	4.3	4.3	3.9	4.3	0	0	0	0	0	0	

P-distance (%) between *Hypnoidus bicolor* forward sequences from different sampling locations in Canada. Colours and numbers (e.g. 'H1') correspond to haplotype. Standard error estimates are shown above the diagonal in italics and were obtained by a bootstrap procedure (1000 replicates). Gaps and missing data were eliminated.

Appendix 5   DNA extraction of tissue using ammonium acetate precipitation

1. Place in a 1.5ml Eppendorf tube 250µl Digsol buffer (see below) and 10µl Proteinase K (10mg/ml). Keep on ice.

2. Remove the legs, head and thorax and cut into small pieces using a sterile blade before adding to the tube.

3. Vortex, then place in a rack in an incubator at 55°C overnight.

4. Add 300µl 4M ammonium acetate to each sample.

5. Vortex several times over a period of at least 15 minutes at room temperature to precipitate the proteins.

6. Centrifuge for 10 minutes at 11337g.

7. Aspirate supernatant (the clear liquid containing the DNA) into clean labelled 1.5ml Eppendorf tubes, discarding the pellet (containing the digested tissue and proteins).

8. Add 1ml 100% ethanol, invert the tubes gently to precipitate the DNA, and place samples in the freezer at -20°C for 20 minutes.

9. Centrifuge for 10 minutes at 11337g.

10. Pipette off the ethanol, taking care not to lose the DNA pellet (stuck to the bottom of the tube.

11. Add 500µl 70% ethanol and invert several times to rinse the pellet.

12. Centrifuge for 5 minutes at 11337g.

13. Carefully pipette off as much of the ethanol as possible without losing the pellet and dry in a heat block at 50°C until fully dry.

14. Add 50µl DNA grade H_2O and flick the tube to dislodge pellet.

15. Place tubes in a heat block at 37°C for 30 minutes to dissolve the pellet, flicking the tube every 10 minutes. Store at 4°C (short term) or -20°C (long term).

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Applied Soil Ecology

The spatial distribution of phytophagous insect larvae in grassland soils

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ABSTRACT

Most research on soil dwelling phytophagous insect larvae has arisen from their functional roles as pests in agricultural systems, but few studies have considered their spatial distribution or the possible interactions between taxa, and none have investigated the effect of scale. In this study the individual and interspecific spatial distribution of wireworms (click beetle larvae – Elateridae), leatherjackets (crane fly larvae – Tipulidae), bibionid flies (Bibionidae) and sciarid flies (Sciaridae) over three scales, and the importance of spatial, biotic and scale variables, was assessed. Soil core samples were collected from 26 sites across six grass fields on a farm in South Devon, UK. Abundance and presence–absence data were analysed over the field, site and soil core scales using variance mean ratio and non-metric multidimensional scaling (NMDS), and deviance partitioning was used to determine the importance of spatial, biotic and scale variables for individual taxa distribution. The spatial distribution of most taxa changed from an aggregated to a random distribution from the field to the core scale. Interspecific distributions of taxa also differed between scales, though some associations held at all scales. Deviance partitioning revealed that scale was the single most important variable influencing taxa distribution, whereas spatial and biotic variables were mostly of minor importance. The results suggest that variation between taxa is likely to be due to interactions between scale-specific environmental, biotic and stochastic factors and individual taxon biology, and emphasises the need to take scale into account when interpreting data from ecological studies.

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1. Introduction

Soil communities have been described as the 'poor man's tropical rainforest' due to the relatively high level of biodiversity and large proportion of undescribed species they contain, and the limited information that is known on community structure and dynamics (Giller, 1996). Ninety percent of insects spend at least some part of their lifecycle in the soil (Klein, 1988), having an influence on for example the diversity of plant communities, competitive interactions among plants and the yield of agricultural systems (Hunter, 2001). Despite this, information on the distribution and abundance of soil dwelling insects is lacking.

Physical, chemical and biotic factors are known to determine the presence, size and survival of invertebrate populations within the soil, causing a patchy distribution (Curry, 1987). However, spatial scale, although long recognised by ecologists as an important component influencing species distributions (Wiens, 1989; Levin, 1992), has been somewhat neglected in ecological studies. It is well recognised that spatial scale of sampling and analysis affects the

observed distributions, but it has often been seen as a complicating factor rather than included as an explanatory variable in its own right, and as such multi-scale experiments are rare (Sandel and Smith, 2009). Spatial structuring, through environmental and community processes, plays a functional role in ecosystems and in order to understand this, modelling spatial patterns at multiple spatial and temporal scales is critical (Borcard and Legendre, 2002; Borcard et al., 2004). Owing to the relatively recent growth in spatial statistical techniques there are now many methods of incorporating spatial location into studies to determine how space affects species presence and/or abundance (e.g. Legendre and Fortin, 1989; Borcard et al., 1992; Coomes et al., 1999; Lichstein et al., 2002; Cocu et al., 2005). In addition, information on the relative importance of biotic, environmental and spatial factors to individual species distributions would be useful in terms of managing pests and biodiversity, and may allow better predictive models to be produced. A combination of methods that describe the spatial distribution and underlying patterns in the data, such as indices of dispersion and multivariate techniques, together with methods that use the spatial location (e.g. geographical coordinates) of samples as an indication of spatial pattern, such as deviance partitioning, might enable a better overall understanding of species distributions to be obtained.

In this study, wireworms (click beetle larvae – Coleoptera: Elateridae), leatherjackets (crane fly larvae – Diptera: Tipuli-

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dae.), sciarid fly larvae (Diptera: Sciaridae) and bibionid fly larvae (Diptera: Bibionidae) were sampled from grassland soil. These taxa are commonly found in abundance in grassland, where they feed on grass roots, but when present in arable land some species can cause considerable damage to crop roots. Therefore research has mainly focused on their role as agricultural pests. Previous studies in the UK have attempted to determine the factors affecting the distribution of wireworms (Salt and Hollick, 1946; Parker and Seeny, 1997) and leatherjackets (McCracken et al., 1995), but without inclusion of either spatial scale or biotic variables, or all three, and studies on interactions between macro-invertebrates in agricultural land and the influence of other species on the distribution of soil insect larvae are lacking. A good understanding of pest distribution and the factors affecting this, is essential to the development of sustainable management strategies, in order that control methods can be targeted at damage causing species at the appropriate scale. For soil living pests, this is particularly important since incorporation of pesticide into the soil can be inefficient, often even with high rates of application, and many of the persistent chemicals which were effective have now been withdrawn from use (Grove et al., 2000).

With these issues in mind, the aims of our study are:

1. To assess the effect of scale of sampling, and the contribution of space, biotic interactions and scale to soil insect larvae distribution.
2. To determine whether there are any interspecific relationships between taxa, and how these relationships vary with the scale of sampling.

2. Methods

2.1. Study site and sampling

As part of a study on the use of water traps to predict the size of leatherjacket populations soil samples were collected from 26 sites over 6 grass fields from Seale Hayne Farm, South Devon, UK, between 15th January and 31st March 2008. Apart from one field which was a permanent ley, all fields were in grass for at least 3 years before sampling, previously being in an informal rotation with cereals and maize. Soils were brown earths of the Highweek and Trusham series. At each site samples were collected from the intersections of 24 radii at 15° intervals with concentric circles at 5, 10, 20 and 40 m from a central point. Soil cores were collected using a 5 cm diameter plastic pipe which was pushed to a depth of approximately 10 cm. In total 96 soil samples were collected from each site, with the exception of four sites which were truncated due to sample points overlapping field boundaries and/or a stream, which equates to an area of 4.86 m² of soil sampled across the study site.

2.2. Extraction and identification of larvae

Larvae were recovered from soil cores using heat extraction (Blasdale, 1974) and placed into separate tubes containing 70% ethanol. Leatherjackets were assumed to be Tipula paludosa Meigen, as no other species had been captured as adults using water traps placed at the sites previously. Humphreys et al. (1993) found this assumption to be correct for the areas surveyed in their study. Bibionid larvae were identified as either Bibio johannis L. or Dilophus febrilis L. based on the posterior spiracles, using a light microscope (Brindle, 1962). Sciarid larvae could not be identified to species by their morphology and so remained unidentified and grouped as Sciaridae. Wireworm DNA was extracted using a standard salt extraction protocol and resuspended in 0.01× TE buffer

(Rice et al., 1992). A terminal restriction fragment length polymorphism (T-RFLP) technique (Ellis et al., 2009) was used to identify wireworms of the species Agriotes obscurus L., Agriotes sputator L. and Agriotes lineatus L. Other species were grouped together as 'non-Agriotes'. There were also instances in which no restriction fragment was produced, possibly due to degraded DNA. These samples have been grouped together as 'unknown wireworms'.

2.3. Data analysis

Ecological data often tend to be spatially autocorrelated, where observations from nearby locations are more similar than would be expected by chance, driven by various environmental and biotic processes (Legendre and Fortin, 1989; Lichstein et al., 2002; Kissling and Gudrun, 2008). This lack of independence violates the assumptions of most traditional statistical tests, resulting in inflation of Type I errors which can affect the interpretation of results. Moran's autocorrelation coefficient (I) was calculated using Spatial Analysis in Macroecology (SAM) software v 3.1 (Rangel et al., 2006) for each species, using geographic distances with 21 distance classes (the default) and default distance class size with equal distances. Due to the low abundance of the taxa sampled, the significance of the Moran's I values were not tested, as when applied to data with many double zeros the degree of autocorrelation may be overestimated (Legendre and Fortin, 1989). Instead, the correlograms, in which autocorrelation values are plotted against distance classes and the Moran's I values alone were checked for spatial autocorrelation. It was determined that no spatial autocorrelation was present and as a result standard statistical tests were used for the analysis.

The variance to mean ratio (VMR) was used as an index of dispersion to determine the distribution of individual taxa at each scale, using species abundance data (Taylor, 1961). This gives an idea of the spatial distribution and underlying patterns in the data, based on the frequency distribution of number of specimens per sample unit. However, it is not an indication of spatial pattern, which uses the spatial location (e.g. geographical coordinates) of samples rather than count data (Binns et al., 2000).

Non-metric multidimensional scaling (NMDS) was computed using species presence–absence data in Brodgar v.2.5.7 (Highland Statistics Ltd., 2006), to visualise the relationships between all taxa at each scale. Jaccard's coefficient, an asymmetrical binary coefficient which excludes double zeros (i.e. where the fields, sites or cores being compared both contain no taxa) was used as a measure of association between species (Legendre and Legendre, 1998). The optimal number of dimensions, or axes m, was determined by selecting the ordination with minimum STRESS, a measure of deviation from monotonicity (Kruskal, 1964); the number of axes was plotted against the STRESS values for each value of m. A clear change in stress (the elbow effect) indicates the optimal value of m (Zuur et al., 2007). For the field scale m = 4, STRESS = 0.0093, for the site scale m = 4, STRESS = 0.0247 and for the core scale m = 4, STRESS = 0.068.

Due to the high number of zero counts geostatistical methods were not used to assess spatial pattern as this would result in violation of the assumptions of these tests. Instead, partial linear regression, using a Generalized Linear Model (GLM) with a binomial distribution for taxa presence–absence data and logistic link function, was used to partition the deviance explained by space (latitudinal and longitudinal coordinates), biotic influences (presence–absence data for all other species) and scale for each taxon (Legendre and Legendre, 1998; Lobo et al., 2002). GLM allows for distributions other than the normal distribution and is also not constrained by the assumption of linearity between dependent and independent variables (Lobo et al., 2002). This makes it a useful technique for analysing ecological data, which is often non-normally distributed. Scale, as a nominal variable, was split into 'field' (numbers 1–6) and 'site' (numbers 1–26) to

Table 1
Number of individuals of each taxon, their percentage of the total 406 insects obtained from 2474 soil cores over 26 sites in 6 fields, and the population density based on an area of 4.85 m² (the total area of all soil cores collected).

Taxa	No. of individuals	%	Population density (m²)
B. johannis	112	28	23
Sciaridae	83	20	17
Leatherjackets	80	20	17
D. febrilis	56	14	12
A. lineatus	35	9	7
A. sputator	15	4	3
A. obscurus	13	3	3
Unknown WW	7	2	1
Non-Agriotes	5	1	1

Table 2
The variance/mean ratio for taxa at the field, site, and core scale.

Taxa	Field scale	Site scale	Core scale
A. obscurus	6.4	2.9	1.1
A. sputator	8	6	1.1
A. lineatus	8	2.5	1
Non-Agriotes	1.6	1.3	1
Sciaridae	22.2	18.9	20.9
B. johannis	64.1	17.6	2.1
D. febrilis	27.8	30	19
Leatherjackets	12.6	3.3	1.3

determine the proportion of deviance explained by the scale of sampling. Eight components of the total explained deviance were calculated: effect of spatial variation, effect of scale, effect of biotic influences, combined effect of space and scale, combined effect of space and biotic influences, combined effect of scale and biotic influences, the joint effect of all three types of variables and the unexplained variation–variation not accounted for by the explanatory variables included in this study. These analyses were carried out using Brodgar v.2.5.7 (Highland Statistics Ltd., 2006).

3. Results

3.1. Abundance and composition of taxa

B. johannis was the most numerous species identified, followed by Sciaridae and leatherjackets which were found in similar proportions (Table 1). D. febrilis was the least numerous dipteran species. The wireworm species were dominated by A. lineatus, whilst A. obscurus and A. sputator were found in similar proportions and the non-Agriotes wireworms comprised a relatively small proportion of all taxa (Table 1). In addition there were a number of individual wireworms that could not be identified, possibly due to degraded DNA (unknown WW; Table 1).

3.2. Variance/mean ratio

The variance/mean ratio generally decreased from the field scale to the core scale (Table 2). A. obscurus, A. sputator, A. lineatus and leatherjackets were aggregated at the field and site scales (VMR > 1) but had a more random distribution at the core scale (VMR = 1). The 'non-Agriotes' wireworms showed a random distribution at all scales, whereas Sciaridae, B. johannis and D. febrilis were aggregated at all scales.

3.3. Non-metric multidimensional scaling

NMDS ordination biplots (Fig. 1) revealed that the distribution of taxa in relation to each other changed between the field, site and core scales. Within the graphical configuration of the biplot, taxa close to each other co-exist in the same fields (a), sites (b) or cores (c) (Zuur et al., 2007). Notably, A. obscurus and non-Agriotes wireworms, and, separately, leatherjackets and Sciaridae are found in close proximity across all scales. The co-existence of other wireworm species is varied across scales, while B. johannis and D. febrilis are somewhat dissociated at the broader field and site scales, but are relatively closely associated at the core scale.

3.4. Deviance partitioning

The combined amount of deviance explained by the explanatory variables ranged from 12% (leatherjackets) to 43% (A. sputator) and the majority of the variation was unaccounted (Table 3). Of the explained deviance, scale was the most important individual component for all taxa, comprising between 10% and 36%, while spatial and biotic variables had a minor influence on most taxa. The biotic and scale variables combined explained slightly more variation than the scale and spatial variables combined, and the combined effect of biotic and spatial variables was relatively small for most taxa. The importance of field and site in terms of explained deviance varied between taxa: the presence of A. obscurus, non-Agriotes, B. johannis and D. febrilis was more affected by field than site, whereas A. sputator, A. lineatus, unknown wireworms and Sciaridae distribution was more related to the site variable. Field and site explained an equal amount of deviance for leatherjackets, which was relatively low compared to the other species, suggesting scale does not considerably influence leatherjacket distribution. Core scale data were not included due to the high number of cores which contained no taxa. Only 14 out of 2474 cores contained two different taxa, which is not significantly different from the six number of occasions of co-occurrence predicted by a chi-square test ($X^2 = 11.76$, $df = 35$,
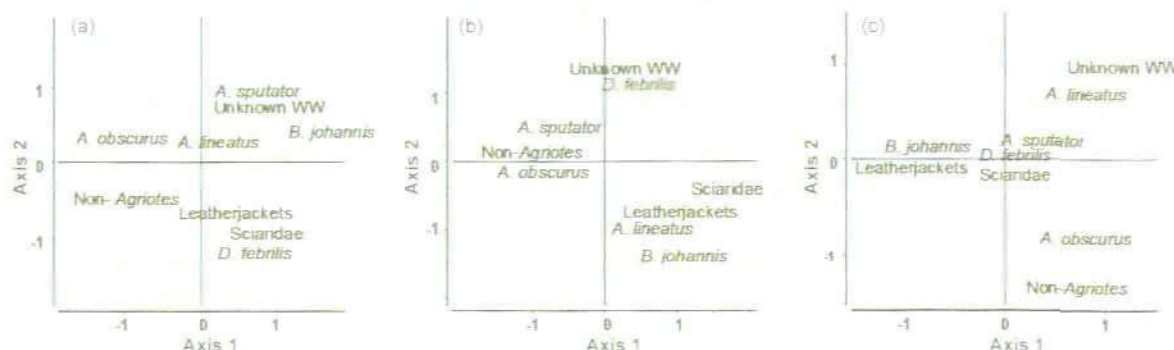
Fig. 1. NMDS ordination biplots (axes 1 and 2) for (a) field scale (b) site scale and (c) core scale. The distances between species represent relative similarity. 'Non-Agriotes' refers to wireworms that are not one of the three UK Agriotes species (A. obscurus, A. sputator and A. lineatus) and 'unknown WW' are wireworms for which no restriction fragment was produced. Bibionid flies were separated to species (D. febrilis and B. johannis), Sciaridae are grouped together as a species complex and leatherjackets are assumed to be Tipula paludosa.

Table 3

The percentage of total explained deviance obtained through deviance partitioning in the dependant variable (species presence-absence) between the three groups of explanatory variables and their combinations with each other, and the deviance unexplained by the variables in this study, for each taxon. See text for definitions of deviance categories.

	A. obscurus	A. sputator	A. lineatus	Non-Agriotes	Unknown WW	Sciaridae	B. johannis	D. febrilis	LJ
Biotic	9	6	4	13	4	3	1	2	2
Scale (all)	30	36	10	26	29	17	30	32	10
Scale (field)	23	12	5	17	11	8	21	18	5
Scale (site)	7	24	10	9	18	9	9	14	5
Space	6	27	1	1	11	2	30	12	2
Biotic and Scale	36	48	18	28	32	18	30	34	12
Biotic and space	16	34	5	15	15	5	2	15	3
Scale and space	33	39	18	27	31	19	30	34	10
All variables	38	43	20	39	34	21	30	36	12
Unexplained	62	57	80	61	66	79	70	64	88

$p > 0.05$), indicating the taxa are distributed randomly in relation to each other at this scale and population density.

4. Discussion

4.1. Abundance and composition of taxa

Only low numbers of insect larvae were recovered during this study when compared with other reports (Table 1). Sciaridae and Bibionidae are reputed to be among the most abundant families of soil dwelling dipteran larvae (Frouz, 1999), though Blackshaw and D'Arcy-Burt (1997) state that Bibionidae only occur 'sporadically and at low population density' in agricultural grassland. Wireworms and leatherjackets can occur in high abundances in grassland soil (e.g. Buckle, 1923; Miles, 1942; Staley et al., 2007; Gongalsky et al., 2009), however, it is likely that large populations are the exception rather than the rule (see Anon., 1948; Blackshaw, 1983). Little is known of species-specific wireworm distribution in grassland in the UK and Europe due to the morphologically cryptic nature of wireworm species. The fact that differences in abundance have been observed here between closely related species underlines the need to separate taxa to species, particularly for potential pests, in order that the damage causing species can be identified. The growing use of molecular techniques, such as DNA barcoding (e.g. Ball and Armstrong, 2006), restriction fragment length polymorphism (RFLP) (e.g. Carew et al., 2003) and T-RFLP (e.g. Ellis et al., 2009) may aid identification of other problematic taxa.

4.2. The effect of scale, spatial and biotic variables on distribution of taxa

The limited importance of biotic and spatial variables, in terms of deviance explained, and the high amount of unexplained variation (between 57% and 88%, Table 3) indicates that other unmeasured factors are more important for determining the distribution of the taxa studied. As well as the effects of scale-dependent environmental factors, reproductive and stochastic effects, which are more difficult to model, may play a role (Giller, 1996). The importance of scale for all taxa implies the effects of chemical, physical and environmental factors, which although not measured directly in this study, are known to differ between fine and broad scales. In turn, differences in taxon preference for these factors affects the distribution observed at different scales of investigation.

Aggregation of wireworms, leatherjackets, B. johannis, D. febrilis and Sciaridae has also been observed in other studies over different scales, and linked to biotic and environmental factors, including soil type, texture and moisture (Cherry and Stansly, 2008; Jackson and Campbell, 1975), amount of organic matter (Salt and Hollick, 1946; D'Arcy-Burt and Blackshaw, 1991), presence of grass species and other soil insects (Salt and Hollick, 1946), distribution of oviposition sites (Doane, 1977; Coulson, 1962; Frouz and Paoletti, 2000),

host plant selection (Jackson and Campbell, 1975) and aggregation pheromones (Blackshaw and D'Arcy-Burt, 1997). In addition, larval dispersal ability may also affect species' distribution. For example, although early instar wireworms remain aggregated, late instar wireworms are better able to disperse, resulting in less aggregation with age (Salt and Hollick, 1946; Doane, 1977). This process is likely to affect larval distribution at smaller scales, and could explain the random distribution of the wireworm species, which were late instar, at the core scale in this study (Table 2). Similarly, Sciaridae larvae (and other soil dwelling Diptera) have been described as 'almost sessile' (Frouz, 1999). Thus, the lack of larval dispersal and possibly adult choice of oviposition site, may be contributing to their aggregation at all scales (Table 2).

An important outcome of the study is that larval distribution differs between species, likely caused by a variety of scale and species-specific factors. For example, scale contributed most to the explained deviance for all wireworm species, but there was variation in the importance of field and site between species (Table 3). Similarly, space explained an equal amount of deviance as scale for B. johannis, but not D. febrilis, indicating possible differences in the biology of these species, especially since pure spatial variation may be associated with biological processes not linked to environmental factors, e.g. growth, predation, competition and social aggregation (Borcard et al., 1992). However, scale and biotic and spatial variables were ineffective at explaining leatherjacket distribution, with 88% of deviance unaccounted for. The equal deviance explained by the field and site scales suggests that scale, and the factors acting at these scales, do not have as much of an influence as, for example, reproductive factors or density dependant feedback mechanisms, such as cannibalism (Blackshaw and Coll, 1999; Blackshaw and Petrovskii, 2007).

4.3. Interspecific interactions across sampling scales

Whereas at large scales species-specific habitat requirements or environmental factors are likely to affect species distribution in relation to each other, at finer scales the effects of competition and predation may have more of an impact (Wiens, 1989). Furthermore, each taxon may have its own characteristic scale. As a result, interactions between two species could depend on the area over which it is measured (Sandel and Smith, 2009). The close association of D. febrilis and B. johannis with leatherjackets observed at the site and core scales (Fig. 1) has been detected in previous studies (D'Arcy-Burt, 1987), but here the dissociation of D. febrilis from these taxa at the field scale contradicts this. It has been noted that patches of damage caused by Bibio species often occur near hedgerows and are related to the occurrence of adult mating swarms, suggesting larval distribution is determined by female behaviour. However, damage associated with D. febrilis is more widely distributed throughout a field (D'Arcy-Burt, 1987; D'Arcy-Burt and Blackshaw, 1991), indicating possible differences

if species feeding and/or habitat preferences. Likewise similarities in biology, for example moisture preferences, may explain the similar distribution of Sciaridae and leatherjackets. The dissociation of wireworm species, apart from *A. sputator*, from the bibionids at the core scale may be related to the effects of predation; it has previously been noted that in captivity *Agriotes* spp. attack and kill bibionid larvae (Andrianov, 1914). Published studies on species-specific wireworm distributions are lacking; however unpublished results of the authors suggest associations at the field scale between some species and not others, linked to differences in soil pH, grass duration and amount of organic matter between fields. This reiterates the importance of identifying individual species and not grouping related organisms together, as has commonly been the case with the *Agriotes* pest complex.

The few taxa sampled here represent only a small proportion of the total biodiversity in soil. Although competitive interactions may contribute to variation in species distributions, predators also play a role. There are known to be large numbers of predators which feed on soil taxa, but their effect on populations in the field is little known (Giller, 1996), with soil food web models concentrating on detritus food webs, despite the fact that root herbivores are important in soil structure and transport processes (Brussaard, 1998). Depending on the questions being addressed another way to describe interactions between soil biota may be through the use of functional groups, using ecological rather than morphological similarities (Giller, 1996). This would overcome the problem of lack of species differentiation in studies on soil communities. However, although this may be appropriate for studies on ecosystem processes and the roles of invertebrates in maintaining soil fertility and decomposition, for example, for others a more detailed approach is needed; in particular pest management and control information on species specific interactions are important for developing selective methods that will target pest species.

5. Conclusions

The use of three complementary approaches in a multi-scale analysis improved our overall understanding and interpretation of species distributions and may be useful for other studies of this kind. Our study emphasises both the need to take scale into account when carrying out ecological studies and that knowledge of the species present and their ecology is important in order to interpret their observed distribution patterns. It would be interesting to establish whether spatial distribution and the biotic and environmental factors affecting spatial patterns changes at higher population densities, which itself might be another scaling factor. For future studies the use of a number of sampling techniques specific to the taxa under study may increase the numbers of nulls data and taxa captured. Geostatistical analysis could then be applied, which has the potential to provide information on the probability of finding taxa between sites based on the spatial distribution of individuals and can also provide insight into the underlying factors affecting spatial patterns.

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The relationship between *Agriotes* click beetles (Coleoptera: Elateridae), wireworms and environmental factors

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Abstract: Three species of soil inhabiting click beetle (Elateridae: Coleoptera) larvae, known as wireworms, are grouped as a crop pest complex in the UK- *Agriotes obscurus, A. sputator* and *A. lineatus*- mainly because these species are morphologically cryptic. In this study a molecular tool, terminal restriction fragment length polymorphism (TRFLP) (Ellis *et al.* 2009), was used to identify wireworms. Principal Components Analysis (PCA) assessed the relationship between adult and larval distributions, which was compared to a study in which wireworms were grouped as a complex. The results suggest that grouping these species as a complex may be inappropriate, and that assessing the distribution of wireworms as separate species may give more information on their distribution in relation to adults and environmental variables.

Key words: Wireworms, click beetles, *Agriotes*, TRFLP, Principal Components Analysis

Introduction

Wireworms, the soil inhabiting larvae of click beetles (Coleoptera: Elateridae), are worldwide pests of many agricultural and horticultural crops, causing loss of crop quality and/or yield. Although research has been carried out for nearly a century on wireworm biology and control, there is still much uncertainty regarding certain aspects of their ecology, and subsequently they continue to be a problem for many farmers. Recently research has focused on click beetle and wireworm ecology, with a view to developing more reliable risk assessment and control methods that do not depend upon pesticides.

In the UK a pest complex of three species are thought to be responsible for the majority of damage; *Agriotes obscurus, A. sputator* and *A. lineatus*. Knowledge of the distributions of individual species, of both adult click beetles and wireworms, and the factors that affect this distribution, is important. However, there have been limited studies in this area so far, and where studies have been carried out they have grouped wireworms together as a complex.

The main reason for this lack of species differentiation is that *Agriotes* wireworms are morphologically cryptic. It has been said to be 'difficult or even impossible' (van Emden 1945) to identify the first instar larvae, and the mandibular structures, an important identification feature, can become worn during feeding. A considerable amount of expertise and time is required to make a reliable identification, particularly with a large number of samples.

Recently a molecular tool, terminal restriction fragment length polymorphism (TRFLP) was developed to identify the three UK species of wireworms (Ellis *et al.* 2009). This method is advantageous in that no expertise is needed and it is relatively easy, quick and reliable in comparison to studying wireworm morphological features.

In this study we used the TRFLP tool to identify wireworms collected as part of a previous study by Hicks (2008). Hicks (2008) collected wireworms, adult click beetles

and environmental data, however wireworms were grouped as a complex in the analysis. We re-analysed this data with individual wireworm species in order to establish whether knowledge of the wireworm species present affects the results of an analysis looking at the relationship between adults and wireworms.

Materials and Methods

Sampling
Wireworms were collected by Hicks (2008) from organic fields in the South Hams, Devon, in 2004, using soil cores (10cm deep 10cm wide) and bait traps (1:1 wheat-Barley seed mixture). Twenty soil cores were sampled from each of 99 fields, and bait traps were placed in a selection of these fields. A range of environmental, chemical, physical and cultural data were obtained for each field, and adult males were obtained using sex pheromone traps (Furlan *et al*. 2001; Toth *et al*. 2003).

Wireworm identification - TRFLP
DNA was extracted using a standard salt/chloroform method from the last abdominal segment of the wireworm. Adult tissue was also extracted in the same way for use as known controls. The TRFLP protocol described by Ellis *et al*. (2009) was used to identify *A. obscurus*, *A. sputator* and *A. lineatus* wireworms.

Multivariate analysis
Principal Components Analysis (PCA) was carried out in Canoco® (version 4.5, Biometris). Data were normally distributed following square root transformation and the 'center and standardize by species' option was chosen so that the impact of the small numbers of larvae were minimised against the large number of adults (Hicks 2008). Otherwise the default options were chosen.
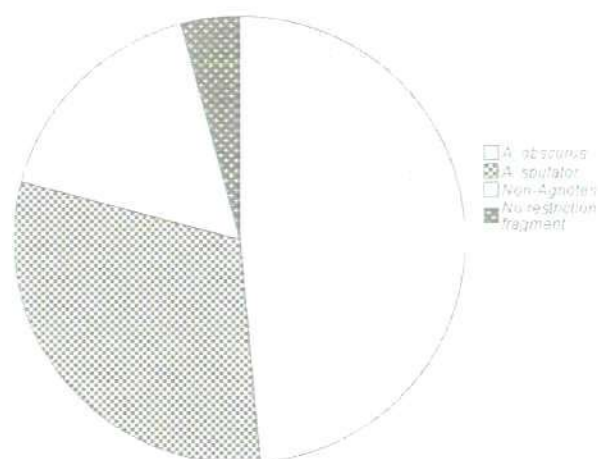
Results and discussion

TRFLP

Figure 1. The relative abundance of *A. obscurus*, *A. sputator*, *A. lineatus* and unknown 'non-*Agriotes*' wireworms.

Of the 72 samples analysed, 35 (49%) were identified as *A. obscurus* and 22 (30%) as *A. sputator*. There were 3 (4%) individuals for which no restriction fragment was

produced, possibly due to degraded DNA. 12 (17%) samples consistently produced an undigested fragment at 400bp (non-*Agriotes*; Figure 1), which were investigated further using direct sequencing. The sequences obtained subsequently failed to align with the published *Agriotes* sequences, or to significantly match any other published click beetle sequences on GenBank, and as of yet remain unidentified. No *A. lineatus* wireworms were identified. These results suggest that grouping wireworms together as a pest complex may not be appropriate, particularly as no *A. lineatus* larvae were collected from an area in which *A. lineatus* adults were found, and a high proportion of other wireworm species were also recovered.

Principal Components Analysis (PCA)

PCA analyses the variability in species composition, and so is useful for investigating the relationship between species and/or life stages. Firstly a PCA was carried out using Hicks' (2008) wireworm complex and adult species data, and then a further PCA was produced combining the wireworm species data collected in this study with the adult data collected by Hicks (2008) (Figure 2).

Figure 2. a) Ordination diagram of PCA axes 1 and 2, with *Agriotes* adults and wireworms and other unknown 'non-*Agriotes*' wireworms as a complex (names abbreviated) b) Hicks' (2008) PCA (axes 1 and 2) with wireworms as a complex.

There is a strong positive correlation between *A. sputator* wireworms and adults, but less of a correlation between *A. obscurus* wireworms and adults. *A. lineatus* adults and the other unknown wireworms (here grouped together) are somewhat

dissociated from each other and the other *Agriotes* species (Figure 2; left). In comparison, the PCA using Hicks' (2008) original data shows that *A. obscurus* adults are strongly associated with the wireworm complex, whereas *A. sputator*, and again *A. lineatus* adults, are dissociated from these species variables. These results reaffirm that grouping wireworms as a complex may not provide reliable information on individual species distributions in relation to adults, particularly for *A. lineatus*, and so the current risk assessment method of pheromone trapping separate species of adults as an indication of overall wireworm infestation may not be reliable.

Multiple regression

Hicks (2008) also carried out stepwise multiple regression, which highlighted significant environmental factors that were different for the wireworm complex and for adults of each species. Out of the large number of variables measured, only five that affected adult prevalence were also selected for the wireworm complex and these were a mixture of chemical, physical and cultural factors. The most interesting finding from the adult stepwise multiple regression was that the factors influencing each species' distributions were different. This suggests that if wireworms were included in the analysis as separate species that there may be species specific differences to environmental conditions that are not apparent when wireworms are grouped as a complex. This information may better enable targeting of existing control strategies and development of new methods that take these factors into account.

Future work will involve identifying the unknown 'non-*Agriotes*' species, identifying wireworms from elsewhere in the UK, Europe and Canada to further assess the proportion and abundance of wireworm species present, and to analyse wireworm and adult species data with the environmental variables collected by Hicks (2008).

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THE DISTRIBUTION OF SOIL INSECTS OVER DIFFERENT SPATIAL SCALES IN GRASSLAND.

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In the UK there are a number of insect taxa whose larvae are associated with grassland, including wireworms (Agriotes spp.), leatherjackets (Tipula spp.), bibionids (Bibionidae) and sciarid fly larvae (Sciaridae). Most research on these groups has arisen from their functional roles as pests in agricultural systems and as such each has been considered separately. Consequently there are no studies that consider the possible interactions between these different taxa. Here we present data on the occurrence of these pest groups. Insect larvae were recovered from soil cores using dry heat extraction (Blasdale 1974). Bibionid larvae were identified from morphological characteristics and Agriotes spp. using a T-RFLP genotyping method (Ellis et al. 2009). Leatherjackets were assumed to be Tipula paludosa, as no adults of other species were found at the sites. Sciarids were not identified to species. Samples were taken from 25 sites over six fields on a farm in south Devon, UK. At each site 96 5cm diameter soil cores were collected from points at the intersections of concentric circles (5, 10, 20 and 40m diameter) and regularly spaced (15o) radii. The distributions of the taxa were compared at three spatial scales: within soil cores, within study sites and within fields. Results are presented and the ecological and pest management implications discussed.