Development and optimisation of PCR-based techniques in predator gut analysis

A thesis presented for the degree of
Doctor of Philosophy

CIARA SIOBHAN DODD

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School of Biosciences
Cardiff University

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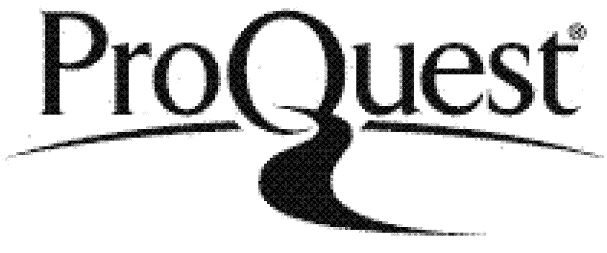

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Full Title of Thesis **Development and optimisation of PCR-based techniques in predator gut content analysis.**

Summary:

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DNA-based detection methods proved suitable for investigating predation by *P. melanarius* on economically damaging slug species. Mitochondrial DNA primers were designed for the 12S ribosomal RNA gene that were specific for *Deroceras reticulatum*, members of the *Arion hortensis* aggregate and the *Arion* genus.

The detection limit and rate of decay of slug DNA in the beetle gut was determined using the slug-specific primers in laboratory-based feeding trials. Slug remains could be reliably detected within beetle guts for at least 24 hours following ingestion, suggesting that this technique would be suitable for detecting predation in the field.

Direct comparison of immunological and DNA-based detection methods revealed that the sensitivity of MAb-ELISA is greater than amplification of prey DNA using the polymerase chain reaction (PCR). The implications of these findings are discussed.

DNA-based detection methods were suitable for detecting predation by beetles on slugs in the field. The number of slug-positive beetles identified using MAb-ELISA was greater than when PCR and prey-specific primers were used. These results are discussed in the context of the availability of target and alternative prey in the field.

Sequence data was also analysed to reveal the population structure and demographic history of *A. hortensis* aggregate in the British Isles. A high level of differentiation between populations was revealed. The rate of molecular evolution in these species is rapid with high levels of inter-specific divergence. The 12s rRNA gene proved especially useful in phylogenetic reconstruction and corroborated previous results based on morphology and enzyme electrophoresis that the congener, *Arion intermedius* belongs to the same subgenus (*Kobelita*) as the aggregate species.

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Chapter 1

General Introduction

1.0 Outline of thesis

Slugs are one of the most economically damaging agricultural pests (Port & Port, 1986) affecting the majority of agricultural and horticultural crops grown in the UK. Primary control methods involving the use of chemical molluscicides have been shown to have adverse environmental effects and can severely affect non-target predatory arthropods due to their strong insecticidal properties. Integrated Pest Management (IPM) promotes methods in agriculture that minimise the use of non-renewable energy inputs (including, but not restricted to pesticides) and protect the biodiversity of the crop community from within which natural enemies can control crop pests (Kogan & Lattin, 1993; Claridge, 1996; Chen *et al.*, 2000). Since carabid beetles are recognised as important predators of slugs, and appear to dominate the soil fauna in temperate agro-ecosystems (Thiele, 1977), interest in their role as bio-control agents is increasing. With the advent of IPM in agricultural practice, certain questions need to be posed in order that effective management practices can be developed and maintained; for example, when studying the role of natural enemies in pest management systems, it is necessary to ascertain trophic interactions within communities of predators and pests. Unless satisfactory methods to quantify these interactions can be found, and a detailed plan of the complexity of the system developed, effective management of the system utilising natural enemies will be difficult. At the current time, the incorporation of biocontrol into IPM programmes is hampered by a lack of basic information on the effectiveness of the natural enemy complex (Chen *et al.*, 2000).

This project focuses on the development of novel techniques for studying the bio-control of slugs by carabid beetles, will help elucidate some of the complexities within arable systems in the UK and will contribute to the augmentation of more effective pest management strategies.

1.1 General biology and ecology of slugs

1.1.1 Taxonomy, evolution and systematics

Slugs belong to the Phylum Mollusca and the Class Gastropoda. Within the gastropoda, the majority of slugs are classified within the sub-class Pulmonata. Evolutionary relationships remain largely unresolved, but approximately 4/5ths of land snails are classified within the suborder Stylommatophora. Three primary divisions of the Stylommatophora (Orthurethra, Heterurethra and Sigmurethra) were proposed by Pilsbury (1900) based on excretory system morphology. A fourth division, Mesurethra, was added later by Baker (1955), but of these groupings, only one (Orthurethra) is still recognised based on the phylogeny of Wade *et al.* (2001) using ribosomal RNA genes.

Classification of slugs based on morphology gives conflicting results primarily because of the long evolutionary history of the group, relatively rapid radiations, convergence and morphological evolution (Wade *et al.*, 2001). The phylogeny of Wade *et al.* (2001) suggests that the different families each appear to be independently derived and this is also reflected in morphological taxonomies (Tillier, 1989; South, 1992). Recent molecular evidence, based on sequence data for ribosomal gene clusters, suggests that the Stylommatophora is monophyletic, but split into two clades, achitinoid and non-achitinoid (Wade *et al.*, 2001). The view posed by Wade *et al.* (2001), of achitinoid and non-achitinoid clades opposes the classical view based on morphology. This implies that Sigmurethra were divided by pedal morphology into the Holopoda and Aulacopoda (Pilsbury, 1900). From the current phylogeny, the achitinoid clade contains only holopod taxa, but the non-achitinoid clade contains both holopod and aulacopod species; however, gastropod phylogenetics remains a matter of debate.

There are 33 species of slug recorded in the British Isles (South, 1992), represented in the families Arionidae, Milacidae, Limacidae, Testacellidae and Boertgerillidae, but the most important crop pests are found within the first three families (Port & Port, 1986). Although not all basal divisions within the non-achitinoid clade are resolved, the

Limacoidea is well supported as a monophyletic group, containing the two British families of slugs, Limacidae and Milacidae. This clade is associated with the Arionidae as a sister group (Wade *et al.*, 2001). As familial groups, the Arionidae are strongly supported, but the Limacidae and Milacidae are less so. However, there is a paucity of genetic data relating to the resolution of deeper level relationships within these families.

1.1.2 Ecology and behaviour

Slugs exhibit variable life cycles both within and between species, with weather conditions and temperature having dramatic effects on the rate of development of both eggs and juveniles (Hunter, 1968; South, 1992). In general, of the three slug families that are important crop pests, the Arionidae have an annual life cycle, the Milacidae a biennial life cycle and the Limacidae a plurennial life cycle (South, 1992). Some species are more flexible in their requirements, such as *Deroceras reticulatum* (Müller), which is capable of reproducing whenever the conditions are suitable and may therefore be able to breed all year round (South, 1989). Arionid slugs such as *Arion distinctus* Mabille are more specialised in their requirements, maturing in early autumn to breed throughout the winter until April (Port & Port, 1986).

Slugs live in intimate contact with the soil and are active at night when conditions are suitable, sheltering by day in resting sites in or on the soil. These resting sites are in frequent use and the slugs often return to them after foraging. This homing behaviour has been demonstrated in a number of different gastropod species (South, 1965; Rollo & Wellington, 1981). *D. reticulatum* shows a distinct preference for soil structure which allows it to lie between soil aggregates, thus keeping most of its body in contact with the soil surface (Duval, 1970; Stephenson, 1975a, b). As slugs have a permeable integument, they are restricted to habitats that remain moist most of the time, e.g. heavy soils and dense vegetation (Gould, 1961). However, *D. reticulatum* is still active at extremes of temperature and humidity, which are limiting for other species (Port & Port, 1986), which undoubtedly contributes to its pest status. Other factors that can affect slug numbers such

as predators, parasites and pathogens (Port & Port, 1986) will be discussed in more detail in Section 1.3.

Most slugs are omnivorous, with the range of species of food plants consumed depending on availability. Slugs are not indiscriminate feeders, but actively select some food plants in preference to others (Paallant, 1972), but this is more usually due to the presence of allelochemicals in non-preferred plants (Cates, 1975; Gouyon *et al.*, 1983). In the case of cereals, barley seems less susceptible to damage than wheat (Duthoit, 1964), and between wheat varieties there seem to be no major preferences, although species with a higher total nitrogen content in the seed are favoured (Ashover unpub data in Port & Port, 1986). However, in the field, winter wheat is at greater risk than spring wheat, due to the cool moist conditions of autumn and winter favouring slug activity (Port & Port, 1986). In addition, the degree of surface compared with below-ground activity of different slug species contributes to their importance as pests and the efficacy of their control. *D. reticulatum,* a surface dwelling species, tends to feed on green, fresh plant material (Lutman, 1978), climbing food plants when conditions are suitable and has been reported as the species most frequently damaging wheat crops (Martin & Kelly, 1986). Soil dwelling species such as *Arion hortensis* Férussac and *Tandonia budapestensis* (Hazay) (Hunter, 1968), which normally remain near the ground to feed, prefer less green material.

1.2 Slugs as pests

Slugs pose a major threat to agriculture across Europe causing millions of pounds worth of damage to crops annually. Several species have extended their geographical distribution as a direct result of agricultural activities (South, 1992). Changes in farming practices as well as milder wetter winters in the UK (Sternberg, 2000) appear to be exacerbating the situation.

The most abundant and damaging species is *D. reticulatum* as it is able to feed and reproduce throughout the whole year in temperate climates (Hunter & Symonds, 1971).

This species together with the *A. hortensis* aggregate and *T. budapestensis* are considered to be the most important pest species in the UK (South, 1992) with the pest status of *Arion intermedius* Normand remaining uncertain, although it has been recorded in large numbers in winter wheat (Glen *et al.*, 1984). The *A. hortensis* aggregate, which was identified as a species complex by Davies (1977, 1979), comprises three species, *A. hortensis, Arion distinctus* Mabille, and *Arion owenii* Davies. Within the aggregate, *A. owenii* has a patchy distribution and it is not generally considered a pest. Of the three species, *A. distinctus* is thought to be the most widespread, occurring across much of Europe (Kerney, 1999). However, the morphological similarity of the species makes identification difficult and consequently the distribution of the three species is probably under-recorded and misrepresented. Another species, *A. intermedius* is not closely related, but is widely distributed across the British Isles.

Other less economically damaging species include *Arion ater* (Linneus), *Arion subfuscus* Draparnaud and *Deroceras panormitaum* (Lesson & Pollonera) (South, 1992). Field populations of slugs often comprise mixed species and age groups since mating and oviposition occur during weather conditions favouring activity (Port & Port, 1986) and reproduction may therefore occur over a prolonged period depending on the species, allowing a prolonged period of attack on the crop. In general, the crops, which sustain the greatest economic loss due to slug damage are winter wheat and potatoes, and to a lesser extent oilseed rape and sugar beet (Port & Port, 1986). Horticultural crops such as lettuce, brassicas and beans are also at risk.

The most important pest attacking winter wheat is *D. reticulatum* because the cool moist conditions in autumn and winter favour slug activity. *D. reticulatum* forages close to the soil surface near the germinating grain causing damage to the plant below ground by hollowing out the seeds and eating through the base of young shoots, preventing germination, and after emergence damage to seed leaves can reduce yield (Martin & Kelly, 1986). Damage to this crop caused by slugs equated to £2.69 million in 1985 (Port & Port, 1986).

In potatoes, the economic losses may still be even more significant, amounting to £7 million at 1985 values (Port & Port, 1986), but the level of damage to the crop is primarily cosmetic. Nevertheless, this still seriously reduces the market value of the crop. The slugs eat holes in the tubers from late summer until harvest whilst they are still in the ground (South, 1992). However, slug damage to the tuber then makes it more susceptible to attack by other pests and pathogens, particularly bacterial rot (Runham & Hunter, 1970).

Other factors contributing to economic loss include the application of molluscicides; Garthwaite & Thomas (1996) estimated that the annual cost in Britain alone is £10 million within agriculture and horticulture. Whether or not this annual expenditure reflects the severity of the problem, it highlights the perception of the problem by the farmer.

1.3 Control methods

1.3.1 Chemical control

Conventionally, slugs have been controlled with chemical molluscicides, the use of which has increased 70-fold since the early 1970s. This is based on an annual application of 4800 tonnes, with usage on arable crops accounting for 99 % of the total treated area (Garthwaite & Thomas, 1996). The products comprising the usage figures were, in 1994, metaldehyde 55 %, methiocarb 40 % and 5 % thiodicarb. Methiocarb used to be considered to be the most effective ingredient for molluscicidal baits (Kelly & Martin, 1989) and was originally developed for its broad spectrum insecticidal and acaricidal properties (Unterstenhofer, 1962). However, a number of field trials revealed little difference between the efficacy of methiocarb and metaldehyde (reviewed in detail in Port & Port, 1986). Recent advances in bait formulations of methaldehyde have resulted in greater control of all slug species (Glen, *pers comms*). Methiocarb is primarily a stomach poison with a low level of contact toxicity and in common with other carbamates is an acetylcholineesterase inhibitor, which in slugs causes paralysis and/or loss of

muscle tone (South, 1992). Metaldehyde works more as a contact poison, being readily absorbed through the foot of the slug, is also effective via ingestion, causing paralysis and excess mucus production eventually leading to dehydration and death (Cragg & Vincent, 1952). Death is usually inevitable if dehydration causes more than 50 percent loss in body weight (Godan, 1999); if less than this, then recovery is possible after exposure to moisture that replenishes body fluids (Jones & Jones, 1974). Additionally, iron and aluminium salts are toxic on contact, but their efficacy following application is dependent on soil type and humidity (Henderson & Martin, 1990).

The control of slugs using chemical methods is more problematic than for insect pests and effectiveness is related to slug behaviour, farming practice and weather conditions, and consequently, crops may even fail after application of chemicals (Glen, 1989). Molluscicide is normally applied, either as pelleted bait together with the cereal seed at sowing or later on after slug damage is observed. Alternative formulations include powder, spray or seed treatments (Ester & Nijenstein, 1996). The efficacy of baits depends on their palatability, attractiveness and toxicity.

As slugs have been observed to ingest smaller meals in the presence of toxic compared with non-toxic meals (Kelly & Bailey, 1996), they do not always ingest a lethal dose of the pesticide (Bailey *et al.*, 1989). To alleviate this problem attractants and phagostimmulants may also be added the bait, particularly to methiocarb (Bowen *et al.*, 1996; Meredith, 1996). As many slugs attack below soil level, they may not be exposed to bait applied to the soil surface (Symondson, 1997), reducing effectiveness to between 10% (Godan, 1999) and 50% (Glen & Moens, 2002). However, environmental conditions can have a profound effect on control success. Slug activity on the surface increases in moist conditions, which means that slugs will be more likely to encounter bait under these conditions. However, the persistence of the bait in the field and its effectiveness in killing the slug may be compromised in these circumstances. As slugs are often immobilised after ingesting a sub-lethal dose of molluscicide, moist conditions will allow the slug to recover without becoming dehydrated, whereas in dry conditions

the production of copious amounts of mucus by the slug, following exposure, will cause it to dehydrate and die (Port & Port, 1986).

The use of molluscicides, especially the carbamates, methiocarb and thiodicarb, have been shown to affect adversely carabid beetle populations (Purvis & Bannon, 1992). Methiocarb-based pellets were chosen in preference to 'less palatable' molluscicides and were toxic to all the carabid species against which they were tested (Büchs *et al.*, 1989), although, it has been argued that the long-term consequences to carabid populations of the use of methiocarb in late autumn has no more harmful effects than routine annual soil cultivation (Purvis, 1996.). Nevertheless, as carabids are important natural enemies of slugs, the wide scale use of carbamate-based molluscicides seems incompatible with the principals of IPM, especially as the effectiveness of such baits is variable (Purvis, 1996). Earthworms (Bieri *et al.*, 1989a, b), ground beetles (Büchs *et al.*, 1989), rove beetles (Wiltshire & Glen, 1989) and birds (Godan, 1999) all show significant effects of methiocarb poisoning. Methiocarb has toxic effects on earthworms, being slightly toxic on contact (Young & Wilkins, 1989) and after ingestion (Bieri *et al.*, 1989a, b). In a review of the environmental effects of metaldehyde, Biere (2003) concluded that it did not show any adverse effects on non-target species.

1.3.2 Cultural control of slugs

The conditions under which a crop is grown can have a profound influence on the degree of damage caused by molluscs. Seedbed preparation and soil condition often determine whether significant slug damage will occur. The incorporation of crop residues into the soil and direct drilling rather than ploughing (Port & Port, 1986; Glen *et al.*, 1988, 1989, 1993; Kendall *et al.*, 1995), all affect the suitability of the crop for slugs. Higher slug numbers have been found when crop residues are left rather than burnt (Glen *et al.*, 1988), indicating that better control of slugs is achieved the more the soil is worked (Hunter, 1967), either by making the soil structure finer and firmer, deterring slug activity (Stephenson, 1975b), or by physically damaging the slugs (Hunter, 1967; Gould & Webley, 1972). When crop residues are incorporated into the soil, slug damage to seeds

and seedlings of the subsequent crop depends on seedbed conditions and the ability of slugs to move through the soil (Glen *et al.*, 1989). Clay soils containing large clods provide ideal shelter for slugs and promote slug damage (Godan, 1983) whereas firm fine tilth is more difficult for slugs to move through (Glen & Wilson, 1995) which may reduce damage. In terms of seedbed preparation, Grant *et al.* (1982) suggested that the worst slug problems were associated with direct drilling (no cultivation), in which the seeds are more accessible to the slugs, which cause damage by hollowing out the grain and by grazing on the young shoots as they appear (Gould, 1961; Duthoit, 1964). Some crops and cultivars (Evans & Spaull, 1996) are more resistant to slug damage than others, for example, spring wheat is less susceptible to attack than winter wheat because it germinates and grows more quickly in conditions that are less favourable to slug activity (Runham & Hunter, 1970). The order in which crops are grown in a rotation can also influence the degree of damage. For example, when oilseed rape is followed in rotation by winter wheat, the latter may suffer a greater degree of damage than would occur if it was not preceded by oilseed rape (Glen *et al.*, 1996). This is because the dense foliage of oilseed rape provides an ideal microhabitat for the development of large slug populations that are then present in the soil ready to attack the next crop.

1.3.3 Biological control

1.3.3.1 Pathogens

In practice, the biological control of slugs does not currently include the use of pathogens (Godan, 1999), although bacteria such as *Moraxella osolensis* are associated with some nematodes that infest slugs. Parasites that attack slugs include protozoa (ciliates e.g. *Tetrahymena* sp.), nematodes (*Phasmarhabditis hermaphrodita*) and mites (*Riccardoella limacum*). The protozoan parasite *Micosporidium novocastriensis* can lead to a reduction in fecundity (Jones & Selman, 1985), longevity and feeding in slugs, but is specific to *D. reticulatum* (Port *et al.*, 2000). However, the most promising parasite in terms of biological control is the nematode *P. hermaphrodita*. This is available commercially as Nemaslug, for the small-scale control of slugs in the home garden market. The nematode enters the body cavity of the slug, introducing bacteria on which the nematode feeds.

This results in characteristic swelling of the mantle, where the nematodes reproduce, causing it to rupture following a build up of pressure. Feeding in slugs infected with the nematode is inhibited and slugs die after a few days (Glen *et al.*, 2000). This nematode has been shown to infect a number of different mollusc species and effectively controls slugs in winter wheat and oilseed rape (Wilson *et al.*, 1994), but currently it is only commercially viable as a method of control in high value produce such as organic farming (Glen & Moens, 2002).

1.3.3.2 The role of predators as bio-control agents

Arthropod predators are the most abundant insect natural enemies in most agroecosystems (Greenstone, 1996). An effective predator complex can reduce the damage caused by phytophagous insect herbivores in agroecosystems, although their effectiveness is influenced by many biotic factors (Barbosa & Wratten, 1998). Plants can have a dramatic impact on the effectiveness of invertebrate predators, even in response to relatively small changes in different crop cultivars (Barbosa & Wratten, 1998). Habitats that are structurally, biologically or temporally diverse usually provide greater microhabitat diversity and a concomitant variety of potential prey and predation opportunities (Barbosa & Wratten, 1998). Predatory invertebrates are usually highly polyphagous, thus any habitat that contains a wide variety and abundance of prey, provides optimal conditions for these species. Most predator species are cryptic, feed infrequently and we know very little about their impact on pest populations (Greenstone, 1996).

A number of invertebrates notably from within the Diptera and Coleoptera are important predators of slugs. The most important families within these are the Lampyridae, Drilidae, Staphylinidae, Carabidae from the Coleoptera, and Sciomyzidae from the Diptera (Stephenson & Knutson, 1966). It has been suggested that many of the larger Carabidae will feed on slugs and Tod (1973) suggested that the larger the beetle the more likely it was to be able to kill slugs due to strongly developed mandibles. Carabid species that have been shown to feed on slugs are *Carabus violaceous* (Linnaeus) (Thiele, 1977;

Paill, 2000), *Pterostichus melanarius* (Illiger) (e.g. Symondson & Liddell, 1993e, 1995; Symondson *et al.*, 1996; Bohan *et al.*, 2000), *Abax parallelepipedius* (Piller and Mitterpacher) (Symondson, 1993; Symondson & Liddell, 1993a), *C. nemoralis, C. problematicus* (Herbst), *P. niger* (Schaller), *Calathus fuscipes* (Goeze) and *Cychrus caraboides* (Linneus) (Tod, 1973). However, the vast majority of research has concentrated on the role of *P. melanarius* as a predator of slugs.

1.4 Biology of carabid beetles

1.4.1 Overview of Carabidae as natural enemies

There are thought to be about 40,000 species of Carabidae world wide, of which 3000 species are distributed across Europe and 350 species occur in Britain. Carabids are mostly polyphagous feeders that consume animal (live prey and carrion) and plant material (Lovei & Sunderland, 1996) and forage actively on the ground surface. Carabids are generally considered to be beneficial predators (since Forbes 1883) in agricultural ecosystems providing an important function in terms of biological control, with very few being pests themselves (Thiele, 1977; Luff, 1987). Agriculture profoundly influences the composition, abundance and spatial distribution of ground beetles through the use of agrochemicals, and changes in habitat structure resulting from cultivation methods and crop type (Stork, 1990; Desender *et al.*, 1994). The timing and type of cultivation can directly affect carabid numbers by interfering or protecting vulnerable life stages although this differs between species (Luff, 1987). Kendall *et al.* (1991) found significantly higher numbers of Carabidae (including *P. melanarius*) in traps on direct drilled plots in October than on ploughed plots, and intermediate numbers in non-inversion tillage fields. A similar situation was observed by Fadl *et al.* (1996) who caught fewer *P. melanarius* in spring cultivated fields than in those that either remained uncultivated or were cultivated in the autumn. It appears that minimum tillage practices are beneficial for carabid abundance and for slug abundance resulting in a greater availability of prey, a factor, which in itself could partially explain the higher number of carabids in minimally cultivated areas. In general, the density of carabids fluctuates in

space and time from <1 in many habitats to >1000 individuals per square meter at suitable overwintering sites (Lovei & Sunderland, 1996).

It has been suggested that carabids can suppress pest outbreaks, but according to (Southwood, 1978) their major beneficial role is to prolong the period between these resurgences i.e. when the pest abundance is in the so-called natural enemy ravine. The stability of carabid numbers implies that they are unlikely to respond rapidly in a numerical way to changes in prey density, but remain as a potentially useful component of environmental resistance to pest outbreaks when pests are absent from the crop (Luff, 1982). To increase the carabids effectiveness, biological control practitioners should consider the general habitat favourability that will keep carabids near their required site of action. A successful application of this technique could be the use of habitat islands to serve as refuges and recolonization foci (Thomas *et al.*, 1991; Lys, 1994).

The majority of carabids are highly polyphagous, and there is increasing evidence that they play a role in the population dynamics of a number of phytophagous insects. A number of reviews have focused on the ecology of carabids on agricultural land (Thiele, 1977; Luff, 1987; Lovei & Sunderland, 1996; Kromp, 1999; Symondson, 2002a). Carabids have been shown to prey upon a variety of insect pests such as aphids and can have a limiting influence on their population growth in the establishment phase (Sunderland & Vickerman, 1980; Hance, 1990; Winder, 1990; Landis & Van der Werf, 1997). For example, three species of carabid showed heavy predation on *Aphis fabae* Scopoli (Hance, 1987) and *Rhopalosiphon padi* Linnaeus (Chiverton, 1986; Ekbom *et al.*, 1992). In experimental enclosures, it was demonstrated that the carabid *P. melanarius* contributed to controlling the rate of recolonisation of the crop canopy by aphids (Winder, 1990). Some of the smaller carabids such as *Bembidion lampros* (Herbst) and *Trechus quadristriatus* (Schrank) have been implicated as important predators of Diptera such as cabbage root fly *Delia radicum* (Linnaeus) (Luff, 1987). Carabids have also been shown to prey upon lepidopteran pests in an agricultural setting in America (Frank, 1971) and in Britain (Frank, 1967). In an agricultural setting, *P. melanarius* is especially

common (Thiele, 1977) and has been shown to be a particularly important predator of slugs (Symondson *et al.*, 1996; Bohan *et al.*, 2000; Symondson *et al.*, 2002) and to a lesser extent of aphids (Chiverton, 1987).

1.4.2 Pterostichus melanarius

P. melanarius is often the commonest large carabid caught in pitfall traps in arable crops in the UK (Thiele, 1977). It is an autumn breeding species in which the larvae overwinter (Paarmann, 1979). Both the larvae and adults are predatory and the range of prey consumed is quite variable. *P. melanarius* is mostly a ground feeding species and does not readily climb vegetation as some other carabids do. Some prey species are likely to be better quality nutritionally than others. In laboratory prey choice experiments, Harwood *et al.* (unpublished) demonstrated that diet had a profound influence on the fitness of *P. melanarius* in terms of weight, numbers of eggs laid, sustainability of egg laying, the period to egg hatching, egg hatching rate and beetle survival. In all cases, the beetles did poorly when fed exclusively on single pest species (slugs or aphids), whereas mixed diets containing earthworms as well as slugs and/or aphids and/or diptera larvae resulted in fitter predators. Similar patterns have been shown for other generalist predators (Pollet & Desender, 1987; Wallin & Ekbom, 1994; Bilde & Toft, 1997a, b, 2001; Harwood *et al.*, unpublished).

1.4.3 Carabid-slug interactions

In general the interactions between carabid predators and their prey are poorly understood, with the majority of studies having a narrow focus, usually only considering a single prey group. Physiological studies are scarce and consequently food choice criteria are poorly understood in terms of diet composition. Other factors such as the degree of true carnivory compared to carrion feeding and the extent of secondary predation has not been adequately determined. It is hoped that the development of molecular techniques for the detection of predation from field collected specimens will elucidate some of the diversity of species that constitute the diet of carabids, information that can then be used to facilitate more effective management of these arable systems.

Evidence is being accrued which highlights the importance of *P. melanarius* as a key predator of slugs from laboratory and field studies. Under laboratory conditions, carabids and their larvae (Thomas, 2002) have been shown to feed on a variety of slugs (Symondson, 1989; Symondson, Liddell, 1993a; McKemey, 2000; McKemey *et al.*, 2001) and snails (Digweed, 1993). In a glass house carabids reduced slugs in a lettuce crop (Symondson, 1993) and grass clover swards (Asteraki, 1993). Analysis of the gut contents of carabids trapped from the field has shown that beetles tested positive for slugs. For example, screening carabid beetles using indirect ELISA with an anti-mollusc haemolymph antiserum (Symondson & Liddell, 1993c, d) revealed that 89.5 % of *A. parallelepipedius* and 42 % of *Pterostichus madidus* (Fabricus) tested positive for mollusc remains in their guts (Symondson & Liddell, 1993a). Symondson *et al.* (1996) showed *P. melanarius* to be an important predator of slugs. The beetles responded directly to the availability of slug prey by concentrating their activity in areas where more and larger slugs were found. More recent research has suggested that *P. melanarius* is also capable of affecting the spatial (Bohan *et al.*, 2000) and long-term temporal (Symondson *et al.*, 2002) distributions of slugs in arable fields. Bohan *et al.* (2000) presented evidence to show that *P. melanarius* formed positive aggregations to areas of high slug density in June. By July, the rate of slug population growth declined in areas of high beetle density, although the beetle population persisted. 11% of these beetles were positive for slug proteins identified by ELISA testing. Bohan *et al.* (2000) suggested that predation was direct and dynamic rather than opportunistic and that significant changes in the spatial distribution and density of the slugs was due to predation on the slugs by the beetles. However, Mair *et al.* (2001) proposed that the relationship between the slug and beetle populations was purely fortuitous and determined by environmental factors with which both were correlated, although Bohan *et al.* (2001) retorted that this alternative hypothesis was insufficient to explain the dissociation of the slug and beetle populations in July, when they had been positively aggregated in June. In a five year study *P. melanarius* was capable of affecting the growth of the slug population between years (Symondson *et al.*, 2002). When slug numbers declined, beetle numbers declined in

parallel the following year. This decrease in the population size of an important predator could allow the slug population to recover in subsequent years. If alternative prey can be managed in adjacent areas to sustain the beetle population at a sufficiently high level, the population of slugs should not be able to increase to a level where they cause serious economic damage. Symondson *et al.* (2000) demonstrated that alternative non-pest prey such as earthworms are important in the diet of *P. melanarius* and that these could potentially help to sustain beetle numbers in the absence of high slug population densities.

1.5 Methods for studying predation

In order to ascertain prey choice by a predator it is necessary either to directly observe the predation event or to determine subsequently prey identity by examining the predators' gut contents or faeces. Direct observation, either in the wild or in experimental enclosures, leaves little doubt about which prey are consumed. However, given the complexity of most ecological processes, it will be difficult in many situations to establish or quantify by direct observation predation events for the full prey range of a generalist predator. Therefore, to overcome difficulties associated with these methods, a range of techniques has been developed to allow the identification of prey remains at the molecular level by distinguishing prey specific proteins or DNA from within the gut of the predator. Methods that have been used to investigate feeding by carabids include exclusion techniques, no-choice feeding trials in the lab, manipulation of prey and predator density, the use of radioactive tracers, isotope labelling prey techniques, gut dissection, various serological techniques, electrophoresis and DNA amplified using the polymerase chain reaction (PCR). These techniques have been comprehensively reviewed by Sunderland (1987, 1988), Greenstone (1996, 1999), Mills (1997) and Symondson (2002a, 2002b).

Most of these techniques have limitations, for example, analysis of gut contents by microscopic examination only enables identification of prey with hard body parts that are less digestible and remain in the gut for longer. This technique is of little use if soft-

bodied prey, such as slugs, are consumed as they are rapidly digested and little more than a proteinaceous soup remains. These proteins can be separated on a gel by electrophoresis (Schelvis & Siepel, 1988; Powell *et al.*, 1996; Paill, 2000). This technique has been little used to investigate predation in carabids, primarily because of the difficulties of interpreting complex banding patterns when a predator has consumed multiple prey species (Symondson, 2002b). In cases where this method has been employed, the prey species could be distinguished from the predator by observing the esterase enzyme banding patterns on a gel (Lovei, 1986). However, when the predator had consumed various different prey items, interpretation of the complex banding patterns was virtually impossible (Walrant & Loreau, 1995). Immunological technologies have now largely replaced the use of electrophoresis.

1.5.1 Detection of predation using immunological methods

Immunological techniques are by far the most widely used for assessing prey choice by carabids, utilising either precipitin tests (e.g., Pickvance, 1970; Boreham & Ohiagu, 1978; Lesiewicz *et al.*, 1982; Dennison & Hodkinson, 1983) or ELISA (Enzyme Linked Immunosorbent Assay) (Symondson, 2002a), although the latter has now largely replaced use of the former. ELISA is much more sensitive and the method of choice for gut content analysis (Symondson & Hemmingway, 1997). ELISA is used in conjunction with antibodies that have been raised to the particular prey item of interest, which are either polyclonal (mixture of antibodies with a range of specificities) (e.g., Crook & Sunderland, 1984; Chiverton, 1987; Symondson & Liddell, 1993c, 1996c; Arnold *et al.*, 1996; Lim & Lee, 1999) or monoclonal (all of which are identical) (e.g., Greenstone & Morgan, 1989; Hagler *et al.*, 1993, 1997; Hagler & Naranjo, 1994; Agusti *et al.*, 1999a; Bohan *et al.*, 2000; Symondson *et al.*, 2000). The use of monoclonal antibodies (MAbs) is the primary method for the detection of prey remains in predator gut samples, although polyclonal antibodies (PAbs) can also prove useful. The heterogenous nature of PAbs means that they often have longer detection periods than MAbs (Symondson & Liddell, 1996b). However, the main disadvantage of using PAbs is that they contain several different antibodies, which means that the chance of cross reactivity with a protein from a

non-target organism is a high. Difficulties may arise if the target protein is attached to a site that is degraded rapidly by digestive processes. One other major disadvantage of Pabs is that once the stock is used, a new antibody needs to be characterised, but as no two polyclonal antibodies are ever identical, the results are not reproducible. MAbs on the other hand can be produced in limitless supply of identical antibodies, which bind to a single epitope on a target protein, as they are clones. As long as the binding site does not occur on other proteins, MAbs provide a highly specific and sensitive probe/assay. Rigorous testing of both polyclonal and monoclonal antibodies is required to determine their specificity through cross-reactivity testing and to confirm that the binding site is to a target protein that is not rapidly digested. PAbs have been raised against molluscs (Symondson & Liddell, 1993c, d), whereas MAbs have been developed to recognise molluscs at various taxonomic levels. Several MAbs have been specifically designed for the slugs, working at the species level for *D. reticulatum* (Symondson & Liddell, 1996a) and *T. budapestensis* (Symondson *et al.*, 1997), for a complex of species, the *A. hortensis* aggregate (Symondson *et al.*, 1999) at the Family level for the Arionidae (Symondson & Liddell, 1993e), to general slug (Symondson & Liddell, 1993b), and for slug eggs (Symondson *et al.*, 1995; Mendis *et al.*, 1996). This ability to target specifically different life stages of potential prey is not possible with DNA based methods and is a significant advantage of this method.

A MAb, or any other type of probe, is only useful if it is able to detect the target, extracted from the guts of predators, for a significant period of time following consumption, but this depends on the antibody, its target site and the predator. In carabids, the minimum requirement is to be able to detect predation for at least the 12 hour period prior to the capture of the predator from the field (Symondson *et al.*, 1996). In general, it has been found that prey remains in carabid crops are usually detectable for longer than for other species, perhaps because in carabids this part of the foregut is thought to be primarily for storage rather than digestion (Symondson, *et al.*, 1999). As a result significantly extended detection periods of prey proteins have been demonstrated

with MAbs, which can range between <2 hours (Hagler & Naranjo, 1997) and >7 days (Harwood *et al.*, 2001).

Although MAbs are very sensitive, and assays using ELISA are fast, their main disadvantage is cost of development in terms of money and time, as it can take up to one year to characterise one MAb (Greenstone, 1996; Symondson, 2002b). In addition, a single antibody can only be used to detect one prey species at a time, although many different antibodies could be used to analyse the same predator gut sample if the whole prey range of a predator was being investigated. However, for the reasons stated above, this is generally unfeasible. The use of MAb coupled with ELISA (MAb ELISA) allows quantification of prey biomass in an individual predator and when this is combined with data on the predator density and detection period it allows an estimation of the rate of prey biomass consumed by a predator (Sopp *et al.*, 1992). However, these results are prone to interpretational error, because digestion and hence detection is affected by the species (Symondson & Liddell, 1993a) and sex of the predator (Symondson *et al.*, 1996, 1999), temperature (Sopp & Sunderland, 1989), quantity of target and presence of non-target remains in gut (Symondson & Liddell, 1995), and changing metabolic rates in response to starvation (Lovei *et al.*, 1985), amongst others.

Symondson *et al.* (1999) found that the detection period of *A. hortensis* remains was approximately 30% longer in male *P. melanarius* than in female beetles. However, the overall rates of decay for male and female beetles were not significantly different. Although there was no significant difference in detection between males and females, or in the presence of alternative prey, the gut weights of females were consistently heavier than for males, probably because female beetles are usually bigger than males and hence have a larger gut. Other studies (Symondson & Liddell, 1993d, 1995) have also shown female foregut weight to be greater than males. This was true whether individuals were starved or provided with alternative prey (Symondson *et al.*, 1999), indicating that females eat more than males. Symondson *et al.* (1999) suggested that feeding on an alternative prey extends detection periods by slowing down the movement of the antigen

out of the foregut into the rest of the digestive system, but also results in faster movement of antigen out of the foregut than the rate of antigen digestion within the foregut. This would lead to significantly extended detection periods for the prey when the predator was fed an alternative prey species in addition to the target species (Symondson & Liddell, 1995). Previous findings for carabid and staphylinid predators found there was no significant difference between the rate of decay in beetles fed an alternative prey and those which were not (Lovei *et al.*, 1990).

Immunoassays cannot be used to distinguish predation and scavenging. As most carabids exploit dead prey when it is available, false estimates of the effect of predation can be obtained. If the item consumed is another predator, itself containing prey, then the detection of this prey could lead to the identification of false trophic links. Secondary predation by generalist predators is known to occur in the field (Chiverton, 1984). It has been frequently suggested that analysis with antibodies may detect secondary predation where the antibody reacts to the proteins in the guts of the initial rather than final predator (Sunderland, 1996). This was found in a polyclonal antiserum against *D. reticulatum* (Tod, 1973), but recent evidence suggests that such food chain errors are unlikely to play a significant role in predation studies (Harwood *et al.*, 2001). Using MAb coupled with ELISA it was only possible to detect the prey from the initial predator immediately after it was fed to the second predator in an aphid-spider-beetle system. As DNA is thought to degrade more rapidly than protein, it is unlikely that this will be a problem with DNA-based detection systems.

Finally, quantification of the number of prey consumed using MAbs is difficult, as the same quantity of antigen may be found in the foregut of a beetle that has either consumed a small quantity of target prey recently, or a large amount some time ago (Symondson, 2002a, b). This issue may be resolved using quantitative PCR based methods, but Zaidi *et al.* (1999) found that the target sequence amplified just as well whether the predator had consumed one or six prey items and had digested them for 0 or 28 hours.

1.5.2 Molecular methods for the detection of predation

The use of DNA techniques to identify the prey range of a predator is a recent approach and has been extensively reviewed by Symondson (2002a, b), although this approach has not been used to investigate predation on the same scale as immunological methods. Molecular techniques have been formerly used to identify the sources of blood meals in haematophagous insects such as mosquitoes (Coulson *et al.*, 1990; Gokool *et al.*, 1993), ticks (Tobolewski *et al.*, 1992) and crab lice (Lord *et al.*, 1998). In addition to these, Johanowicz & Hoy (1996) attempted to identify an endosymbiont occurring in the gut of the prey species, but not in the predator. The first replicated, timed laboratory feeding trials were conducted by Asahida *et al.* (1997), in which long fragments of multiple copy mitochondrial DNA (mtDNA) were targeted using general fish primers to investigate feeding on stone flounder *Kareius bicoloratus* (Basilewsky) by sand shrimp *Cragon affinis* (De Haan). The longest fragments could be detected immediately following ingestion, but not after four hours, and ones that were smaller, in 50 % of predators five hours following ingestion (Asahida *et al.*, 1997). When nuclear esterase genes, in which the copy number had increased to 40-50 fold, were targeted from two insecticide resistant strains of mosquito, better results were achieved (Zaidi *et al.*, 1999). Sequences of 146 bp and 263 bp from these genes could be detected from the gut of a carabid predator (*Poecilus cupreus* (Linnaeus)) for 28 hours post-feeding. The shorter fragment was detected in 100% of the predators after this time, regardless of the period of digestion or the number of mosquitoes eaten. This work suggested that short fragments were better targets for extended detection periods.

In contrast, two studies by Agusti *et al.* (1999b, 2000) could not reliably detect single copy nuclear (scn) genes in the gut of the predator. In the first (Agusti *et al.*, 1999b), sequence amplified characterised region (SCAR) primers amplifying 1100, 600 and 254 bp sequences were used. The 1100 bp fragment could not be detected even immediately following consumption of the prey (*Helicoverpa armigera* (Hübner) Lepidoptera: Noctuidae) and the 600 bp segment was only detectable in 50 % of predators (Miridae and Anthocoridae) after this time. The best results were achieved with the shortest

fragment, which was detectable in 75 % of specimens immediately following consumption of the prey, falling to 45 % 4 hours after ingestion. In the second study (Agusti *et al.*, 2000), again targeting scnDNA, large RAPD sequences of 2100 bp were undetectable, whereas a smaller fragment of 310 bp was detected in 80 % of predators (Miridae) immediately following consumption of whitefly (*Trialeurodes vaporariorum* (Westwood)), dropping to 60 % after 4 hours.

Recent studies now appear to be focusing on the use of multiple copy genes, either mitochondrial (Chen *et al.*, 2000; Agusti *et al.*, 2003a, b) or ribosomal gene clusters (Hoogendoorn & Heimpel, 2001), and targeting relatively short amplicons. Chen *et al.* (2000) detected the remains of cereal aphids within the guts of polyphagous predators. In their study, mitochondrial cytochrome oxidase II (COII) amplicons ranging from 77-386 bp were amplified from six species of aphid, which could be distinguished within the guts of coccinellid and chrysopid predators. A 198 bp fragment was detected in C*hrysoperla plorabunda* (Fitch) for 3.95 hours, and in *Hippodamia convergens* Guerin for 8.78 hours following ingestion. The predators were each fed a single aphid (*R. padi*) then with six aphids of a different species (*Rhopalosiphon maidis* (Fitch)). In a more recent study, Agusti *et al.* (2003a) achieved a detection limit of 32 hours with a small 188 bp fragment of psyllid mtDNA amplified from within the gut of a heteropteran predator. A longer fragment was only detected for a maximum of eight hours. Again, these results indicate that shorter fragments survive digestion for longer. Preliminary results also indicate that this approach is suitable for studying predation by spiders (Agusti & Symondson, 2001).

Only one study (Hoogendoorn & Heimpel, 2001) has explored the usefulness of multiple copy ribosomal gene clusters for the molecular detection of predation. In this case, they again found that the shorter fragments could be detected for the longest time, achieving 50 % detection success of lepidopteran DNA from within the guts of a coccinellid predator ten hours following ingestion. However, ribosomal gene clusters may not be the best targets for this type of study as considerable intra-species and intra-individual variation can occur in sequence length.

In contrast to studies on predation by invertebrates using molecular targets, diet in vertebrates has almost entirely examined predators from the field rather than in laboratory experiments. Taberlet & Fumagalli, (1996) successfully identified a 54 bp nuclear DNA and 307 bp mtDNA fragment from rodent and insectivore skulls in owl pellets. However, as determining species identity from skulls is usually a reliable method, using a molecular approach may not be necessary for this type of predation study. Microsatellites have been used to distinguish remains of eider ducks from swans and geese from the gastrointestinal tract of glaucus gulls (*Larus hyperboreus* Gunnerus) (Schribner & Bowman, 1998). Using this technique gulls were identified as potentially important predators of emperor geese (*Chen canagica* (Sevastianov)), but not of eider ducks. Microsatellites have also been used to assess filial cannibalism by the tessellated darter (*Etheostoma olmstedi*) and other fish (DeWoody *et al.*, 2001). DNA from cannibalised embryos was analysed, but only those that were relatively undigested yielded useful data, which showed that male *E. olmstedi* were eating their own offspring. Reed *et al.* (1997) used a combination of microsatellites and other nuclear DNA to determine the species, sex and identity of seals from their faeces. Prey remains in the faeces were identified visually so that patterns of predation could be related to individual seals.

The remaining studies have used mtDNA to investigate predation by vertebrates. Two studies have analysed plant remains in faeces of brown bears (*Ursus arctos* Linneus) (Höss *et al.*, 1992) and extinct ground sloths (Poinar *et al.*, 1998). Sutherland (2000) and Casement (2001) examined predation by blue tits (*Parus caeruleus* Linneus) and great tits (*Parus major* Linneus) on lepidoptera and by swallows (*Hirundo rustica*) on diptera. Prey DNA was extracted from faeces and amplified using mitochondrial 12S rRNA primers. Amplicons were either cloned and species identified from RFLP banding patterns or they were sequenced and compared with sequences for known prey species. The results showed that swallows caught a wider diversity of prey over organic compared with conventionally managed farms (Sutherland, 2000), and that blue and great tits preyed on the same lepidopteran species, but in significantly different ratios (Sutherland,

2000; Casement, 2001). DNA from faeces was used again by Farrell *et al.* (2000) to study the diets of sympatric carnivores in Venezuela. The identity of the species from which the faeces came was determined by comparing amplified sequences, 146 bp in length, with those for known predators. The prey species were identified morphologically from remains in the faeces and matched to the particular predator.

Only two studies have applied molecular technology to detecting predation by invertebrates in the field, neither of which has investigated predation by carabids. Both studies targeted mtDNA using PCR-based methods. Hoogendoorn & Heimpel (2003) successfully identified *Ostrinia nubialis* Hübner DNA from the guts of six out of 562 coccinellid predators that were analysed over a two-year period. They suggested that low densities of egg masses and low encounter rates by predators accounted for the low number of positive results. In the second study, Agusti *et al.* (2003b) amplified Collembola DNA from the guts of spiders. By comparing the ratios of the Collembola species in the field with the numbers of spiders that gave positive results they were able to determine that the spiders were exercising prey choice, i.e., the species most frequently identified in spider guts was the least numerous of the three target species of Collembola identified from the field.

From the current research it appears as though multiple copy genes are the most useful targets for detecting prey remains in the guts of predators. The evidence also suggests that detection is most successful when small fragments are amplified rather than ones that are larger. This is most likely due to degradative digestive processes, which readily break down the prey DNA into small pieces. Essentially the type of DNA that is being amplified in these studies can be described as ancient, as the term ancient DNA (aDNA) covers any DNA from a dead organism of part of it, which has undergone autolytic or diagenetic (breakage due to loss of bases and crosslinks) processes of any kind (Herrmann & Hummel, 1994). Therefore, aDNA is always fragmented with a typical size range of 100-500 bp and is present in low copy number (Hoss *et al.*, 1996), with inhibitors being co-purified with the DNA, which may impede PCR amplification of the

target DNA (Hummel & Hermann, 1994). As only small amounts of DNA are usually extracted in these circumstances, there is a greater chance of amplifying the target sequence if multiple copy genes, such as those in mtDNA, are chosen rather than single copy nuclear DNA (scnDNA), because the sequence produced by the PCR will be derived from a larger number of templates.

However, before prey remains can be detected within the gut of a predator, specific primers need to be developed that amplify the prey species in question. If sequences for the given species already exist e.g. in the GenBank database, then designing primers is a relatively quick task. However, if no previous sequence data exists then this needs to be generated, which brings into question which gene or genes to use to give the required level of discrimination. Genes that evolve too quickly will lose their ability to unambiguously resolve the phylogeny of anciently diverged taxa (Lunt *et al.*, 1996). Conversely, a gene that is too conserved will not provide sufficient information to determine the requisite relationships, especially for recently diverged taxa.

When choosing a gene for a given level of divergence, favoured molecules are those where the level of variability is such that the number of different species sharing the same sequence will be minimized, while maximizing the number of nonhomoplasious shared character states, i.e., those which reflect evolutionary processes. For distantly related species, slowly evolving genes in which few nucleotide positions are likely to vary are the most useful (Kocher *et al.*, 1989). Conversely, for comparisons of closely related species genes that evolve rapidly and have many variable nucleotide positions will be the most valuable. This is because only the most rapidly evolving nucleotide sites will have gained substitutions, whereas slowly evolving nucleotide sites will have escaped the masking effects of multiple substitutions at the same site (Simon *et al.*, 1994).

In studies of invertebrate systematics, it has been suggested that the use of ribosomal genes for closely related species could be problematic whereas protein coding genes have fewer difficulties (Simon *et al.*, 1994). At deeper levels of divergence, protein coding

genes may be saturated at the amino acid level, in which case, highly conserved regions such as ribosomal RNAs, including 12S, may be more useful. The evolutionary rate in the rRNA 12S subunit varies between species, but in general, this is a highly conserved gene. It has been suggested that the use of the 12S gene, especially the third domain, is of limited phylogenetic usefulness for closely related species (Thomas *et al.*, 1989; Ballard, 1994), but at more distant levels may be valuable for developing specific primers which will distinguish between Orders, Sub-Orders or Families of invertebrates. In this instance, it is likely that homoplasy caused by multiple substitutions will not be a problem (Simon *et al.*, 1990, 1994). In this study, we have targeted the third domain of the 12S rRNA subunit. The target gene was chosen as rRNA genes commonly contain insertions and deletions (Kjer, 1995) and general primers are available which anneal to conserved flanking regions on either side of the third domain of this gene, allowing easy amplification across wide taxonomic groups (Simon *et al.*, 1994). In molluscs, it appears that 12S rRNA is very variable with insertions and deletions occurring between species and genera (Dodd *et al.*, this thesis (Chapter 6)), although this is not observed in many arthropods, such as aphids, which exhibit little variation in this gene (*pers obs*; Simon *et al.*, 1994).

1.6 Aims of the study

1. Predation on slugs in the field can be monitored using DNA-based detection methods

The use of MAb ELISA allows quantification of prey biomass in an individual predator and when this is combined with data on the predator density and detection period it allows an estimation of predation rates (Mills, 1997). Monoclonal antibodies have been used, for example, to study the spatial dynamics of predation by *P. melanarius* on slugs in the field (Bohan *et al.*, 2000) and predation by *P. melanarius* larvae in experimental enclosures (Thomas, 2002). Detection methods suitable for use in field studies must be sufficiently sensitive to detect the target for a significant period of time following consumption. In carabids, the minimum requirement is to be able to detect predation during the 12 hour period prior to the capture of the predator from the field (Symondson *et al.*, 1996). The suitability of DNA-based approaches to detect predation by carabids in field studies has not been tested. The hypothesis that DNA techniques are suitable detection methods for use in field studies was investigated by analysing the foregut contents of beetles caught in the field with slug specific mtDNA primers. The same beetles were also tested for slug protein using MAb ELISA to allow comparison of the two methods in field studies.

2. Mitochondrial 12S rRNA and COI genes in the Arion hortensis *aggregate are sufficiently variable to provide species-specific markers*

The relationships between closely related species are important in predation studies when it is necessary to design primers working at these taxonomic levels. This is particularly true where species complexes occur, e.g. *Arion hortensis* aggregate, as morphological differentiation is often uncertain and can be influenced by factors such as diet (Jordaens *et al.*, 2001) climate and age (Backeljau & de Bruyn, 1990). The lack of homology of these characters means that their usefulness in resolving phylogenetic relationships between taxa is questionable. Therefore, in order to resolve these ambiguities, and allow the design of specific and robust primers, phylogenetic separation of these species needs to occur, based on molecular genetic data. Previous attempts at systematic separation of

the *A. hortensis* aggregate, using enzyme electrophoresis (Backeljau, 1985, 1987) confirmed that they were indeed distinct species. Voss *et al.* (1999) were able to separate species within and between genera using DNA fingerprinting techniques, but only *A. hortensis* was studied from within the species aggregate. This thesis investigates the molecular evolution of mitochondrial 12S rRNA and COI in the *A. hortensis* aggregate and presents a molecular phylogeny of the aggregate based on 12S rRNA and COI sequence data. These sequences will be utilised for the design of robust species-specific and genus level primers.

2. Mitochondrial haplotypic diversity within the A. hortensis *aggregate requires sampling of geographically separated populations in order to design species-specific primers*

The degree of intra-specific variation in gene sequences is important for primer design, particularly in predation studies, where it is necessary to be able to identify the haplotypes within a species within the geographic area of the predation study. It is quite likely that more than one haplotype will occur within a population therefore the haplotypic diversity needs to be assessed before robust primers are designed. The use of mtDNA as a tool for addressing phylogeographic relationships is well documented. Most molecular analyses within the Class Mollusca have only examined large scale relationships (e.g. Tillier *et al.*, 1992; Boore & Brown, 1994; Rosenberg *et al.*, 1997; Yamazaki *et al.*, 1997; Davis *et al.*, 1998) and nothing has been published on the phylogeography of the *A. hortensis* aggregate. Although previous studies using allozymes have reliably separated species, little regional geographic variation within each species has been revealed. This thesis investigates the genetic diversity and genetic structure of the *A. hortensis* aggregate within the British Isles using mitochondrial sequence data from 12S rRNA and COI genes.

4. Detection of prey DNA from the foregut of P. melanarius *is determined by amplicon size*

The use of DNA techniques to identify the prey range of a predator is a relatively recent approach. Previous research has indicated that in order to detect predation using DNA-based techniques, it is most appropriate to target multiple copy DNA and short amplicons (Zaidi *et al.*, 1999; Chen *et al.*, 2000; Hoogendoorn & Heimpel 2001; Agusti *et al.*, 2003b). For example, sequences of 146 bp and 263 bp from esterase genes, in which the copy number had increased 40-50 fold, could be detected from within the gut of a carabid predator (*Poecilus cupreus* (Linneus)) for 28 hours post feeding (Zaidi *et al.*, 1999). As mitochondrial genes have a naturally high copy number within cells, mtDNA is an ideal candidate for the molecular detection of predation. In order to test the hypothesis that short DNA fragments amplify in preference to those that are larger, the rate of decay of slug DNA within the gut of *P. melanarius* was investigated using mtDNA 12S rRNA primers producing amplicons between 99-294 bp.

5. DNA-based detection methods offer an efficient alternative to protein-based detection systems

Immunological techniques are the most widely used for assessing predation on molluscs by carabids. Several MAbs have been specifically developed against slugs, working at the different taxonomic levels (Symondson & Liddell, 1993d, e, 1996; Symondson *et al.*, 1997, 1999; Thomas, McKemey, Symondson, unpublished), and developmental stages (Symondson *et al.*, 1995). Although MAbs are very sensitive, and assays using ELISA are fast and allow rapid screening of large sample sets, the major drawback with their use is the cost and time of development. Specialised tissue-culture facilities are required and it can take up to one year to develop and characterise one MAb (Greenstone, 1996; Symondson, 2002a). Thus, the cost of investigating predation on numerous prey species would be prohibitive. DNA-based detection methods can be more cost- and time-effective relative to MAbs. Direct comparisons of DNA- and MAb-based approaches, however, have not previously been attempted. The hypothesis that DNA and MAb

techniques are equivalent in sensitivity was investigated by directly comparing the rates of decay of slug DNA and protein from within the guts of the same beetles.

This thesis has been written as a series of papers to be submitted for publication therefore the format of some chapters may differ, according to which journal the paper is to be submitted. This format also means that there will be a degree of repetition for some sections between chapters, particularly where the same methods or samples have been used, but for different analyses and applications.

1.7 References

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Chapter 2

General methods and development of PCR-based techniques for gut content analysis.

2.1 General Methods

2.1.1 Culturing Techniques

2.1.1.1 Carabid beetles

All *Pterostichus melanarius* (Illiger) specimens were obtained by pitfall trapping at IACR-Long Ashton, Bristol. Beetles were maintained individually for long-term use on peat in clear plastic containers with lids (9 cm x 5 cm) and kept in a controlled temperature room at 16 °C with an 18 hour light: 6 hour dark regime. Beetles were fed once every two weeks on two dead *Calliphora* larvae.

2.1.1.2 Slugs

Adult slugs (*Deroceras reticulatum* (Müller) and *Arion hortensis* Férussac) were collected from various locations including Bristol (IACR-Long Ashton), Lincolnshire and West Glamorgan. The slugs were maintained on moist cotton wool in clear plastic containers with lids (9 cm x 5 cm) and kept in a refrigerator in the dark at 4 °C with a maximum of ten slugs per container. Slugs were fed once per week on a mixture of potato, carrot, lettuce and wheat seeds.

2.1.2 DNA Extraction Methods

2.1.2.1 Salt extraction with Livak Buffer

Approximately 25 mg of tissue from individual slug (head and tentacles) and beetle (thoracic muscle) specimens was homogenised in 100 µl of a high salt extraction buffer containing 0.08 M NaCl, 0.16 M sucrose, 0.06 M EDTA, 0.5 % SDS, 0.1 M Tris-base (pH 8.6). The homogenate was incubated at 65 °C for 30 min, after which 14 µl 8 M potassium acetate was added to a final concentration of 1 M and incubated on ice for a further 30 min. Samples were centrifuged at 13,000 rpm for 15 min at room temperature. The supernatant was transferred to a clean 1.5 ml Eppendorf tube and centrifugation repeated. Again, the supernatant was transferred to a clean 1.5 ml Eppendorf tube and 200 µl ice-cold 100 % ethanol was added. Following incubation at –20 °C for 1 hour, samples were centrifuged at 13,000 rpm for 15 min at room temperature. The supernatant

was discarded and the pellet washed in 100 µl ice-cold 70 % ethanol. After centrifugation for 5 min at 13,000 rpm at room temperature, the pellet was vacuum dried for 10 min and resuspended in 100 µl TE (Tris-EDTA) buffer (pH 8.0) by heating (65 °C for 10 min). Extracted DNA was stored at –20 °C.

2.1.2.2 QIAamp® DNA Mini Kit

Individual predator foregut samples were extracted using the QIAamp® DNA Mini Kit (Qiagen Ltd). Beetles were thawed to room temperature and the foregut was removed as described in Symondson *et al.* (2000). The foregut was weighed and homogenized in a 1:19 w:v ratio with 1X PBS (phosphate buffered saline). The homogenates were centrifuged at 8,000 rpm for 15 min at room temperature. The majority of the supernatant was transferred to a clean 1.5 ml Eppendorf tube and stored at –20 °C for subsequent analysis using MAb ELISA (Dodd *et al.*, this thesis (Chapter 5)).

The particulate remains and 80 µl of the supernatant were retained for DNA extraction using the QIAamp® DNA Mini Kit in accordance with manufactures instructions. Extracted DNA was stored at –20 °C.

2.1.3 Enzyme Linked Immunosorbent Assay (ELISA)

All predator gut samples from *Deroceras* decay rate experiments and from field caught specimens were screened by indirect ELISA at room temperature using two different monoclonal antibodies (MAbs), the anti-Deroceras MAb, DrW-1G4 (Symondson and Liddell, 1996) and the general anti-mollusc MAb AiW-1C9 (RS Thomas, A McKemmy, DM Glen and WOC Symondson unpublished).

Each ELISA plate included a 1.5X dilution series of *D. reticulatum* standards diluted 1:20,000 (w/v) in 1X PBS and stabilized with heterologous protein also diluted 1:20,000 (w/v) in 1X PBS. This provided slug protein concentrations between 315 and 9 ng/200 µl. Heterologous protein consisted of starved beetle gut diluted to a concentration of 1:20,000 (w/v). The results of this dilution series were plotted on a graph against

absorbance to produce a standard concentration curve. The regression equations obtained from the standards on each plate were used to calculate slug protein concentration equivalents in the predator gut samples. Slug protein concentrations were calculated following a protein assay using the BioRad Protein Assay System (Bio-Rad Laboratories GmbH) (see section 2.1.4).

The homogenised individual gut samples were diluted to a final concentration of 1:20,000 (w/v) in 1X PBS. A Falcon Pro-Bind™ microtitre plate (Becton Dickinson Labware) was loaded with duplicate 200 µl samples and incubated overnight at room temperature. All wells of the ELISA plate were washed three times with PBS-Tween (0.05 % Tween 20) (Sigma-Aldrich). The antibody DrW-Ig4 was used at a concentration of 1:5,000 and AiW-1C9 at a concentration of 1:250 diluted in PBS-Tween. In each case, 200 µl of either antibody was added to the first duplicate well and 200 µl of PBS-Tween was added to the second. The plates were incubated for two hours at room temperature to enable the antibody to bind to the antigen. The plates were then washed three times with PBS-Tween, coated with 200 µl of goat antimouse IgM (µ-chain specific) horseradish peroxidase enzyme conjugate (Sigma-Aldrich) that had been diluted 1:2,500 for DrW-1G4 and 1:2,000 for AiW-1C9 with PBS-Tween. The plates were incubated for 1 hour at room temperature so that the conjugate could bind to the mouse antibodies. The plates were again washed three times with PBS-Tween, and 200 µl of the enzyme substrate *o*-phenylenediamine in a citrate-phosphate buffer was added to the wells. The plates were incubated at room temperature in the dark for 20-30 min to allow colour development. The reaction was then stopped by adding 50 µl of 2.5 M H_2SO_4 to each well. Absorbance readings were recorded at 490 nm using an ELISA plate spectrometer (Thermomax Plate Reader, Molecular Devices). The effects of non-specific binding by the conjugate were eliminated by subtracting the absorbance readings for the wells to which no antibody had been added, from the reading for wells containing antibody (Symondson *et al.*, 2000).

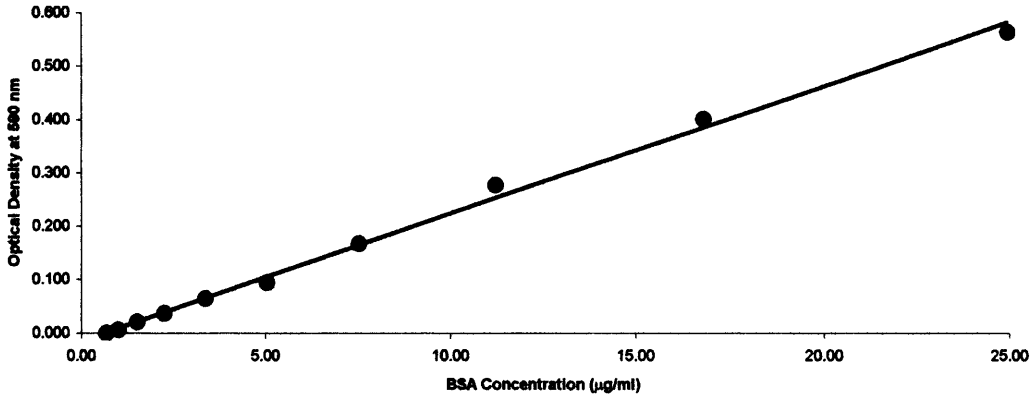
2.1.4 Protein Assay

Protein concentrations of homogenised *D. reticulatum* were used for the protein assay.

A 1.5 dilution series of Lyophilised Bovine Serum Albumin (BSA) (Bio-Rad Laboratories GmbH) diluted with 1X PBS provided final concentrations of BSA ranging from 25 μg/ml to 0.68 μg/ml. A similar dilution series of *D. reticulatum* was also prepared. To 800 μl of each dilution, 200 μl of Bio-Rad Dye Reagent Concentrate (Bio-Rad Laboratories GmbH) was added in a 1.5 ml Eppendorf tube. The samples were briefly vortexed left for 20 min to allow colour development, after which, 200 μl of each was added in duplicate wells onto a Falcon Pro-Bind™ microtitre plate (Becton Dickinson Labware). The plate was read using an ELISA plate spectrometer (Thermomax Plate Reader, Molecular Devices) at an optical density of 590 nm. Readings were corrected for reagent blanks containing PBS only. The OD_{590nm} was plotted against BSA protein concentration (Figure 2.1) and *D. reticulatum* protein concentration was calculated from the regression equation and corrected for each time it was diluted.

A protein concentration of 315.41 ng/200 μl was calculated for *D. reticulatum*, which was then used to calculate the protein concentration equivalents of all samples tested by indirect ELISA, by comparing the absorbance reading of the sample with those of a dilution series of this standard on each plate.

Figure 2.1: Standard curve for Bio-Rad Protein Micro Assay. Optical densities (read at 590 nm) for dilutions of Bovine Serum Albumin (BSA) at concentrations of 0.68 to 25.00 μg/ml. Regression equation $y = 0.0238x - 0.0142$; $R^2 = 0.9959$.



2.2 Developmental methods and results for DNA-based predator gut sample analysis

2.2.1 Introduction

Predation on slugs by beetles has not been previously been detected using DNA-based methods. Preliminary feeding experiments were conducted to determine whether slug DNA could be detected from within the foregut of the beetle *P. melanarius*, and if so, for what period following ingestion.

2.2.2 Methods

2.2.2.1 DNA samples, extraction, amplification and sequencing

Beetles (*P. melanarius*) and slugs of agricultural importance (*D. reticulatum, A. hortensis, Arion distinctus* Mabille, and *Arion intermedius* Normand) were collected from an established field site at IACR Long Ashton, Bristol, UK for DNA extraction and sequencing. Additional specimens were also collected from sites in Lincolnshire and North and South Wales so that genetic variation in these species could be assessed before primers were designed. Total genomic DNA was isolated from the head and tentacles of slugs and from thoracic muscle tissue of beetles, which had been freshly frozen, using a high salt extraction method modified by Collins *et al.* (1987) from Livak (1984) (section 2.1.2.1). The third domain of the mitochondrial 12S rRNA gene (400 bp) was amplified by polymerase chain reaction (PCR) using the general invertebrate primers SRN14588 and SRJ14233 (Simon *et al.*, 1994) in a Perkin-Elmer 9700 Automated Thermocycler. These primers were also used for sequencing.

Amplification of the 12S rRNA gene sequence was carried out in a 25 µl reaction containing approximately 200 ng total genomic DNA, 1X PCR reaction buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl (InvitrogenGibco, Life Technologies®), 4 mM $MgCl_2$, 0.025 mM each of dGTP, dATP, dTTP and dCTP, 0.5 µM each primer and 0.625 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were as follows: initial

denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 45 s, 72 °C for 1 min 15 s, then a final extension step of 72 °C for 10 min.

PCR products were purified for sequencing using the GeneClean Turbo for PCR Kit (Bio101) following manufacturers instructions. Each sample was sequenced in both forward and reverse directions on an ABI Prism Model 377 semi-automated DNA Analyser using the Prism Big Dye Terminator v2 ready reaction kit (Perkin Elmer Applied Biosystems). The sequencing reaction contained 2 µl DNA, 1 µl of either forward or reverse primer (1.6 pmol/µl), 1 µl Big Dye and 1 µl sterile water (Sigma-Aldrich).

GenBank Accession numbers for the 12S rRNA sequences are for *D. reticulatum* (AY423668), *A. distinctus* (AY423654-63), *A. hortensis* (AY423632-53), *A. owenii* (AY423664-66), *A. intermedius* (AY423667), and *P. melanarius* (AY423669).

2.2.2.2 Sequence alignment and primer design

Following a species-specific secondary structure analysis for each ribosomal gene, the method for which is described elsewhere (Dodd *et al.*, this thesis (Chapter 3)), the predator (beetle) and prey (slug) sequences were aligned according to a secondary structure template (Hickson *et al.*, 1996). Potential primer sites were first identified by eye in regions where the predator and prey sequences differed. Primer pairs were then tested for primer-primer binding and secondary structure formation using Omiga v2 (Accelerys Inc.). Five primers were designed for *D. reticulatum* (two forward and three reverse) which in combination gave rise to five primer pairs (A-E) and one primer pair for each of the species *A. hortensis* (F), *A. distinctus* (G) and *A. intermedius* (H), as well as an *Arion* genus-specific pair (I) (Table 2.1). Large DNA fragments have been found to digest more quickly than small ones in predator guts (Zaidi *et al.*, 1999; Agusti *et al.*, 1999, 2000, 2001), therefore primer pairs were chosen that generated small amplicons ranging in size from 99-294 bp. The five *Deroceras* primers pairs were used to test if

there was any difference in amplification success of a range of fragments, below 300 bp, with increasing time since ingestion of the target DNA.

2.2.2.3 PCR amplification with slug primers and cross-reactivity tests

For each primer pair, the target species and a negative control was amplified in a 10 µl reaction volume containing approximately 200 ng total genomic DNA, 1X buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl (InvitrogenGibco, Life Technologies®), 2 mM $MgCl_2$, 0.025 mM each of dGTP, dATP, dTTP and dCTP, 1.0 µM each primer and 0.04 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 44-56 °C for 45 s, 72 °C for 1 min, then a final extension step of 72 °C for 10 min.

Once optimised for the target species, all primer pairs were tested for cross-reaction against at least three individuals of each of the predator, other related species and potential prey species. Full details of cross-reactivity tests are given in Chapter 5. PCR products were separated on a 1.7 % agarose gel for both the cross-reactivity tests and the decay rate experiment. A PCR was considered positive by the presence of a band of the target size on the gel.

Table 2.1: Primer sequences, optimal PCR annealing temperatures, and amplicon sizes for *Deroceras reticulatum* species specific primers (A-E), *Arion hortensis* (E), *A. distinctus* (F), *A. intermedius* (H) and *Arion* genus (I) specific primers.

Name	Primers	Sequence 5'-3'	Annealing temperature (°C)	Amplicon size (bp)
A	DR11F	CTATACACAATTTTTAAATAAGC	50.4	109
	DRF29RC	GTCTCTGGTTTATCTATTATTGGT		
B	DR100F	CTGAGTTTAACTTCAGGGGAACTTACC	52.2	165
	DR16MR	GCCAGTAACTTATTTGGT		
C	DR11F	CTATACACAATTTTTAAATAAGC	45.1	226
	DR16MR	GCCAGTAACTTATTTGGT		
D	DR100F	CTGAGTTTAACTTCAGGGGAACTTACC	56.1	233
	DR50R	AAATTTACTTTCAAGTCCAGC		
E	DR11F	CTATACACAATTTTTAAATAAGC	50.0	294
	DR50R	AAATTTACTTTCAAGTCCAGC		
F	AH1MF	CACCAAGATACTCAACCCAC	55.0	130
	AH2R	CGAACGCCCACTTATAGG		
G	AD1F	CGTTAAAAAAGGACCCATAAAGGG	60.4	128
	AD2R	GGTCAATTCYACCTATCGCTGGA		
H	Ai1F	CACATAAATGATAGTCACC	48.4	99
	Ai2R	TAATAGAAACACAAAAACCCC		
I	Ai1F	CACATAAATGATAGTCACC	44.4	204-221
	AR2R	ATACTTACAAGTCCATCTTT		

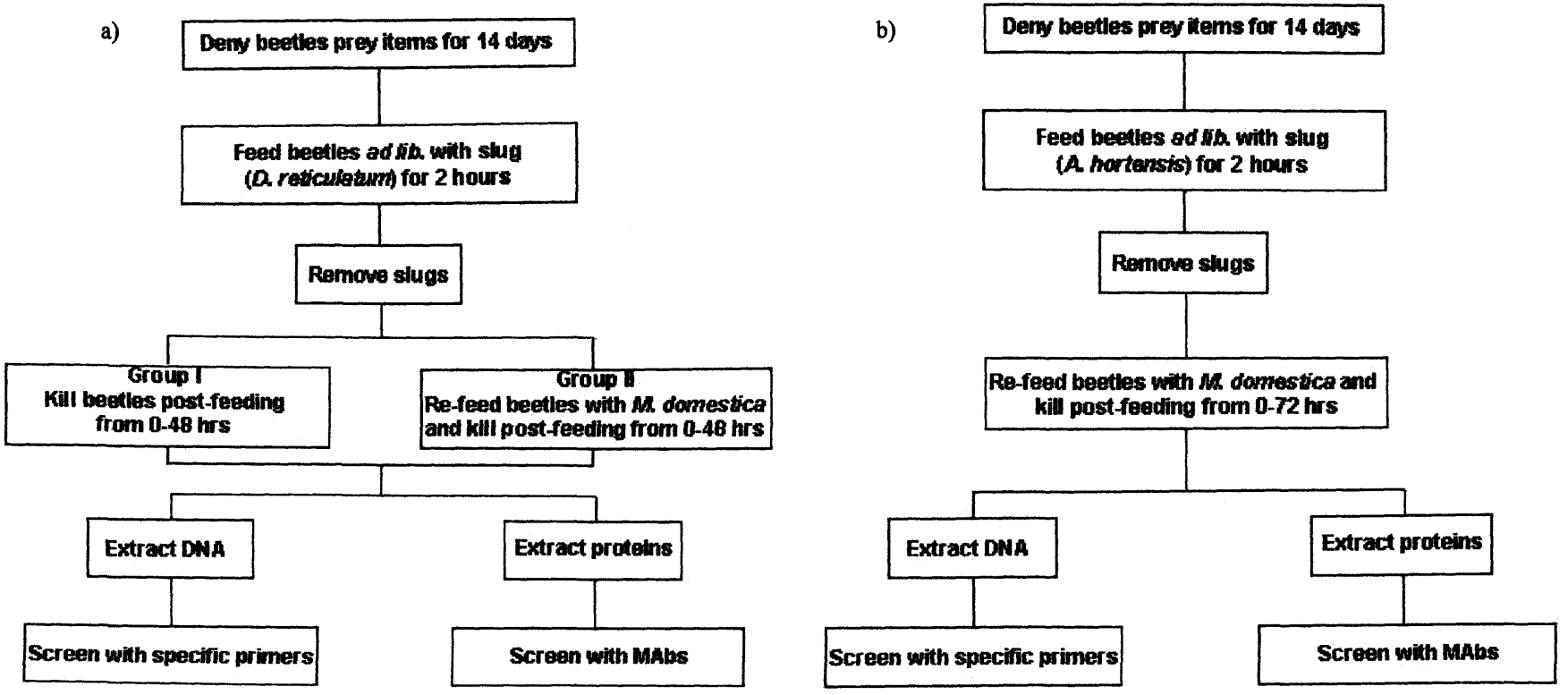
2.2.2.4 Feeding studies

A timed DNA decay rate experiment was conducted for *D. reticulatum* and for *A. hortensis*, the structures of which are outlined in Figures 2.2a and b, respectively, and are briefly described below.

Single *P. melanarius* were placed in individual petri dishes (2 cm x 5 cm) containing moistened filter paper. The beetles were each fed a single blow fly larva (*Calliphora* sp.) and allowed to feed *ad lib* for 24 hours, after which time any beetles that had not fed were removed from the experiment. Approximately 400 beetles were selected for the *D. reticularum* and 200 for the *A. hortensis* experiment. The beetles were then denied further prey and maintained in a controlled temperature room at 16°C under a 16 hour light: 8 hour dark regime for 14 days. Ten of these beetles (five male and five female) were

killed and frozen as negative controls. The remaining beetles were then transferred to clean petri dishes containing moist filter paper and allowed to feed on an immobilized whole wounded *D. reticulatum* or *A. hortensis* for two hours. Only beetles that had been observed feeding during that time were retained. The selected beetles were then divided into two groups each containing an equal number of males and females. The beetles in Group I (re-fed) were each provided with half a house fly larva (*Musca domestica* Linneus) every 24 hours, to mimic the availability of alternative prey that would be accessible in the field; beetles in Group II (not re-fed) were not provided with any alternative prey. Samples of 10 beetles from each group (5 male and 5 female) were killed and frozen at 0, 2, 4, 8, 12, 18, 24, 36 and 48 hours after removal from the slug meals. As the beetles could have ingested the slug meals at any point during the two-hour feeding period, the mid-point (after 1hour) was considered as the time of consumption. Therefore, one hour has been added to each time interval in the subsequent analyses (Harwood *et al.*, 2001). The *Arion* decay rate experiment was carried out as described above, except that only female beetles were used as insufficient male beetles were caught for them to be used in the experiment, all beetles were provided with alternative prey, five female beetles were killed at each time period and the experiment was conducted for 72 hours. Timed decay rate experiments for *A. distinctus* or *A. intermedius* were not conducted, although primers were developed for these species since it was not possible to collect enough slugs for such an experiment.

Figure 2.2: Structure of DNA and antigen decay rate experiments: a) Beetles in Group I were firstly fed *ad lib* with *Deroceras reticulatum* for 2 hours and then re-fed with an alternative prey (*Musca domestica*), and in Group II were fed *ad lib* with *Deroceras reticulatum* for two hours, but were not provided with alternative prey, before being killed at 0-48 hours post-feeding; b) Beetles were fed *ad lib* with *Arion hortensis* for two hours and then re-fed an alternative prey (*Musca domestica*), before being killed at 0-72 hours post-feeding.



2.2.2.5 Extraction of predator gut contents

Beetles were thawed to room temperature and the foregut was removed as described in (Symondson *et al.*, 2000). The foregut was weighed and homogenized in a 1:19 w:v ratio with 1X phosphate buffered saline (PBS). The homogenates were centrifuged at 8,000 rpm for 15 min at room temperature. The majority of the supernatant was transferred to a clean 1.5 ml Eppendorf tube and stored at –20 °C for subsequent analysis using MAb ELISA (Dodd *et al.*, this thesis (Chapter 5)). The particulate remains and 80 µl of the supernatant were retained for DNA extraction using the QIAamp® DNA Mini Kit (Qiagen Ltd.) in accordance with manufacture's instructions (section 2.1.2.2). Extracted DNA was stored at –20 °C.

2.2.2.6 DNA analysis

The DNA extractions from the *Deroceras* decay rate experiment were screened by PCR with the specific *D. reticulatum* primers (A-E) and from the *Arion* decay rate experiment with the *A. hortensis* (F) and general *Arion* (I) primers using the PCR and thermocycling conditions described in section 2.2.1.3. PCR amplicons were run on a 1.7 % agarose gel stained with 0.1 % ethidium bromide so that percentage PCR success could be assessed.

2.2.2.7 Statistical analysis

Linear regression analysis was used to determine the rates of decay of the different amplicons. Analysis of covariance (ANCOVA) was used to ascertain the relationship between the decay rates within each experiment according to i) beetle sex, ii) provision of alternative prey and iii) amplicon size (i & ii refer to the *Deroceras* experiment only). Detection half-life was calculated for each amplicon according to the regression equation, $y = mx+c$, for the rate of decay of each amplicon, which is rearranged so that $x = y-c/m$, where $y = 50$ %.

2.2.3 Results for DNA based detection methods

2.2.3.1 Primer specificity

Sequences, optimal annealing temperatures and fragment sizes for the specific primers are shown in Table 2.1. The results of the cross-reactivity tests showed that two pairs of primers (A & C) designed for *D. reticulatum* were specific for *D. reticulatum* with the remaining pairs (B, D, E) also amplifying a congener, *Deroceras panormitanum* (Lessonna and Pollonera); only one primer pair (D) showed any significant cross-reactivity with other mollusc species (*Tandonia budapestensis* (Férussac), *Cepaea hortensis* (Müller) and *Cepaea nemoralis* (Linneus)), although not all individuals of these species amplified. Primers designed for *A. hortensis* (F), *A. distinctus* (G) and *A. intermedius* (H) were specific and did not co-amplify other closely related species and the genus level primer pair (I) amplified all members of the Genus *Arion* that were tested. None of the primers amplified the predator or alternative prey. Details of the cross-reactivity tests are given in Dodd *et al.* (this thesis (Chapter 5)).

2.2.3.2 Rate of decay of slug DNA within the gut of Pterostichus melanarius

2.2.3.2.1 Deroceras reticulatum

Using the primers pairs (A-E), *D. reticulatum* DNA was detected from within the guts of carabid predators. It was possible to detect specifically slug DNA in the presence of the DNA of an alternative species (*M. domestica*). The percentage PCR success for each amplicon is summarised in Table 2.2. Feeding the beetles an alternative prey as well as the target species appeared to increase the detection period of the slug DNA within the beetle gut compared with beetles that were provided with no alternative prey. When the beetles were provided with alternative prey, slug DNA could be detected in 10 % of individuals at 37 hours for all amplicons, but in 0 % at 49 hours following ingestion. In contrast, the longest detection period for the beetles that were provided with no alternative prey was 25 hours following ingestion for all fragments except one (D), where 10 % of the beetles tested positive for slug remains at 49 hours and one (B) where 40 % were positive at a maximum of 19 hours following ingestion of the prey. When the data for Groups I and II were combined, slug DNA was detected from the foreguts of 5 % of

the beetles from all of the amplicons at 37 hours following ingestion. Only one fragment (B) could be detected in 5 % of the beetles at 49 hours after ingestion of the prey.

The rate of decay of slug DNA within the guts of male and female beetles was compared for each amplicon in Groups I (re-fed) and II (not re-fed), using analysis of covariance (ANCOVA). In all analyses, the slope of each regression line was significantly different from zero at $P<0.001$. When the rate of decay of slug DNA was compared in male and female beetles for each amplicon, there was no significant difference between the rates at which the slopes or the y-axis intercepts declined within Groups I and II (Table 2.4a). Therefore, for subsequent analyses, the data for male and female beetles were combined. When the rates of decay of each amplicon were compared between Group I and II using ANCOVA, no significant difference was found between the slopes or y-axis intercepts of the regression lines for either group for each amplicon (Table 2.4b). In order to obtain an overall decay rate for each amplicon, and to enable the decay rates of the different amplicons to be compared, the data for Group I and Group II were combined to provide a single regression for each amplicon. When the decay rates for each amplicon were compared, no significant difference between the rates at which the slopes declined ($F_{4,35} = 0.12$; $P>0.05$) or the y-axis intercepts ($F_{4,35} = 0.15$; $P>0.05$) was found. The R^2 values for the decay rates of these fragments were highly significant, showing that the ability to detect the slug DNA within the gut of the predator decreases in a linear manner with time since ingestion (Figure 2.3a-e).

The detection half-life of amplicons (A-E) was calculated using the regression equation for the rate of decay of each amplicon, shown in Figure 2.3a-e. Detection half-lives ranged between 19.64 to 21.25 hours (Table 2.2).

Table 2.2: Percentage PCR success for *Deroceras reticulatum* remains following ingestion by *Pterostichus melanarius* in the presence (Group I) and absence (Group II) of alternative prey, and in the two groups combined (Group I+II), for different sized amplicons, and DNA decay half-life for each amplicon.

Time since ingestion (hours)	Amplicon	A (109bp)			B (165bp)			C (226bp)			D (233bp)			E (294bp)		
	Group	I	II	I+II	I	II	I+II	I	II	I+II	I	II	I+II	I	II	I+II
1		100	100	100	100	100	100	100	100	100	100	100	100	90	90	90
3		100	100	100	100	90	95	80	90	85	90	100	95	90	100	95
5		80	70	75	90	70	80	90	80	85	90	70	80	80	70	75
9		90	70	80	70	60	65	70	60	65	90	60	75	80	60	70
13		70	80	75	80	60	70	70	70	70	70	70	70	70	70	70
19		60	40	50	70	40	55	90	40	65	70	50	60	70	40	55
25		20	20	20	30	0	15	50	20	35	30	20	25	30	20	25
37		10	0	5	10	0	5	10	0	5	10	0	5	10	0	5
49		0	0	0	0	0	0	0	0	0	0	10	5	0	0	0
DNA decay half-life (hours)		-	-	20.57	-	-	19.64	-	-	21.05	-	-	21.25	-	-	19.74

Table 2.3: Percentage PCR success for *Arion hortensis* remains following ingestion by *Pterostichus melanarius* determined using species specific and genus specific primers and DNA decay half-life.

Time since ingestion (hours)	Amplicon	
	F (130bp)	I (204-221bp)
1	100	100
3	60	80
5	80	40
9	80	60
13	100	80
19	40	40
25	20	60
37	40	40
49	20	0
73	0	0
DNA decay half-life (hours)	20.56	17.17

Table 2.4: Rates of decay of amplicons of *Deroceras* DNA within the guts of *P. melanarius*, compared using ANCOVA a) males vs. females in the presence (Group I) and absence (Group II) of alternative prey; and b) Group I vs. Group II in which data for male and females is combined within each group.

		Slope		y-axis intercept	
a) Male vs. female	Amplicon	$F_{1,14}$	P	$F_{1,14}$	P
Group I	A	0.37	>0.05	0.03	>0.05
	B	0.17	>0.05	1.40	>0.05
	C	0.17	>0.05	0.49	>0.05
	D	1.47	>0.05	0.22	>0.05
	E	0.88	>0.05	0.00	>0.05
Group II	A	0.04	>0.05	0.03	>0.05
	B	0.00	>0.05	0.01	>0.05
	C	0.05	>0.05	0.03	>0.05
	D	0.47	>0.05	0.37	>0.05
	E	0.20	>0.05	0.23	>0.05
b) Group I vs. Group II	A	0.65	>0.05	0.14	>0.05
	B	0.01	>0.05	0.93	>0.05
	C	0.61	>0.05	0.01	>0.05
	D	0.04	>0.05	0.64	>0.05
	E	0.62	>0.05	0.02	>0.05

2.2.3.2.2 Arion hortensis and Arion genus

Using the *A. hortensis* specific (F) and general *Arion* primers (I), we were able to detect *A. hortensis* DNA from within the guts of carabid predators. The percentage PCR success is summarised in Table 2.3. Slug DNA could be detected from the foreguts of 40 % and 20 % of the beetles for up to 37 and 49 hours, respectively following ingestion, using the *A. hortensis* specific primers and in 40 % and 0 % using the general *Arion* primers. Prey remains could not be detected in predators 73 hours following ingestion with either set of primers.

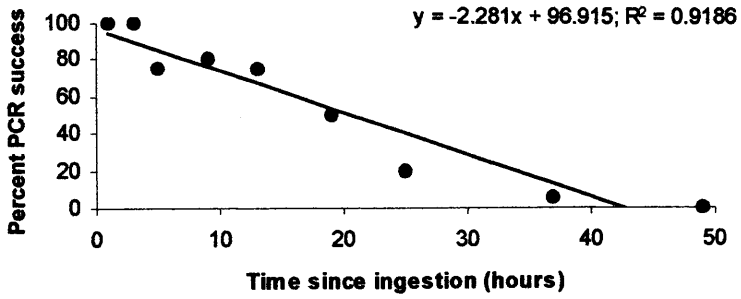
Slug DNA within the guts of the beetles decayed in a significant linear manner (Figure 2.4a-b) with increasing time since ingestion. In all analyses, the slope of each regression line was significantly different from zero at $P<0.001$. When the rate of decay of the

different amplicons (F & I) was compared, no significant difference between the rates at which the slopes declined ($F_{1,16} = 0.14$; $P>0.05$) or the y-axis intercepts ($F_{1,16} = 0.05$; $P>0.05$), was found.

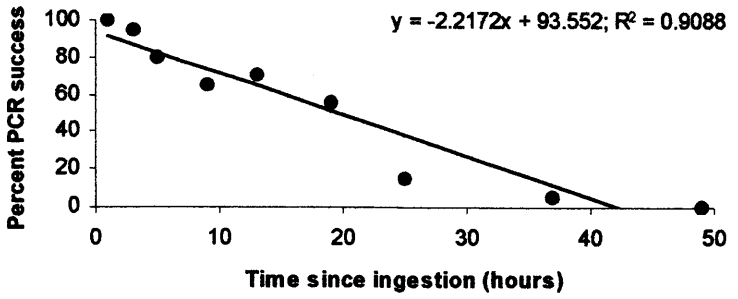
The half-lives of the different amplicons were calculated using the regression equation for the decay rate of each fragment (Figure 2.4a-b). The half-life for the *A. hortensis* specific amplicon was 20.56 hours and for the general *Arion* amplicon was 17.17 hours (Table 2.3).

Figure 2.3a-e: Rate of decay of five amplicons (A-E) of *Deroceras reticulatum* DNA within the gut of *Pterostichus melanarius*, expressed as percent PCR success.

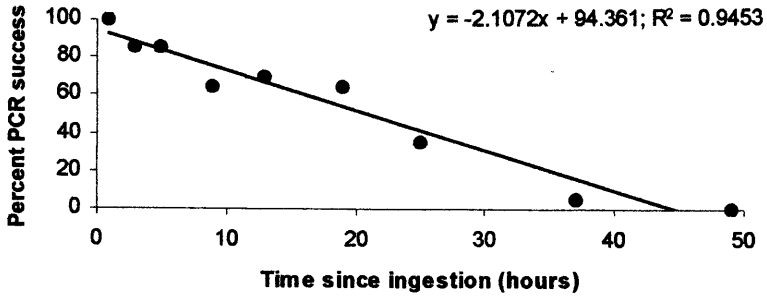
a) *D. reticulatum* amplicon A (109bp)



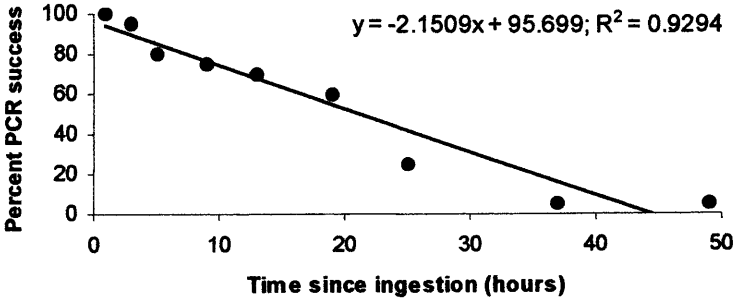
b) *D. reticulatum* amplicon B (165bp)



c) *D. reticulatum* amplicon C (233bp)



d) *D. reticulatum* amplicon D (226bp)



e) *D. reticulatum* amplicon E (294bp)

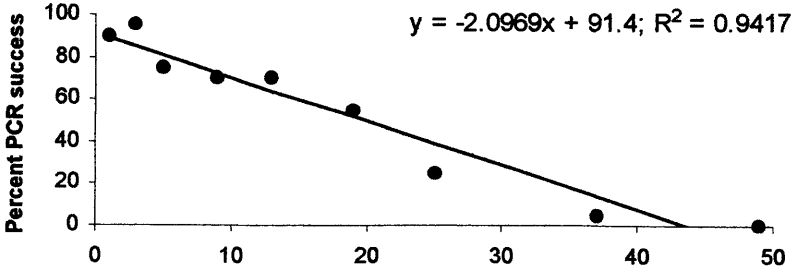
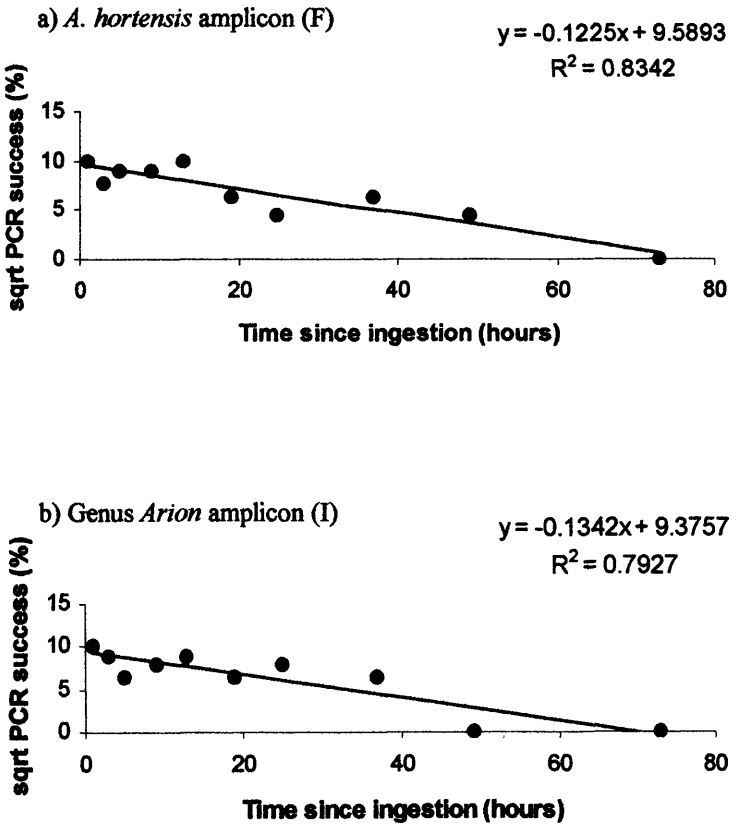


Figure 2.4: Rate of decay of *Arion hortensis* DNA within the gut of *Pterostichus melanarius*, expressed as percent PCR success using a) *A. hortensis* specific and b) *Arion* genus specific primers.



2.2.4 Discussion and Conclusions of Initial Results

These preliminary results have demonstrated that it is possible to identify the remains of slug mtDNA in the guts of arthropod predators in timed laboratory feeding trials using species specific and genus specific PCR primers. Primers were designed to amplify small fragments of mitochondrial DNA (99-294 bp) as these are more likely to be detected in the gut of the beetle following degradative digestive processes, which break down the prey DNA into small pieces. By targeting mtDNA, which has a naturally high copy number within cells, there is a greater chance of amplifying the target sequence because the sequence produced by the PCR is likely to be derived from a larger number of templates, compared with single copy DNA. Previous studies (Chen *et al.*, 2000) showed greater amplification success for short compared with longer fragments. However, we found no significant difference in the rates of decay of our different sized amplicons and therefore it is possible that a critical size limit exists, below which amplification should be successful.

In this study, two fragments (D & F) of slug DNA were amplified from 5 % and 20 % of carabid guts 49 hours following ingestion by the beetle, with all the other fragments (A-C, E & I) being amplified in 5-40 % of predators for a maximum of 37 hours. These results are the longest detection periods of prey DNA from within predator guts to date. A similar study (Agustí *et al.*, 2003), targeting the mitochondrial COI gene in a pear psyllid-heteropteran system, achieved 5-15 % amplification success after 32 hours with 271 bp and 188 bp fragments respectively. Implementation of a model system, in which insecticide resistant mosquito larvae were used as the prey and a carabid beetle as the predator, demonstrated a detection limit of 28 hours following ingestion of the prey (Zaidi *et al.*, 1999), but in this case multiple copy nuclear esterase genes rather than mitochondrial genes were targeted. However, amplification of single copy nuclear (SCN) genes was less reliable, at best resulting in detection of a 310 bp fragment in 60 % of predators 4 hours following ingestion (Agustí *et al.*, 1999). Chen *et al.* (2000), targeted aphid mtDNA from within the gut of coccinellid and neuropteran predators, found a detection period not exceeding 9 hours. However, these studies and our research

suggest that multiple copy genes, such as those found in mtDNA, are probably the most sensitive targets for the molecular detection of predation.

The detection half-lives of the amplicons obtained using the *Deroceras* and *Arion* primers ranged between 17.17-21.25 hours. In the beetles used for these experiments, 40-65 % of individuals tested positive for slug remains within this period. In carabids, the minimum requirement for a detection system is to recognize the target prey for at least 12 hours, which will allow detection of predation in the field during the previous night (Symondson *et al.*, 1996). The results of this preliminary study demonstrate that these primers are capable of reliably amplifying the target species within the period of time required for testing field collected specimens, indeed, the results of our analysis demonstrate that this should be a successful system for the analysis of predation in the field.

In this study, we did not observe a statistically significant difference in detection of *D. reticulatum* remains within the guts of male and female beetles. However, (Symondson *et al.*, 1999) found that prey remains could be detected for approximately 30 % longer in males than in females, but the overall regression slopes and hence rates of decay for male and female beetles were not significantly different. In *Pterostichus madidus*, there was no significant difference between haemolymph concentration equivalents in male and female beetles (Symondson & Liddell, 1993).

These results indicate that a system has been designed which is capable of detecting the remains of *D. reticulatum* and *Arion* spp. within the foreguts of carabid predators for sufficient periods of time that will enable the analysis of predation on these species in the field by *P. melanarius* (Chapter 6). The rates of decay of DNA amplicons produced by these primers are compared directly, using the same samples, with the rates of decay of slug proteins detected using monoclonal antibody ELISA, so that the sensitivity of the two techniques can be evaluated (Chapter 5). In addition, fluorescent and standard PCR is compared to ascertain whether there is any advantage in using one technique rather than the other (Chapter 5).

2.3 Cloning of non-target amplicons from PCR reactions with field trapped beetles

2.3.1 Introduction

Following successful development of the primers described above (Section 2.2; Table 2.1), five were modified with a fluorescent label to determine whether using a fluorescent system to detect predation was an improvement over the standard agarose-gel/ethidium bromide methodology described above. The results of this comparison are presented in Chapter 5. Additionally this fluorescent system was tested with beetles trapped from the field (Chapter 6). As a result of this analysis, a number of non-target amplicons were discovered in field sample PCRs. These products could not be identified as amplicons of non-target species by comparing them with the results of the cross reactivity tests and fell outside the expected amplicon size range for this set of primers. Therefore, they were cloned, as described below, to determine their identity and relevance to the subsequent analysis.

2.3.2 Methods

A large proportion of non-specific PCR products falling outside the target range of the primers were obtained when screening the field, but not in the decay rate samples for the general *Arion* primers. In order to confirm the identity of these products, samples exhibiting strong non-target amplification were cloned into the pCR®2.1-TOPO® (InvitrogenGibco, Life Technologies®) plasmid vector using the method of Sambrook *et al.* (1989). Nine reactions were undertaken, representing five field collected beetles and a feeding experiment control. After confirming the presence of a correctly sized insert in the extracted plasmid DNA by *EcoR1* digestion and agarose gel electrophoresis, the inserts were sequenced, as previously described, using M13 forward and reverse primers. The sequences were compared with known sequences for the predator and other potential mollusc prey available at the field site, and were aligned with related sequences retrieved by a BLAST search on the NCBI gene bank using default settings.

2.3.3 Results

Within a given amplicon size, regardless of the PCR from which it was obtained, the cloned insert fragments aligned, without variation, but between different sized amplicons, homology was not observed. One of the cloned fragments (97 bp) was identified as primer-dimer. Following BLAST searches, only one fragment (314 bp) resulted in significant alignment with the database. The most significant alignments for this fragment were obtained with *Lactobacillus sake* (AF036968; Morel-Deville *et al.*, 1997.) and *L. plantarum* (AL935259.1; Kleerebezem *et al.*, 2003) (Figure 2.5). The 314 bp fragment was observed in 10.2 % of field samples between 21/08/01-11/09/01. This amplicon did not occur when the PCR reaction was positive for slug DNA and *vice versa* and was not observed in timed DNA decay rate samples. The BLAST results for all the other amplicons, resulted in no significant alignments and were attributed to PCR artefacts.

2.3.4 Discussion and implications for further research

We found a large proportion of non-target amplicons when screening the field samples with the general *Arion* primers, the most frequently occurring of which were cloned in order to confirm their identity. The results of cloning, suggest that at least one of the non-target amplicons (314 bp) could be accounted for by the presence of *Lactobacillus* spp. within the guts of the beetles collected from the field. The gut microflora of many insects is very diverse (Bignell, 1984), in many instances, the association between microorganisms and insects are casual and transitory, and are probably acquired from the diet upon which the insect feeds. However, many insects also possess obligate microbial endosymbionts that are in some way beneficial to the insect (Douglas, 1989). Therefore, the beetles could have indirectly acquired the bacteria from consuming infected prey. However, no Lactobacilli were found in healthy or diseased slugs, when their microbial flora was cultured (Amos, 2000), although many other species of gut bacteria were identified.

No significant alignments were obtained for the other amplicons when they were BLAST searched. It is suspected that these fragments are chimeric sequences formed as a result of PCR-jumping giving spurious sequence information, due to the random nature, short length and non-significant alignment hits from the database. The formation of chimeric sequences in a single PCR product is a common phenomenon in ancient DNA (aDNA) amplification. In such circumstances, the aDNA template is often present in low numbers and these are frequently sheared and fragmented (Pääbo *et al.*, 1989, 1990; Lawlor *et al.*, 1991), a condition in which we would expect to find DNA that has undergone digestive processes.

In general, it is assumed that PCR faithfully replicates the target DNA. However, the theory of PCR recombination violates this assumption by generating chimeric sequences derived from two or more homologous templates, a phenomenon that can occur frequently. Wang & Wang (1996) demonstrated that up to 30 % of the products generated during coamplification of similar templates were chimeric and Cronn *et al.* (2002) found that chimeras represented 31 % of amplicons screened and comprised up to 89 % of the amplicons in individual reactions. In our samples, non-target chimeric amplicons that were identified from cloning reactions were observed in 22.5 % of individual PCR reactions.

In vitro chimera formation requires that a minimum of two highly similar non-identical template sequences are present in a single PCR reaction which has been primarily attributed to the periodic formation of incompletely extended PCR products (Saiki *et al.*, 1988; Myerhans *et al.*, 1990; Shammas *et al.*, 2001). In the presence of two similar templates, prematurely terminated products can anneal to non-identical templates and be extended to completion in the next cycle, the resulting recombinant molecules are propagated during subsequent rounds of PCR where they are subject to additional rounds of recombination (Liesack, 1991). In our samples, we would expect to find multiple short fragments of DNA originating from different sources such as the predator, innumerable potential prey, and gut microflora. Therefore, it is not surprising that

chimeric amplicons are able to form, particularly with the general *Arion* primers the annealing temperature (44.4 °C) of which is at the lower advisable limit. Therefore, to a certain extent the experimental conditions used in the PCR reaction may promote chimera formation.

The frequency of occurrence of PCR mediated intergenic recombination can be reduced by adopting long extension times (up to 6 min/kb; Judo *et al.*, 1998); reducing the number of amplification cycles to the absolute minimum for evaluation (Odelberg *et al.*, 1995); or adding PCR additives such as betaine or DMSO which reduce the frequency of recombination (Shammas *et al.*, 2001). All these minimise frequency of chimera formation, but none will entirely eliminate the problem (Cronn *et al.*, 2002). Shearing or partial digestion of the DNA template allows the non-target template to migrate into unexpected size fractions and may permit divergent amplifiable templates to recombine during amplification (Cronn *et al.*, 2002). This situation is unavoidable with prey DNA obtained from gut samples. As none of the non-specific amplicons were of mollusc origin, and the majority were identified as PCR artefacts, it was decided that amplicons falling outside the expected target range for the primers should be discounted when analysing the electropherogram results. In addition, the primers were specifically designed to only amplify a narrow range of target species, for which a consistently sized amplification product was produced. Extensive cross-reactivity testing showed that these primers do not amplify non-target species within the expected size range when sufficient target DNA is present in the reaction and the cloned amplicons were never present in the results of these PCRs. When the correct amplicon was present in field sample PCRs, then the non-target amplicons were absent. Products falling outside the expected amplicon range for the primers should probably be ignored, even if they are potential positives as they will be of little use if their identity cannot be confirmed.

Figure 2.5: Sequence alignment of cloned DNA amplified from within the guts of *Pterostichus melanarius* with *Lactobacillus* sequence hits from the NCBI gene bank database.

```
CLONE 314BP               -------------CACATAAAT-GATAGTCACCAGCCTTATATAATTAAGTCTATTACA-
L.sake(AF036968)          AAATTAATTTTAACAAGCGTGTCAAGATTCGTCAATTCAACGTGGTAAATTTCATAGCTT
L.plantarum (AL935295.1)  ------------------------------------------------------------

CLONE 314BP               CGTAATATAGTAGCAAGTTTTTTGTGATTATTATAGAGTTATTTTAGAAGAAATATA---
L.sake(AF036968)          CTTCATATTTAATTCATCCGCCATTGGTTAAGATACTGTATAATAAGAATATAATCACAC
L.plantarum (AL935295.1)  ------------------------------------------------------------


CLONE 314BP               GAAATTGGAGTTTTATATATGAAAATATTAATGATTGAAGATAATAAATCAGTATCAGAA
L.sake(AF036968)          AAAAGAGAAGGAATTCTAATGAAAATATTAATGGTTGAAGATAATAAGTCTGTCTCTGAA
L.plantarum (AL935295.1)  -----------------TATGAAATTATTAATGATTGAAGATAATAAATCGGTCTCTGAA

CLONE 314BP               ATGATGGGAATGTTTTTCCAAAAGGAAGATTGGGATGCTACATTTTCATATGATGGAAAT
L.sake(AF036968)          ATGATGGGCATGTTCTTTCAAAAAGAAGCGTGGGATGCCCACTTTGCGTATGATGGTAAT
L.plantarum (AL935295.1)  ATGATGGCCATGTTTTTTAAGAAGGAAAAGTGGGATGC----------------------

CLONE 314BP               GATGCCGTCGAAGTATTTAATCAAGATCCTGAAAGTTGGGATATGATTACATTGGATTTG
L.sake(AF036968)          GAAGCGGTTGAACAATTTAGTGTTGATCCTGATAGTTGGGATATTATTACGTTAGATTTG
L.plantarum (AL935295.1)  ------------------------------------------------------------

CLONE 314BP               AATTTGCCTGGTAAAGATGGACTTGTAAGTAT--------------------
L.sake(AF036968)          AATTTACCAGGAATGGATGGGATGCAGGTCGCCCAAAAAATTCGGGAGCAAT
L.plantarum (AL935295.1)  ------------------------------------------------
```

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Chapter 3

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Molecular evolution and phylogenetic analysis of the *Arion hortensis* aggregate (Pulmonata: Arionidae) based on mitochondrial 12S rRNA and cytochrome oxidase subunit I genes.

Ciara S Dodd, William OC Symondson, Sian Moore, David M Glen* and Michael W Bruford.

Cardiff School of Biosciences, Main Building, Cardiff University, Park Place, Cardiff, CF10 3TL, UK

* Styloma Research Ltd., Phoebe, The Lippiatt, Cheddar, BS27 3QP, UK

Corresponding Author: Ciara S Dodd
Biological and Ecological Processes Group
Cardiff School of Biosciences
Main Building
Park Place
Cardiff University
Cardiff
CF10 3TL
UK

Tel: 029 20875776
Fax: 029 20874305

Doddcs@cardiff.ac.uk

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3.1 Abstract

The molecular evolution and taxonomic status of the *Arion hortensis* aggregate was investigated using mitochondrial sequences from the cytochrome oxidase subunit I and domain III of the 12S ribosomal RNA genes. The rate of molecular evolution in the aggregate species appears to be rapid, with high levels of interspecific divergence, a feature common to some other species of mollusk. The presence of conserved motifs in the 12S rRNA sequences greatly facilitated alignment of the sequences. Overall, the structure of the 12S rRNA molecule was more conserved than the sequence. A significant A+T bias existed in both genes, with this bias being greater in loops than stems. Plotting transitions and transversions against uncorrected sequence divergence revealed that saturation was not evident in either data set. Neighbor joining and maximum likelihood trees for both genes separated the three aggregate species confirming taxonomy based on morphology and enzyme electrophoresis. The 12S rRNA gene proved to be more useful in resolving intraspecific relationships and indicated that *Arion intermedius* and the *Arion hortensis* aggregate belong to the same *Kobelita* subgenus, as suggested by morphological and electrophoretic data.

3.2 Introduction

Members of the family Arionidae are widely distributed and several are considered major agricultural pests. However, the taxonomy and phylogenetic relationships of these animals are still poorly understood. Members of the genus *Arion* are morphologically very similar, mainly differing in their color. Color in terrestrial slugs is highly variable and can often reflect factors unrelated to taxonomy, so that considerable variation may exist between individuals within and between populations, which may be influenced by external factors such as diet (Jordaens *et al.*, 2001), climate (Albonico, 1948) and age (Backeljau & de Bruyn, 1990). However, color varieties within a species may be of value in identification as some varietal forms are taxon-specific (Noble & Jones, 1996). Indeed many varieties can be diagnostic markers at different taxonomic levels, e.g. for distinguishing members of the *A. ater* and *A. lusitanicus* aggregates (Noble & Jones, 1996) and these, and especially varietal forms, have a reliable genetic basis (Williamson, 1959; Noble & Jones, 1996). Anatomical features are more diagnostic, but are often subtle and difficult to interpret especially with respect to phylogenetic significance (Backeljau *et al.*, 2001). The determination of phylogenetic relationships within the Mollusca based on morphological differentiation can be hampered by homoplasy (Gosliner, 1991; Mikkelsen, 1993), primarily caused by the independent and repeated reduction or loss of characters during evolutionary history. For example, the slug body form has evolved on several separate occasions from different lineages of snails (Solem, 1974; Tillier, 1984). The presence of variable and/or mixed breeding systems (outcrossing and selfing) within species of arionid slugs is an additional complicating factor (Backeljau *et al.*, 1997). For example, many species are thought to regularly reproduce by non-apomictic selfing, even though some offspring are produced by outcrossing, as in *Arion ater* (Williamson, 1959).

Several *Arion* species are thought to be complexes of cryptic taxa (Cameron *et al.*, 1983; Noble & Jones, 1996). Revision in the taxonomy of *A. hortensis* Férussac, based on anatomical features, identified this species as an aggregate comprising three species;

Arion distinctus Mabille, *Arion hortensis* and *Arion owenii* Davies (Davies, 1977, 1979). The taxonomic validity of this aggregate was supported using enzyme electrophoresis (Backeljau, 1985a, b, 1987; Dolan & Fleming, 1988). In particular, the use of polyacrylamide gel electrophoresis of albumen gland proteins and isoelectric focusing of non-specific digestive gland esterases showed species specific banding patterns (Backeljau (1985a, b, 1989). DNA fingerprinting techniques, simple sequence repeat anchored PCR (SSRa PCR) and random amplified polymorphic DNA (RAPD) have been applied to separate slug species within and between genera, but only *A. hortensis* was studied from within the species aggregate (Voss *et al.*, 1999). None of these studies examined evolution in the *A. hortensis* agg. at the sequence level. However, most recently, the ribosomal gene cluster (Wade & Mordan, 2000; Wade *et al.*, 2001) demonstrated strong support for monophylly of the Family Arionidae.

Most other analyses based on molecular data have examined divergent evolutionary relationships within the Class Mollusca (Boore & Brown, 1994; Rosenberg *et al.*, 1997; Yamazaki *et al.*, 1997; Davis *et al.*, 1998; Harasewych & McArthur, 2000). There is a paucity of published data specifically relating to the molecular phylogeny or molecular evolution of pulmonate gastropods, particularly slugs.

In this paper we present mitochondrial 12S rRNA and cytochrome oxidase subunit I (COI) gene sequences for the *Arion hortensis* aggregate. The use of mitochondrial DNA as a tool for addressing phylogenetic relationships at various levels is well documented. The 12S rRNA gene is one of the most conserved regions in the mitochondrial genome, however mitochondrial ribosomal genes evolve more rapidly than their nuclear counterparts (Mindell & Honeycutt, 1990; Simon *et al.*, 1994) and they have proved useful at lower taxonomic levels from genera to populations (Moritz *et al.*, 1987; Simon *et al.*, 1994). COI is one of the most conserved protein coding genes in mitochondrial genome. It is appropriate for determining species-level relationships and thus could complement 12S rRNA where the latter may lack resolution (Medina & Walsh, 2000). To obtain reliable phylogenetic reconstruction based on rRNA sequences it is necessary to

construct a secondary structure model. Here we present secondary structures for domain III of the 12S rRNA subunit for all the species included in the analysis. Our principal aims were to examine the molecular evolution of the 12S rRNA gene within this aggregate, to elucidate the phylogenetic relationships within the *Arion hortensis* aggregate using mtDNA sequence data, and to compare this with morphological and enzyme-based taxonomic separation of these species.

3.3 Materials and methods

3.3.1 Sample collection

119 individuals from the three aggregate species were collected from ten locations in the British Isles although the restricted distribution of some species meant that not all species were collected from all the sites listed (Table 3.1). Specimens were identified according to external morphological characters and tentacle color was the primary diagnostic feature used to distinguish *A. hortensis* from *A. distinctus* (Cameron *et al.*, 1983). The congener *Arion intermedius* and *Deroceras reticulatum* were used as outgroup species.

3.3.2 DNA extraction, amplification and sequencing

Total genomic DNA was isolated from the head and tentacles of slug specimens using a salt precipitation method modified by Collins *et al.* (1987) from Livak (1984). The third domain of the mitochondrial 12S rRNA (approximately 400 bp) was amplified by PCR in a Perkin-Elmer 9700 Automated Thermocycler using the general invertebrate primers SRN14588 and SRJ14233 (Simon *et al.*, 1994). A 700 bp fragment of the mitochondrial cytochrome oxidase subunit I gene was amplified using the general invertebrate primers COF14 and COR722 (Folmer *et al.*, 1994).

Amplification of the 12S rRNA gene sequence was carried out in a 25 µl reaction containing approximately 200 ng total genomic DNA, 1X PCR reaction buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl (InvitrogenGibco, Life Technologies®)) 4 mM $MgCl_2$, 0.025 mM each of dATP, dCTP, dGTP, dTTP, 0.5 µM each primer and 0.625 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 45 s, 72 °C for 1 min 15s, then a final extension step of 72 °C for 10 min.

Amplification of COI was carried out in a 25 µl reaction containing approximately 200 ng total genomic DNA, 1X buffer (20 mM Tris-HCl (pH 8.4); 50mM KCl

(InvitrogenGibco, Life Technologies®), 1.5 mM to 4 mM $MgCl_2$, 1mM each of dATP, dCTP, dGTP, dTTP, 0. 4 μM each primer and 1.25 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 95 °C for 1 min 30 s, then 40 cycles of 95 °C for 1 min, 47 °C for 1 min 20 s, 73 °C for 1min 10 s (plus 1 second per cycle), followed by final extension at 74 °C for 5 min.

PCR products were purified for sequencing using the GeneClean Turbo for PCR Kit (Bio101) following the manufacturer's instructions. Each sample was sequenced in both forward and reverse directions using the primers described above and electrophoresed on an ABI Prism 377 semi-automated DNA Analyser using the Prism Big Dye Terminator v2 Ready Reaction Kit (Perkin Elmer Applied Biosystems). The sequencing reaction contained 2 μl DNA, 1μl of either forward or reverse primer (1.6 pmol/μl stock), 1 μl Big Dye and 1 μl sterile water (Sigma-Aldrich). The thermocycling reaction consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min.

GenBank Accession numbers for the 12S rRNA sequences are for *D. reticulatum* (AY423668), *A. distinctus* (AY423654-63), *A. hortensis* (AY423632-53), *A. owenii* (AY423664-66) and *A. intermedius* (AY423667). GenBank Accession numbers for the COI sequences are for *D. reticulatum* (AF239734), *A. distinctus* (AY423692-701), *A. hortensis* (AY423670-78), *A. owenii* (AY423702-04) and *A. intermedius* (AY423705).

3.3.3 Sequence alignment and 12S rRNA secondary structure

COI sequences were aligned using Sequencher v3.02 (GENECODES™) and translated into amino acid data with the invertebrate genetic code, using the first reading frame. The reading frame was obtained by aligning the existing COI sequence for *D. reticulatum* (AF239734) (Prasher & Bernon, unpublished) from GenBank to our sequences.

The 12S rRNA sequences were aligned using the default settings of Clustal X version 1.81 for Macintosh (Thompson *et al.*, 1997) in profile alignment mode. The alignment was subsequently refined by hand based on the structural template of Hickson *et al* (1996; 2000). The presence of conserved motifs occurring in both paired and unpaired sections of the molecule simplified alignment of these regions. In areas where there was no conservation of sequence, the alignment was refined manually using the structural template as a guide as well as the model of Medina & Walsh (2000) for *Aplysia cervina.* Flanking regions outside the area covered by the Hickson model were aligned with the aid of the secondary structure model of Page (2000). The use of secondary structure models greatly improved the accuracy of alignment, which allowed a more precise determination of homologous characters for phylogenetic analysis (Kjer, 1995).

Non-canonical (non-Watson-Crick) base pairing was allowed in the model between G•U and A•C bases, which were considered to form stable purine-pyrimidine bonds (Hickson *et al.*, 1996). The G•U pair forms two hydrogen bonds and is considered almost as stable as an A•U pair (Chastain & Tinoco, 1991). On a few occasions other non-canonical pairs (e.g. U-U) were permitted to maintain a structure similar to the template, thus forming stable symmetrical bulges (Hickson *et al.*, 1996). Stems (helices) were required to contain a minimum of two paired bases (Hickson *et al.*, 1996), and hairpin loops a minimum of three bases to avoid distortion of the helix (Zuker, 1989). It was necessary to insert gaps in some sequences, to accommodate insertions and deletions, which are common in rRNA sequences (Kjer, 1995) so that the best alignment could be obtained and structure preserved, since this is more conserved than the nucleotide sequences (Kjer, 1995).

3.3.4 Molecular evolution of the 12S rRNA and COI genes in slugs

Nucleotide composition, and pairwise uncorrected and corrected sequence divergences were calculated in PAUP 4.0b10 (Swofford 2001). The number of transitions (Ts) and transversions (Tv) and the Ts/Tv ratios (R) between pairs of sequences were calculated in MEGAv2.01 (Kumar *et al.*, 2001). To explore the degree of saturation, the number of

substitutions (Ts and Tv) were plotted against uncorrected sequence divergence (*d*) for 12S rRNA, and for COI, first and second codon positions were combined and the third position was plotted separately.

Substitutions in stem regions were divided in to four types in accordance with Springer *et al.* (1995), as follows: Type I (complementary to complementary), Type II (complementary to noncomplementary), Type III (noncomplementary to complementary) and Type IV (noncomplementary to noncomplementary). The number of each substitution falling into these categories is shown in Table 3.2.

3.3.5 Phylogenetic analysis

Phylogenetic analysis was conducted using PAUP 4.0b10 (Swofford, 2001) on each gene separately and on the combined data set. Gaps were considered a fifth character state in all analyses. The most likely evolutionary model was determined using Modeltest v3.06 (Posada & Crandall, 1998). Distance trees were derived using NJ analysis with 1000 bootstrap replications using corrected distances, the model for which was determined using Modeltest. The distance measure varied depending upon the gene being used to create the tree. The best evolutionary model chosen for COI TVM+G ($\Gamma = 0.2197$); for 12S was K81UF+G ($\Gamma = 0.4722$). The shape of the gamma parameter and rate matrix were determined using PAUP and were employed using these models in subsequent heuristic searches to obtain the best ML trees for the data set. Heuristic bootstrap analysis using 100 bootstrap replications was performed for each tree generated.

3.4 Results

3.4.1 Rates and patterns of nucleotide substitution

Complete sequences comprising the two gene fragments contained a total of 962 base pairs, 386 in 12S rRNA and 576 in the COI. Structural alignment for 12S rRNA sequences is shown in Figure 3.1 and sequence alignment for COI is shown in Appendix 1 and the amino acid translation is shown in Figure 3.2. Mean pairwise nucleotide frequencies are given in Table 3.3. There is a clear compositional bias in both 12S rRNA ($\chi^2 = 19.54$, $P<0.05$) and COI ($\chi^2 = 11.17$, $P<0.05$) genes, with adenine (A) and uracil (U) occurring most frequently. Within the 12S rRNA gene, loops show a higher average A+U bias (80.1%) than stems (61.9%; $\chi^2 = 20.95$, $P<0.05$) or the complete sequence (71.1%; $\chi^2 = 27.02$, $P<0.05$). The frequency of guanine (G) and cytosine (C) is markedly less in loops (19.8%) compared with stems (28.9%; $\chi^2 = 27.15$, $P<0.05$) or the entire gene (37.1%; $\chi^2 = 21.57$, $P<0.05$). The alignment of the 12S rRNA gene required the insertion of gaps at 86 sites among the sequences (22.3% of the aligned 12S sequence length), resulting in a gap frequency of between 6.5-14% per species. Compensatory substitutions occurred in 12S helicies and are summarized in Table 3.2.

A+T bias varied within codon positions of the COI (57.8 % for first positions, 58.9 % for second positions, 73.7 % for third positions; $\chi^2 = 13.80$, $P<0.05$). The third codon position in the COI sequences was the most variable accounting for 21.5 % of the overall variation, with first and second positions accounting for 4.5 % and 0.9 % respectively.

Ts/Tv ratios are given in Table 3.4. The degree of saturation was confirmed by plotting the number of Ts and Tv against *d* (Figure 3.3). Saturation was not evident for 12S rRNA or COI genes or at first and second or third codon positions. For the aggregate as a whole, there is a greater frequency of Tv per site in loops (0.094 ± 0.013) compared with stems (0.054 ± 0.010) whereas the rate of transitions is higher for stems (0.066 ± 0.011) than loops (0.037 ± 0.009). The ratio of Ts/Tv is greater in stems (1.162 ± 0.385) than loops (0.327 ± 0.105). In the COI gene, Ts/Tv ratio is greatest in the first codon position

(4.852 ± 3.585), although substitutions occur frequently at third codon positions. Amino acid translation showed that 31.8 % of codons were invariant, and 68.4 % were variable, of which 60.9 % of substitutions were synonymous and 6.8 % were nonsynonymous.

Corrected (K81UF+G (Γ = 0.4722) determined by Modeltest) and uncorrected sequence divergence among taxa is shown in Appendix 2a and 2b, for 12S and COI gene fragments respectively. For the rRNA data set, variation within the ingroup taxa ranged from 0.28-23.37 % using uncorrected distances and between 0.29-50.07 % using corrected distances. For the COI data set, variation within the ingroup taxa ranged from 0.17-21.35 % using uncorrected and between 0.17-67.66 % using corrected distances

3.4.2 12S secondary structure

12S secondary structures derived for each species are shown in Figure 3.4. The region between stem 32 and 32' are based on the model of Hickson *et al.* (1996) with the remaining sequence based on the model of Page (2000). The slug sequences aligned well with the template especially in the regions where conserved sequence repeats occurred with discrepancies being relatively minor, for example differences in the lengths of stems and loops (Figure 3.1). The frequency of non-cannonical base pairs, i.e. G•U or A•C, ranged between 10-12 per species, with G•U pairs occurring more often than A•C.

Helices 42, 45, 47, 48 differed the most in sequence composition from the template and structural models, however these were identified after the backbone of the structure had been established. The hairpin loop of helix 31 was highly variable requiring 3-6 indels to obtain the best alignment. The loss of one base was required between helices 40' and 39' and in the hairpin loop of helix 35 to maintain the structure, resulting in an alteration of the conserved template motifs in these regions. An indel occurred in stem 34' for *D. reticulatum* although this was an unpaired base within the helix and in stem 47' for *A. hortensis*. Additional indels in stem 48 resulted in the formation of helical bulges in *A. hortensis, A. owenii* and *A. intermedius*. All other indels were situated in loop regions. Large indels of between 6-11 bases and 1-12 bases were required in the hairpin loops of

helices 42 and 48 respectively. Stable helical bulges were necessary to maintain structure in helix 48 for *A. distinctus*, and *A. intermedius*, in helix 34 for *A. hortensis* and *A. distinctus* and in helices 35 and 40 for *D. reticulatum*. In general, loop regions were more variable than stems.

3.4.3 Phylogenetic analysis

NJ trees for both 12S and COI (Figure 3.5) show strong support for the three species within the *A. hortensis* aggregate. Surprisingly, little intra-species variation is shown with COI compared with 12S, although these internal branches are not well supported. In the NJ tree for 12S rRNA , *A. intermedius* is not well supported as an out group species, falling in the same clade as *A. owenii*. This is not the case with the COI tree, where both *A. intermedius* and *D. reticulatum* fall together as outgroup species. The ML trees (Figure 3.6) support the tree aggregate taxa as distinct species. Again, the ML trees show that with the 12S rRNA sequences, *A. intermedius* falls in the same clade as *A. owenii*, where as with the COI sequences it falls as an outgroup together with *D. reticulatum*.

3.5 Discussion

3.5.1 Phylogenetic relationships within the A. hortensis *aggregate*

There are few phylogenetic studies of Limacoidea or its member families based on DNA sequence data. This is surprising since classification, particularly of slugs, based on morphological characters, such as color, is renowned for being complicated by factors unrelated to taxonomy. The taxonomy of British slugs has undergone several revisions at the species level, making reliable identification more difficult. Several species within the Arionidae are know or suspected to be complexes of cryptic species, for example, *A. hortensis* agg., *A. ater* agg. and *A. fasciatus* agg. Previous systematic separation of the *A. hortensis* agg. based on morphological features (Davies, 1977, 1979) was confirmed by enzyme electrophoresis (Backeljau, 1985a, b, 1987). The results of our analyses using on mtDNA sequence data support this classification. Both NJ and ML trees support separation of the species within the aggregate.

Different topologies within and among species were revealed according to the gene used to generate the tree. Both NJ and ML trees for COI revealed little or no intra-species variation, whereas surprisingly the NJ tree based only on the 12S rRNA data set showed considerable internal branching particularly for *A. distinctus*, although these divisions were not strongly supported. Nevertheless, this suggests that the 12S rRNA gene may well be suitable for defining intra-specific relationships within the Arionidae. However, Simon *et al.* (1996) suggest that the use of 12S rRNA for comparisons of closely related species e.g. *Drosophila* spp. in phylogenetic reconstruction may not be useful. They noted that the 3' end of this gene of which the majority comprises domain III, is under extreme constraint in some species and therefore should not be chosen in preference to protein coding genes for closely related species. It seems unlikely however, that this is a problem in mollusks. The level of sequence variation within and between species of the *A. hortensis* agg. was far higher than that observed in *Drosophila* (Simon *et al.*, 1996). Indeed, the mitochondrial 12S rRNA gene of other mollusks, e.g. *Mandarina* sp. (Chiba, 1999) has been shown to exhibit similar high levels of divergence between species within

the genus. Tree topologies based on COI clearly delineate the three aggregate species and place *A. intermedius* outside the aggregate. However, in the 12S trees, *A. intermedius* grouped together with *A. owenii.* A similar close relationship was shown between these two species based on hepatopancreas esterase banding patterns (Backeljau, 1985a). It has been suggested that *A. hortensis* agg. and *A. intermedius* belong to the same subgenus, *Kobeltia* based on morphology (Davies, 1979) and enzyme electrophoresis (Backeljau, 1985a). Our results would lend validity to this suggestion, although phylogenetic analysis of the entire genus would be required to substantiate this supposition. In addition, it would be prudent to extend the analysis to include nuclear markers. Analysis of mtDNA alone will provide a gene genealogy, but may present an incomplete history of the organisms if hybridization has taken place and several species of Arionid slugs are known to hybridize readily.

3.5.2 Molecular evolution

The 12S rRNA gene is one of the most conserved mitochondrial genes (e.g. Jacobs *et al.*, 1988), but this is not always the case in comparisons of closely related species (Simon *et al.*, 1996). We observed a high level of within and between species variation in both the 12S and COI sequences. If this represents real variation in a functional copy of the 12S rRNA gene then the patterns of substitution should reflect natural selection occurring on functional constraints of the sequence, such as base pairing and highly conserved sequence motifs (Hickson *et al.*, 1996). The highly conserved sequence motifs identified by Hickson *et al.* (1996) were present in all our sequences, with the majority of the variation lying outside these regions. Although there were no indications that we were not amplifying a mitochondrial copy of the 12S sequences, it has been noted that nuclear copies of mitochondrial sequences (Numts) can exhibit striking concordance with the 12S rRNA secondary structure template with little indication of their pseudogene status (Olson & Yoder, 2002). Therefore using structural criteria and identifying conserved sequence motifs is not always a reliable method for discriminating 12S rRNA pseudogenes. A similar high level of divergence within and between species was observed in slug COI sequences and here amino acid translation identified these

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sequences as functional COI copies. Such a high level within species variation has been reported in other mollusks such as *Mandarina* sp. (Chiba, 1999) and crustaceans where divergence of 9.6 % between two species of shrimp (*Penaeus* sp) was observed (Palumbi & Benzie, 1991). However, in limpets of the genus *Patella*, only one substitution distinguished two closely related species (Koufopanou *et al.*, 1999). It appears that the mitochondrial genome of *A. hortensis* aggregate species is evolving rapidly. The level of overall sequence divergence (uncorrected *p*) between members of the aggregate is 12.2 % for 12S rRNA and 10.7 % for COI. These values are of a similar order of magnitude as those reported for *Mandarina* sp. Chiba (1999) states that the level of divergence between their species is comparable with the level of divergence between horses and humans. The level of divergence between our species is approximately half of this value; nevertheless, the degree of divergence is higher than would be normally expected among closely related individuals. Intra-specific divergence ranged from 2.3 % to 4.9 %, with *A. distinctus* exhibiting the highest level of diversity. These values are equivalent to the level of divergence between different genera or families in other taxa. Differences in evolutionary rates of 12S between taxa can be caused by differences in mutation rate or in molecular structural and functional constraints (Kimura, 1987). These results suggest an exceptionally high evolutionary rate in *A. hortensis* agg., although if these species have invaded the British Isles on several occasions, this may not be the case. Founder events can accelerate the rate of mtDNA evolution (DeSalle & Templeton, 1987) as can the frequent contraction and expansion of populations. Slugs have a long history in the British Isles and Arionid shell granules are regularly found in postglacial deposits (Solem, 1974). Dodd *et al.* (this thesis Chapter 4) suggest that *A. hortensis* agg., populations have undergone rapid expansion in the British Isles possibly as a result of bottlenecks or founder events and that populations of these species may have undergone multiple contraction and expansion from isolated refugia as a result of the glacial history of the northern hemisphere.

Analysis of 12S rRNA and COI sequences seems to indicate that molecular evolution in the *A. hortensis* agg. is rapid. Investigation of sequence feature revealed a marked bias in

nucleotide frequence and Ts/Tv ratios. The marked A+T bias in our sequences is unsurprising as invertebrate (e.g. Simon *et al.*, 1994) and mollusk (e.g. Medina & Walsh, 2000) mtDNA frequently shows this compositional bias. Within the 12S rRNA gene, the A+T content of the *A. hortensis* agg. (71 %) is comparable with that of other invertebrates which ranged from 70-84 % (Hickson *et al.*, 1996). We found that loops had a higher A+T bias than stems, which has also been observed in other mollusks (Medina & Walsh, 2000). In COI sequences, the A+T bias was greatest at the third codon position. In loops, A is much more abundant than in stems or COI, a pattern that has been observed in vertebrate and invertebrate (Vawter & Brown, 1993) 12S sequences, but not in mammals (Springer & Douzery, 1996). A possible explanation for this was posed by Gutell *et al.* (1985) who suggested that A is the least polar of the four bases and may therefore facilitate hydrophobic interactions with proteins. Although a slight A+T bias exists in stems, it is much less pronounced than in loops and consequently, the G+C content is considerably higher in stems (37.1 %) than in loops (19.8 %). Again, this finding is consistent with the characteristics of 12S rRNA in other taxa and has been predicted on the basis of free energy calculations (Turner *et al.*, 1988). Compositional bias means that parsimony will favor changes from the common to the rare, for example A→G over G→A, if A is more abundant than G (Collins *et al.*, 1994). This is the case in the entire 12S data set, where A is more common than G and transitions from A→G occur more commonly than from G→A.

It is evident that there is a Ts/Tv bias in loops with an approximate 1:1 ratio of Ts/Tv in stems. Similar patterns have been observed in other groups of closely related taxa such as fish (Orti *et al.*, 1996; Bakke & Johansen, 2002), primates (Hixon & Brown, 1986) and other mammals (Douzery & Catezeflis, 1995; Springer & Douzery, 1996). However, in comparisons of vertebrate and invertebrate taxa the opposite pattern was found (Vawter & Brown, 1993). It seems likely that patterns of Ts/Tv ratios therefore depend on levels of phylogenetic divergence. We found a transversion bias in loops compared with stems, which may indicate saturation of multiple substitutions at the same site (Simon *et al.*, 1990), although saturation of transitions or transversions was not observed in the12S

sequences. In common with studies on Acari (Norris *et al.*, 1999), mammals (Springer *et al.*, 1995) and crustaceans (Taylor *et al.*, 1996) the majority of transversions in the 12S slug sequences were accounted for by changes between A↔T. Within the *A. hortensis* agg., COI sequences, showed a transversion bias at the third codon position, with Ts/Tv ratio being greatest in first then third position.

In terms of substitution frequency, a higher proportion of unambiguous nucleotide changes occurred in stems compared with loops. Wheeler & Honeycut (1988) suggested that substitutions are more likely to occur in stems as there is a non-random pattern of paired differences in these regions which indicates that positive selection drives fixation of changes that restore base complementarity and hence conserve structure. Dixon & Hillis (1993) and Wheeler & Honeycut (1988) recommended down weighting stems, but (Hickson *et al.*, 1996) suggested that this is sometimes inappropriate as unpaired regions can be also very well conserved due to functional constraints such as interactions with protein, tRNA or rRNA (Noller *et al.*, 1990).

3.5.3 12S rRNA secondary structure

Domain III of the 12S rRNA gene has constrained secondary structure, many conserved motifs and most of the 12 helices are more or less fixed in length (Hickson *et al.*, 1996). Rate variation between taxa may be due to constraints caused by interactions with these molecules and ribosomal proteins (Moritz *et al.*, 1987; Simon *et al.*, 1996). The identification of conserved motifs greatly facilitated alignment of the primary sequence particularly in highly variable regions. Some variation was evident in the length of both stems and loops, and in common with other studies (Hickson *et al.*, 1996; Medina & Walsh, 2000), we found that the most variable regions were in the vicinity of helices 42, 45, 47 and 48. The sequence that aligned most consistently with the template was around the central helices 32, 33, 34, 36 and 38. Norris *et al.* (1999) found similar variation in loop and stem length between species of hard ticks, to the extent that stem 47 was entirely absent from these sequences. No helices were missing from *A. hortensis* aggregate or outgroup sequences and variation in stem length was within the parameters

stated by Hickson *et al.* (1996) for each helix. The terminal loops of stem 42 and 48 contained the greatest number of indels between slug species. The loop of helix 42 was reported in Hickson *et al.* (1996) as being the most variable in birds, containing indels of up to 10 bp. The shortest stems in our structure were stems 35 and 40, both of which were only 2 bp in length. In the Hickson *et al.* (1996) model, stem 35 is between 2-4 bp and stem 40 is between 2-3 bp, but is usually three. In *A. owenii*, an A•C pairing is required to form stem 40, in common with amphibia and the earthworm *Aporrectodea rosea* (Hickson *et al.*, 1996). Evidence of compensatory substitutions was evident in all but four stems (Table 3.2), with the vast majority of substitutions between variable bases being of Type I (complementary-complementary). Springer *et al.* (1995) also found that Type I substitutions occurred most frequently in mammalian 12S rRNA sequences and Hickson *et al.* (1996) provides evidence of different types of compensatory substitutions in stems between avian and odonate taxa. In our data set, the majority of paired bases show no change between members of the *A. hortensis* aggregate and it has been suggested that compensatory changes in rRNA may not occur for substantial periods of time (Kraus *et al.*, 1992). Therefore, we would expect to see a greater proportion of base pair changes between more distantly related taxa.

3.5.4 Conclusions

The rate of evolution in the *A. hortensis* aggregate appears to be relatively rapid, in common with evolutionary rates in some other mollusk lineages. Neighbor joining and maximum likelihood trees for both 12S rRNA and COI genes supported separation of the species within the aggregate. However, the 12S rRNA gene appears to provide a greater degree of resolution within species and supports the notion posed by morphological and electrophoretic data that *A. intermedius* and the *A. hortensis* aggregate belong to the same subgenus *Kobelita*.

3.6 Acknowledgements

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3.8 Tables

Table 3.1: Location, grid reference and number of samples analyzed for each species within the *Arion hortensis* aggregate.

A. hortensis	*A. distinctus*	*A. owenii*	Location	Grid reference (Latitude/Longitude)
9	-	-	Blackpool	53 49 14 N 3 2 29 W
8	-	-	Hull	53 51 1 N 0 24 46 W
16	7	-	Lissington	53 20 10 N 0 20 16 W
1	2	1	Gamlingay	52 9 25 N 0 11 46 W
4	4	-	Mold	53 9 48 N 3 8 47 W
14	8	1	Glyn Ceiriog	52 56 17 N 3 11 7 W
-	5	-	Cowbridge	51 25 32 N 3 26 2 W
7	9	-	Cardiff	51 29 18 N 3 13 3 W
10	6	-	Long Ashton	51 25 33 N 2 40 17 W
-	-	7	Bodmin	50 26 39 N 4 42 16 W

Table 3.2: Number of nucleotide substitutions in pairwise comparisons between taxa falling into each substitution category. Type I (complementary-complementary), Type II (complementary-noncomplementary), Type III (non-complementary-complementary), Type IV (noncomplementary-noncomplementary) (Springer *et al.*, 1995).

	Type of Substitution				
	No Change	**Type I**	**Type II**	**Type III**	**Type IV**
Helix 31	12				
Helix 32	15	12			
Helix 33	15				
Helix 34	20	3	2	4	
Helix 35	6				
Helix 36	8	8	2		
Helix 38	31	2			
Helix 39	10	8			
Helix 40	4	1	2		
Helix 42	6				
Helix 45	6	1			
Helix 47	4	2			
Helix 48	11	1	4	2	1
Total	**148**	**35**	**10**	**6**	**1**

Table 3.3: Mean frequency of nucleotide substitutions (%) in 12S rRNA and COI sequences for the *Arion hortensis* aggregate. Standard deviation is given in brackets().

	Stems only				Loops only				Stems + Loops				COI			
Taxon	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
A. hortensis	28.9 (0.31)	17.5 (0.00)	20.8 (0.29)	32.8 (0.12)	52.3 (0.27)	10.8 (0.00)	8.9 (0.14)	27.9 (0.22)	39.7 (0.21)	14.4 (0.00)	15.3 (0.16)	30.6 (0.13)	23.3 (0.14)	18.8 (0.11)	19.6 (0.15)	38.3 (0.14)
A. distinctus	29.5 (0.07)	14.5 (0.03)	20.5 (0.19)	35.6 (0.08)	52.6 (0.56)	8.3 (0.31)	11.4 (0.03)	27.7 (0.38)	40.9 (0.26)	11.4 (0.16)	16.0 (0.11)	31.7 (0.20)	26.8 (0.15)	16.7 (0.10)	17.5 (0.11)	39.0 (0.19)
A. owenii	28.9 (0.00)	16.1 (0.00)	20.0 (0.00)	35.0 (0.00)	51.5 (0.00)	13.5 (0.00)	7.4 (0.00)	27.6 (0.00)	39.7 (0.00)	14.9 (0.00)	14.0 (0.00)	31.5 (0.00)	27.8 (0.00)	15.2 (0.10)	16.8 (0.00)	40.2 (0.10)
***A.hortensis* agg.**	29.1 (0.36)	16.5 (0.86)	20.6 (0.35)	33.8 (0.89)	52.3 (0.55)	10.2 (1.97)	9.6 (1.06)	27.8 (0.56)	40.1 (0.58)	13.5 (1.42)	15.4 (0.56)	31.0 (0.56)	24.7 (1.84)	17.8 (1.30)	18.7 (1.12)	38.7 (0.61)

Table 3.4: Number of Transitions (s), transversions (v) and transition/transversion ratio (R) for the *Arion hortensis* aggregate for 12S rRNA and COI sequences calculated using uncorrected-*p* distances.

SP		12S +COI	12S stems and loops	12S stems	12S loops	COI	1ST	2ND	3RD
A. hortensis	**R**	1.295 ± 0.555	0.282 ± 0.134	0.095 ± 0.061	0.043 ± 0.038	2.182 ± 0.858	n/c	0.000 ± 0.000	2.331 ± 1.064
	s	0.003 ± 0.001	0.002 ± 0.001	0.003 ± 0.002	0.001 ± 0.001	0.004 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.011 ± 0.003
	v	0.001 ± 0.000	0.002 ± 0.001	0.000 ± 0.000	0.004 ± 0.002	0.001 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.001
A. distinctus	**R**	1.909 ± 0.966	0.450 ± 0.474	n/c	0.450 ± 0.465	1.772 ± 1.050	2.100 ± 1.330	1.100 ± 0.778	1.342 ± 0.864
	s	0.004 ± 0.001	0.003 ± 0.002	0.000 ± 0.000	0.007 ± 0.004	0.004 ± 0.001	0.003 ± 0.002	0.002 ± 0.001	0.006 ± 0.004
	v	0.001 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.002 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.002 ± 0.001
A. owenii	**R**	n/c	n/c	n/c	n/c	n/c	n/c	n/c	n/c
	s	0.003 ± 0.001	0.004 ± 0.003	0.007 ± 0.005	0.000 ± 0.000	0.002 ± 0.002	0.003 ± 0.003	0.000 ± 0.000	0.003 ± 0.003
	v	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
***A. hortensis* aggregate**	**R**	1.059 ± 0.146	0.623 ± 0.117	1.005 ± 0.306	0.366 ± 0.126	1.29 ± 0.181	4.852 ± 3.585	0.988 ± 0.764	1.129 ± 0.183
	s	0.055 ± 0.004	0.053 ± 0.007	0.061 ± 0.010	0.040 ± 0.009	0.056 ± 0.006	0.038 ± 0.008	0.003 ± 0.002	0.127 ± 0.013
	v	0.060 ± 0.004	0.072 ± 0.008	0.057 ± 0.010	0.087 ± 0.013	0.053 ± 0.005	0.009 ± 0.005	0.003 ± 0.003	0.146 ± 0.014

3.9 Figure legends

Figure 3.1: Structural alignment for domain III of the mitochondrial 12S rRNA gene sequences of *Arion hortensis* aggregate (Ah – *A. hortensis*, Ad – *Arion distinctus*, Ao - *A. owenii*) and two outgroup species *A. intermedius* (Ai) and *Deroceras reticulatum* (Dr). Shaded areas show regions of complementary base pairing within stems, labeled according to the numbers. Bold type indicates conserved motifs within the 12S rRNA gene (Hickson *et al.*, 1996) and the bases highlighted in black show regions where sequence is missing for this part of the motif.

Figure 3.2: Amino acid translation for *Arion hortensis* aggregate and outgroup species for the mitochondrial COI gene using the first reading frame.

Figure 3.3: Genetic distance (uncorrected p) verses number of transitions (•) and transversions (◊) for the mitochondrial 12S rRNA and COI genes. The first and second codon positions have been grouped together and the third position is plotted separately.

Figure 3.4: Secondary structures for domain III of the mitochondrial 12S rRNA gene for a) *Arion hortensis*, b) *A. distinctus*, c) *A. owenii*, d) *A. intermedius* and e) *Deroceras reticulatum*. Numbers indicate stems and follow the system of Hickson *et al.* (1996).

Figure 3.5: Neighbor joining trees for the *Arion hortensis* aggregate for a) 12S rRNA and b) COI genes. Numbers indicate bootstrap support following 1000 replications. The distance estimator used for 12S rRNA sequences was K81UF+G (Γ = 0.4722) and for COI was TVM+G (Γ = 0.2197).

Figure 3.6: Maximum likelihood trees for the *Arion hortensis* aggregate for a) 12S rRNA and b) COI genes. Numbers indicate bootstrap support following 100 replications. The distance estimator used for 12S rRNA sequences was K81UF+G (Γ = 0.4722) and for COI was TVM+G (Γ = 0.2197).

3.10 Figures

Figure 3.1

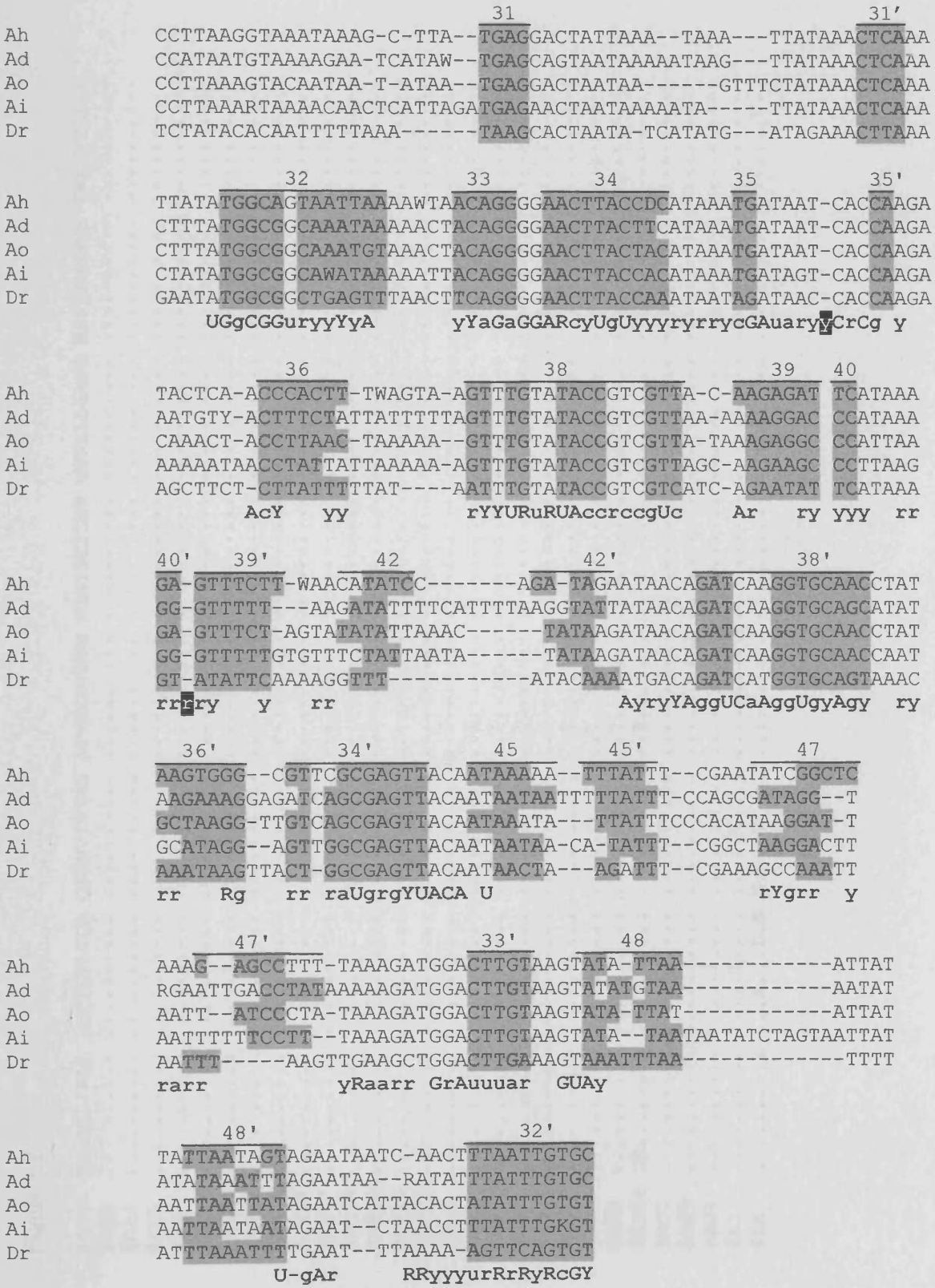
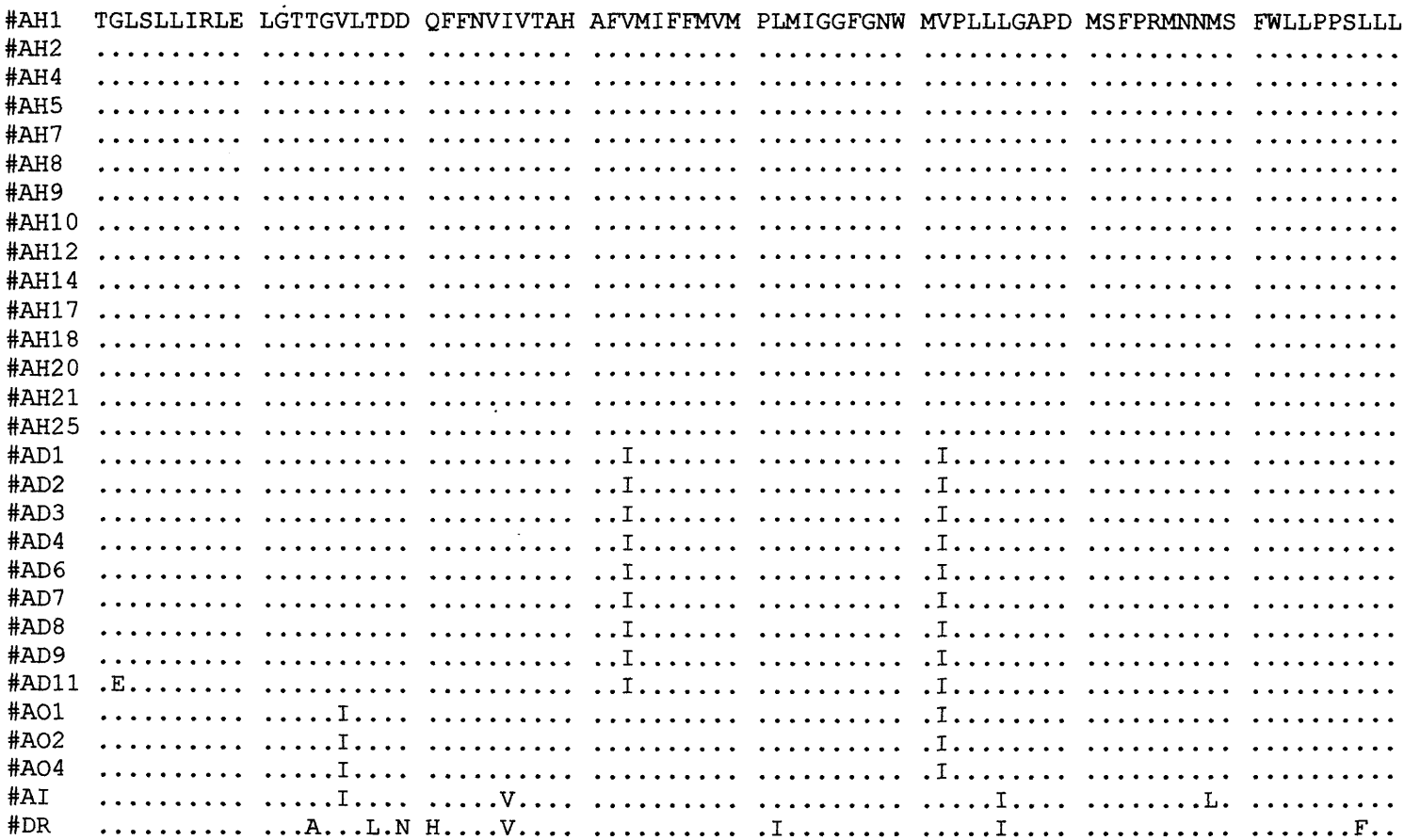


Figure 3.2

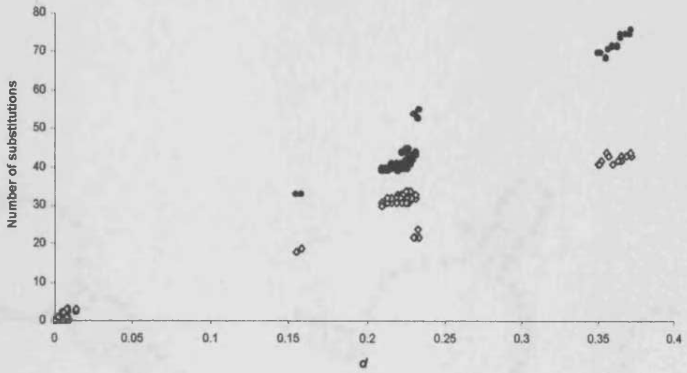

#AH1 TGLSLLIRLE LGTTGVLTDD QFFNVIVTAH AFVMIFFMVM PLMIGGFGNW MVPLLLGAPD MSFPRMNNMS FWLLPPSLLL
#AH2 ....
#AH4 ....
#AH5 ....
#AH7 ....
#AH8 ....
#AH9 ....
#AH10 ....
#AH12 ....
#AH14 ....
#AH17 ....
#AH18 ....
#AH20 ....
#AH21 ....
#AH25 ....
#AD1 ....I....... .... .I........ ....
#AD2 ....I....... .... .I........ ....
#AD3 ....I....... .... .I........ ....
#AD4 ....I....... .... .I........ ....
#AD6 ....I....... .... .I........ ....
#AD7 ....I....... .... .I........ ....
#AD8 ....I....... .... .I........ ....
#AD9 ....I....... .... .I........ ....
#AD11 .E........ ....I....... .... .I........ ....
#AO1 ....I.... .... .I........ ....
#AO2 ....I.... .... .I........ ....
#AO4 ....I.... .... .I........ ....
#AI ....I.... .....V.... ....I.... ....L. ....
#DR ....A...L.N H....V.... .... .I........ .....I.... ....F..

```
#AH1  LIVSSMVEGG AGTGWTVYPP LSSIMAHSGA SVDLAIFSLH LAGMSSILGA INFITTIFNM RPKVLTFERL SLFVWSILIT
#AH2  .......... .......... .......... .......... .......... .......... .......... ..........
#AH4  .......... .......... .......... .......... .......... .......... .......... ..........
#AH5  .......... .......... .......... .......... .......... .......... .......... ..........
#AH7  .......... .......... .......... .......... .......... .......... .......... ..........
#AH8  .......... .......... .......... .......... .......... .......... .......... ..........
#AH9  .......... .......... .......... .......... .......... .......... .......... ..........
#AH10 .......... .......... .......... .......... .......... .......... .......... ..........
#AH12 .......... .......... .......... .......... .......... .......... .......... ..........
#AH14 .......... .......... .......... .......... .......... .......... .......... ..........
#AH17 .......... .......... .......... .......... .......... .......... .......... ..........
#AH18 .......... .......... .......... .......... .......... .......... .......... ..........
#AH20 .......... .......... .......... .......... .......... .......... .......... ..........
#AH21 .......... .......... .......... .......... .......... .......... .......... ..........
#AH25 .......... .......... .......... .......... .......... .......... .......... ..........
#AD1  ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AD2  ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AD3  ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AD4  ..C....... .......... ...S...... .......... .T........ .......... ......LK.M .V.A......
#AD6  ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AD7  ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AD8  ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AD9  ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AD11 ..C....... .......... ...S...... .......... .......... .......... ......L..M ..........
#AO1  ..C....... .......... ...SL..... .......... .......... .......... .........M ..........
#AO2  ..C....... .......... ...SL..... .......... .......... .......... .........M ..........
#AO4  ..C....... .......... ...SL..... .......... .......... .......... .........M ..........
#AI   ..C....... .......... ..GS...... .......... .......... .......... ......L..M ..........
#DR   ..C....... .......... ..GPLG.A.. .......... .......... .......... .SPGMSM... ........V.
```

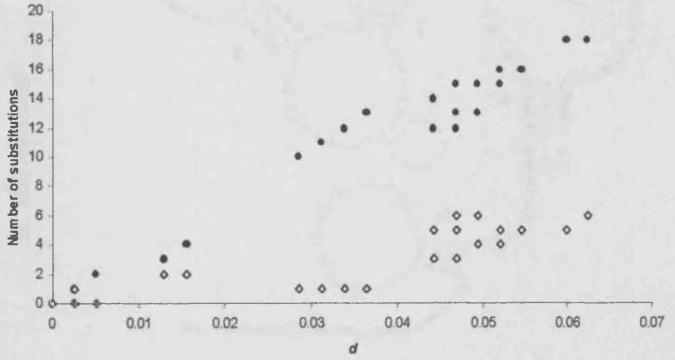
```
#AH1  VFLLLLSLPV LAGAITMLLT DRNFNTSFFD PA
#AH2  .......... .......... .......... ..
#AH4  .......... .......... .......... ..
#AH5  .......... .......... .......... ..
#AH7  .......... .......... .......... ..
#AH8  .......... .......... .......... ..
#AH9  .......... .......... .......... ..
#AH10 G......... .......... .......... ..
#AH12 .......... .......... .......... ..
#AH14 .......... .......... .......... ..
#AH16 .......... .......... .......... ..
#AH17 .......... .......... .......... ..
#AH18 .......... .......... .......... ..
#AH20 .......... .......... .......... ..
#AH21 .......... .......... .......... ..
#AH25 .......... .......... .......... ..
#AD1  .......... .......... .......... ..
#AD2  .......... .......... .......... ..
#AD3  .......... .......... .......... ..
#AD4  G......... .......... .......... ..
#AD6  .......... .......... .......... ..
#AD7  .......... .......... .......... ..
#AD8  .......... .......... .......... ..
#AD9  .......... .......... .......... ..
#AD11 .......... .......... .......... ..
#AO1  .......... .......... .......... ..
#AO2  .......... .......... .......... ..
#AO4  .......... .......... .......... ..
#AI   .......... .......... .......... ..
#DR   .......... .......... .......... ..
```

Figure 3.3

a)



b)



c)

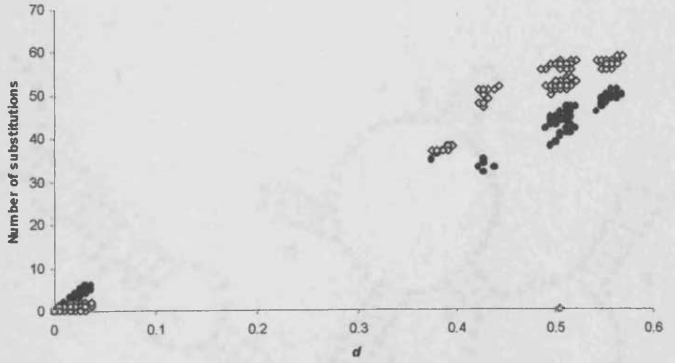
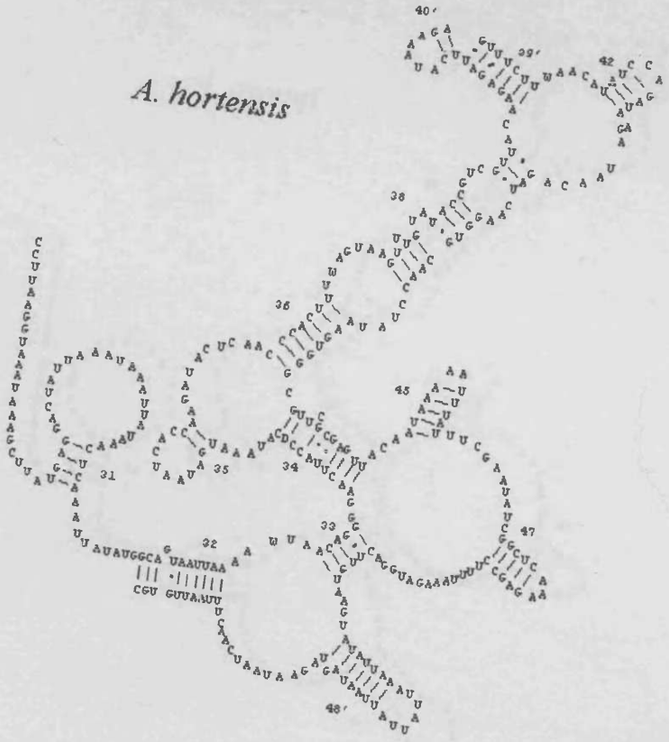
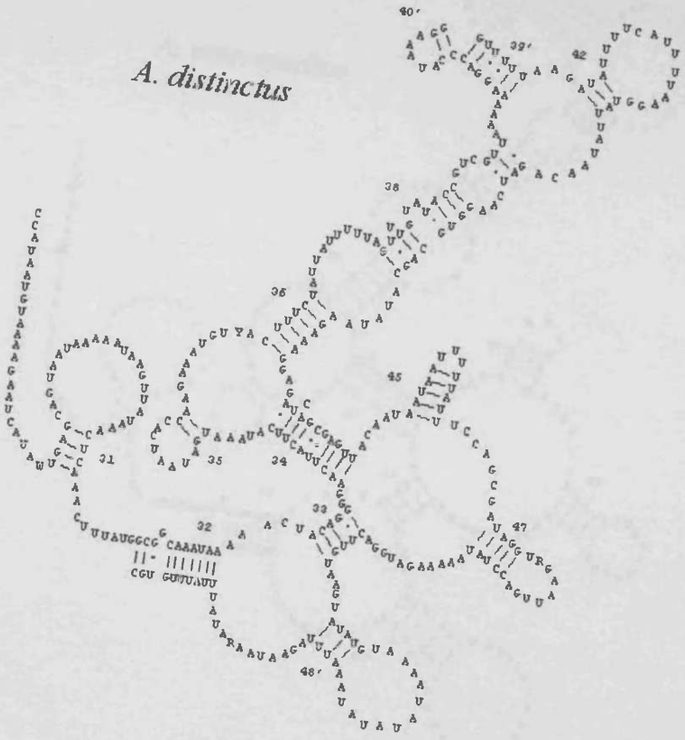


Figure 3.4

a)

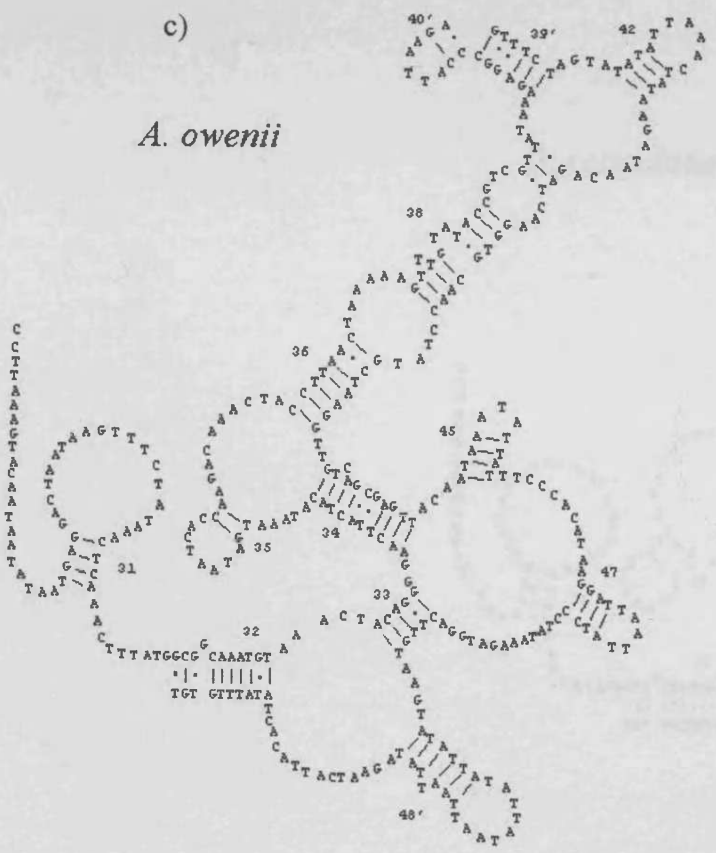


b)



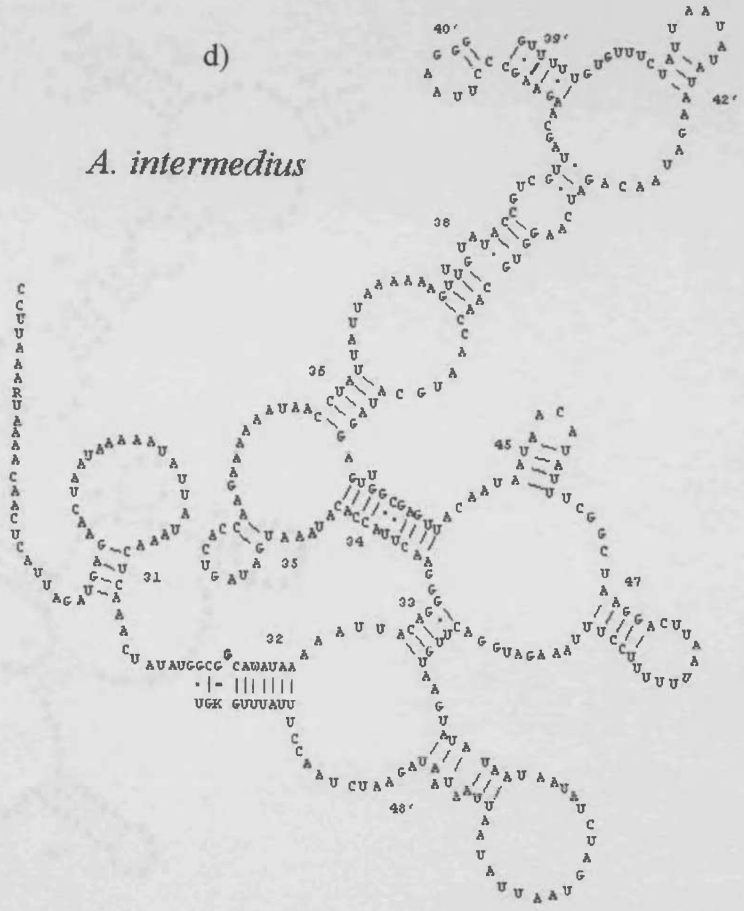
c)

A. owenii



d)

A. intermedius



e)

D. reticulatum

Figure 3.5

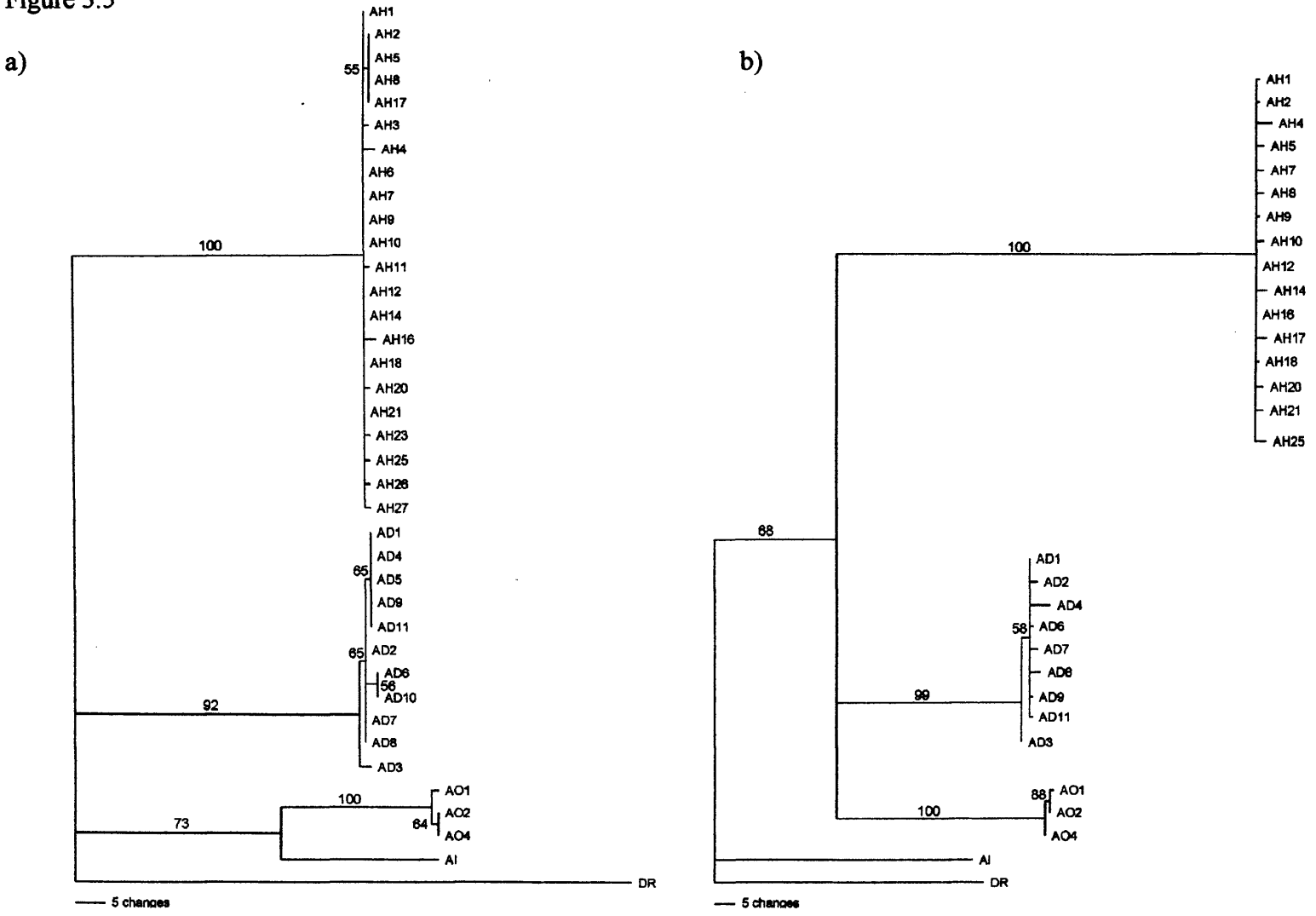
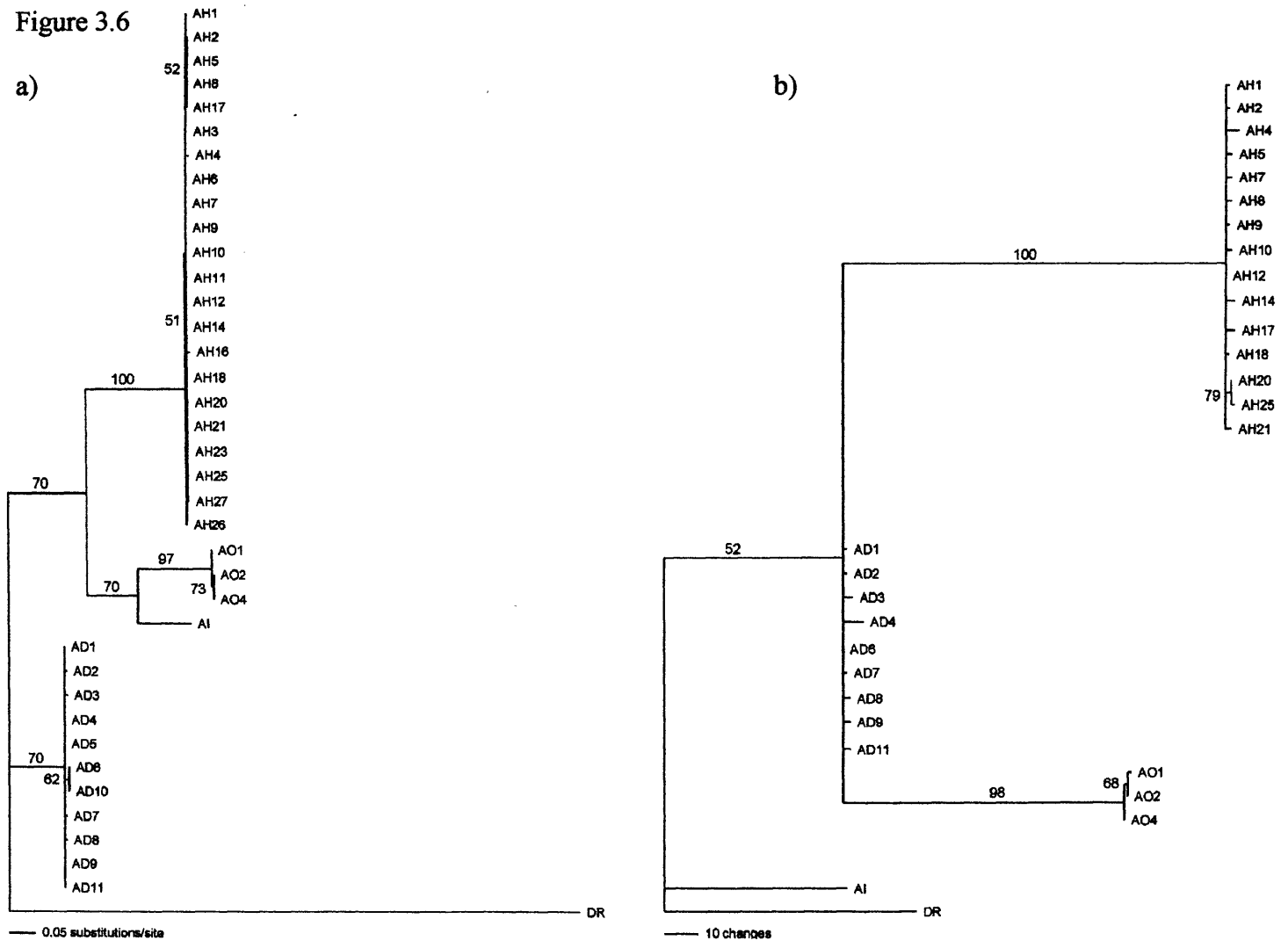

a)
AH1
AH2
AH5
AH8
AH17
AH3
AH4
AH6
AH7
AH9
AH10
AH11
AH12
AH14
AH16
AH18
AH20
AH21
AH23
AH25
AH26
AH27
AD1
AD4
AD5
AD9
AD11
AD2
AD6
AD10
AD7
AD8
AD3
AO1
AO2
AO4
AI
DR
5 changes
100
55
65
65
56
92
100
64
73
b)
AH1
AH2
AH4
AH5
AH7
AH8
AH9
AH10
AH12
AH14
AH16
AH17
AH18
AH20
AH21
AH25
AD1
AD2
AD4
AD6
AD7
AD8
AD9
AD11
AD3
AO1
AO2
AO4
AI
DR
5 changes
100
68
58
99
88
100

Figure 3.6


a)
AH1
AH2
AH5
AH8
AH17
AH3
AH4
AH6
AH7
AH9
AH10
AH11
AH12
AH14
AH16
AH18
AH20
AH21
AH23
AH25
AH27
AH26
52
51
100
70
97
70
73
AO1
AO2
AO4
AI
AD1
AD2
AD3
AD4
AD5
AD6
AD10
AD7
AD8
AD9
AD11
70
62
DR
0.05 substitutions/site
b)
AH1
AH2
AH4
AH5
AH7
AH8
AH9
AH10
AH12
AH14
AH17
AH18
AH20
AH25
AH21
100
79
52
AD1
AD2
AD3
AD4
AD6
AD7
AD8
AD9
AD11
98
68
AO1
AO2
AO4
AI
DR
10 changes

Chapter 4

Modified version to be submitted for publication in Molecular Ecology

(format according to journal specifications)

Genetic variation and phylogeography of the *Arion hortensis* aggregate in the British Isles based on mitochondrial 12S rRNA and COI gene sequences.

Ciara S Dodd, William OC Symondson, David M Glen* and Michael W Bruford

Cardiff School of Biosciences, Main Building, Cardiff University, Park Place, Cardiff CF10 3TL, UK

* Styloma Research Ltd., Phoebe, The Lippiatt, Cheddar, BS27 3QP, UK

Corresponding Author:
Ciara S Dodd
Biological and Ecological Processes Group
Cardiff School of Biosciences
Main Building
Park Place
Cardiff University
CF10 3TL
UK

Tel: 029 20875776
Fax: 029 20874305

Doddcs@cardiff.ac.uk

Key words: *Arion hortensis* aggregate ,12S rRNA, cytochrome oxidase I, phylogeography, demographic history

4.1 Abstract

Slugs of the *Arion hortensis* aggregate were collected from across the British Isles to investigate intraspecific diversity, population differentiation and demographic history. Individuals were sequenced using PCR primers for the mitochondrial cytochrome oxidase subunit I (COI) gene and the third domain of the 12S rRNA gene. High levels of intra-specific polymorphism were revealed. Overall, haplotype diversity was greatest for *Arion hortensis* Férussac, with *Arion distinctus* Mabille and *Arion owenii* Davies exhibiting similar levels of a similar order. The results of nested AMOVA indicated that for *A. hortensis* approximately half of the variation observed was within populations and half between populations within groups (north vs. south and east vs. west). For *A. distinctus*, the majority of the variation lay within the populations. However, no geographic clines were evident for either species. Moderate to high levels of genetic differentiation between populations was revealed, although a Mantel test revealed no spatial structure between populations and the results of nested clade analysis were inconclusive. Mismatch distributions indicated that *A. distinctus* has the longest history in the British Isles, *A. hortensis* the shortest, with *A. owenii* being intermediate between the two. All three species showed evidence of population expansion and contraction following bottlenecks or founder events. Initial rapid population expansion followed by a sudden decrease in population size may have been as a result of climatic cooling. Subsequent secondary expansion may have been due to recolonisation from refugia in warmer interglacial periods.

4.2 Introduction

The *Arion hortensis* aggregate is a complex of three cryptic species (*Arion hortensis* Férussac, *Arion distinctus* Mabille and *Arion owenii* Davies). Prior to the 1970s the separate species were not recognised and were all considered *A. hortensis* (Kerney, 1999). Following a taxonomic revision (Davies, 1977, 1979), the three species were separated; two of these, *A. hortensis* and *A. distinctus* agreed with the original nomenclature and a third, new species (*A. owenii*) was identified. The current information on the distribution of these species in the British Isles is incomplete (Kerney, 1999) and it is likely that these species are under-recorded, especially *A. owenii.* All three species are thought to be native (i.e. not introduced) to the British Isles. They all have similar habitat requirements, preferring to live amongst herbage, ground litter and in soil crevices in both natural and humanly disturbed habitats, although *A. distinctus* has a slight preference for base-rich soils and is reportedly absent from harsh upland terrain (Kerney, 1999).

Northern hemisphere regions have been colonised by terrestrial molluscs in relatively recent post-glacial times from a few refugia (Solem, 1985), but within the British Isles, slugs have a long history. Arionid shell granules are frequently found in Pleistocene and post-glacial deposits (Evans, 1972). The influence of human activity on the distribution of certain agriculturally important slugs, such as *Deroceras* sp. has been reflected by a steady increase in the occurrence of their remains in post-glacial deposits (Evans, 1972). Both *A. distinctus* and *A. hortensis* are considered agricultural pests and are readily spread by man. Of the three species *A. distinctus* has the widest recorded distribution and is likely to occur throughout most of the British Isles, whereas *A. hortensis* has a more patchy distribution, is locally common in southern England and Ireland, and there is evidence that it is extending its northerly distribution as a result of human activity (Kerney, 1999). *A. owenii* is thought to be much rarer and is a clearly local species, being common in certain areas such as Devon, and it has not been detected by intensive mapping elsewhere (e.g. Cardigan, Bedfordshire, Suffolk) (Kerney, 1999).

The population genetics of many species of terrestrial molluscs such as *Helix aspersa* Müller (Arnaud *et al.*, 1999) exhibit an "isolation by distance" structure within their populations (e.g. Pfenninger *et al.*, 1996) as they are subdivided in numerous demes with limited migration between them (Guiller *et al.*, 2000). Geographic isolation of subpopulations over time results in the establishment of marked genetic isolation by distance and striking local spatial patterns of genetic variation within and among populations (Arnaud *et al.*, 2001). Such low levels of gene flow between established populations can allow the genetic traces of colonisation to remain for many generations (Davison & Clarke, 2000). Stepping-stone and isolation-by-distance models predict that gene flow declines monotonically with increasing geographical distance between spatially discrete and continuous populations respectively, as dispersal is usually constrained by distance and gene flow is most frequently achieved between neighbouring colonies.

Allozymes have been frequently used to assess genetic structure and gene flow in molluscs, although they often reveal little variation either within or between populations, indicating that they may not be sufficiently variable to disclose underlying levels of population structure (Arnaud *et al.*, 2001). Although inter-specific variation in the *A. hortensis* aggregate has been investigated using enzyme based methods (Backeljau, 1985 a,b, 1987, 1989; Backeljau *et al.*, 1988; Dolan & Flemming, 1988) and confirmed the taxonomic validity of the species complex, little intra-specific regional or geographic differentiation has been shown. However, albumen gland polymorphisms in *A. owenii* suggested that the geographically isolated populations might represent distinct forms or genetic strains (Backeljau *et al.*, 1988) which have also been found in other arionid slugs (Jordaens *et al.*, 1996; Backeljau *et al.*, 1997).

There are remarkably few studies of molecular genetics in terrestrial mollusc populations. Considerable genetic variation was found using mitochondrial DNA (mtDNA) markers between north African populations of *H. aspersa* (Guiller *et al.*, 2001), and in *Cepaea*

nemoralis (Linnaeus) across its European range although only two common and two rare mtDNA haplotypes were found in Britain (Thomaz *et al.*, 1996). Noble & Jones (1996) used random amplified polymorphic DNA (RAPD) to investigate north-west European populations of the large Arionid slugs, and more recently, Brookes (2002) has demonstrated population differentiation in British populations of *Deroceras reticulatum* (Müller) and *Arion intermedius* Normand using mitochondrial single strand conformation polymorphism (SSCP) haplotypes and microsatellites. However, to the best of our knowledge there are no published studies relating to the phylogeography of British and European slug populations at the sequence level.

Using mitochondrial sequence data from 12S ribosomal RNA and cytochrome oxidase subunit I (COI) loci, the genetic diversity and genetic structure of the *Arion hortensis* aggregate were examined within the British Isles. Previous studies have shown little intra-specific variation in allozyme loci. In this study, we investigate the distribution of mitochondrial haplotypes in Britain and use these data to examine the history of the *A. hortensis* aggregate in the British Isles, examine the population structure and the effects of population subdivision at the macro geographic scale.

4.3 Materials and Methods

4.3.1 Sample collection

119 individuals from the three aggregate species were collected from ten locations in the British Isles (Figure 4.1) although the restricted distribution of some species meant that not all species were collected from all the sites listed. *A. hortensis* is locally common in the south of England and Ireland, although is probably under-recorded; *A. distinctus* is the commonest of the three species, occurring throughout most of the British Isles; *A. owenii* appears common in Devon, Dumfrieshire, Cumbria and parts of Northern Ireland, although remains seriously under-recorded (Kerney, 1999).

4.3.2 DNA extraction, amplification and sequencing

Total genomic DNA was isolated from the head and tentacles of slug specimens using a high salt extraction method modified by Collins *et al.* (1987) from Livak (1984). The third domain of the mitochondrial 12S rRNA gene (385 bp) was amplified by polymerase chain reaction (PCR) in a Perkin-Elmer 9700 Automated Thermocycler using the general invertebrate primers SRN14588 and SRJ14233 (Simon *et al.*, 1994). A 700 bp fragment of the cytochrome oxidase subunit I gene (COI) was amplified using the general invertebrate primers COF14 and COR722 (Folmer *et al.*, 1994).

Amplification of the 12S rRNA gene sequence was carried out in a 25 µl reaction containing approximately 200 ng total genomic DNA, 1X PCR reaction buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl (InvitrogenGibco, Life Technologies®), 4 mM $MgCl_2$, 0.025 mM each of dGTP, dATP, dTTP and dCTP, 0.5 µM each primer and 0.625 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 45 s, 72 °C for 1 min 15 s, then a final extension step of 72 °C for 10 min.

Amplification of COI was carried out in a 25 µl reaction containing approximately 200 ng total genomic DNA, 1X buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl (InvitrogenGibco, Life Technologies®), 1.5 mM to 4 mM $MgCl_2$, 1 mM each dNTP, 0. 4 µM each primer and 1.25 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 95 °C for 1 min 30s, 40 cycles of 95 °C for 1 min, 47 °C for 1 min 20 s, 73 °C for 1 min 10 s (plus 1 second per cycle), followed by final extension at 74 °C for 5 min.

PCR products were purified for sequencing using the GeneClean Turbo for PCR Kit (Bio101) following manufacturers instructions. Each sample was sequenced in both forward and reverse directions on an ABI Prism 377 semi-automated DNA Analyser using the Prism Big Dye Terminator v2 Ready Reaction Kit (Perkin Elmer Applied Biosystems). The sequencing reaction contained 2 µl DNA, 1 µl of either forward or reverse primer (1.6 pmol/µl), 1 µl Big Dye and 1 µl sterile water (Sigma-Aldrich). The thermocycling reaction consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min.

GenBank Accession numbers for the 12S rRNA sequences are for *D. reticulatum* (AY423668), *A. distinctus* (AY423654-63), *A. hortensis* (AY423632-53), *A. owenii* (AY423664-66) and *A. intermedius* (AY423667). GenBank Accession numbers for the COI sequences are for *D. reticulatum* (AF239734), *A. distinctus* (AY423692-701), *A. hortensis* (AY423670-78), *A. owenii* (AY423702-04) and *A. intermedius* (AY423705).

4.3.3 Sequence alignment and analysis

The COI sequences were aligned using Sequencher v3.02 (GENECODES™) and translated into amino acid data using the invertebrate genetic code, using the first reading frame.

The 12S rRNA sequences were aligned using the default settings of Clustal X v.1.81 for Macintosh (Thompson *et al.*, 1997) in profile alignment mode. The alignment was subsequently refined manually based on the structural template of Hickson *et al.* (1996, 2000) and Page (2000). Details of secondary structures for these sequences can be found in Dodd *et al.* (this thesis Chapter 3).

A minimum spanning network was designed for each species, illustrating equally parsimonious haplotype connections using TCS v.1.13 (Clement *et al.*, 2000) and Minspnet (Excoffier & Smouse, 1994). F-statistics do not use temporal information on allelic variations (Templeton, 1998) and therefore, nested clade analysis (Templeton *et al.*, 1992) was used to accommodate the temporal component of the data. TCS was used to produce a nested cladogram based on the minimum spanning network. The nested clade analysis was implemented using Geodis v2.0 (Posada *et al.*, 2000), which separates population structure from population history by performing significance tests upon an estimated nested cladogram through a Monte Carlo procedure (Templeton *et al.*, 1992). A minimum of 1000 random permutations was performed to provide a 5 % level of significance. These results were interpreted using the inference key in Templeton (1998).

Haplotype ($\hat{h}$) and nucleotide diversity (π) were calculated for each population and species using Arlequin v.2.000 (Schneider *et al.*, 2000). Pairwise comparisons of populations and a nested analysis of molecular variance (AMOVA) were used to determine partitioning of variation within and between populations and within and between regions of the British Isles for each species, using Arelquin v.2.000 (Schneider *et al.*, 2000). Populations were partitioned into regions according to a N/S and E/W split, as a north-south cline in enzyme allele frequency changes has been suggested for *C. nemoralis* and *Cepea hortensis* (Müller) (Ochman *et al.*, 1987) and an east-west cline in common 16S rRNA mtDNA haplotypes in *C. nemoralis* (Davison, 2000) in the British Isles. Gene flow between population pairs was calculated using $Nm = 0.5[(1/Fst)-1]$ (Takahata & Palumbi, 1985). Significance values were obtained from a minimum of 1000 permutations. Since not all data points were independent, the relationship between

genetic distance (Fst) and geographic distance (*d*) was determined using a Mantel Test (Smouse *et al.*, 1986; Smouse & Long, 1992) with significance values obtained using 1000 permutations in Arlequin v.2.000 (Schneider *et al.*, 2000). Great circle distances (Km) between populations were determined from latitude and longitude coordinates using the programme Earth (Byers, 1999).

Mismatch distributions were calculated for each species using Arlequin v.2.000 (Schneider *et al.*, 2000). This method calculates the distribution of pairwise nucleotide differences among DNA sequences by estimating τ (expansion time), θ_0 and θ_1 (mutation parameters) based on a model of sudden expansion (Rogers & Harpending, 1992; Schneider & Excoffier, 1999). The sum of square deviations (SSD) test of goodness of fit tests the validity of the estimated mismatch parameters by comparing observed and expected mismatch distributions (Schneider & Excoffier, 1999). The following equations were then used to calculate t (time in generations since expansion) and N_0 (effective population size before expansion): $t = \tau/2u$, where $u = 2\mu k$, k is the number of nucleotides in the sequences being compared and μ is the mutaion rate; $N_0 = \theta_0/2u$ (Rogers & Harpending, 1992). A mutation rate of 2.8 %-10 %/site per Myrs based on the entire mitochondrial genome of *Partula* (Murray *et al.*, 1991) and 12S and 16S rRNA genes of *Mandarina* (Chiba, 1999) and a generation time of 1 year were used (Davies, 1977). The estimated population size after expansion (N_1) was not calculated, as it is not reliably recovered from mismatch distribution (Schneider & Excoffier, 1999). Confidence intervals of 95 % were calculated for all parameters with 100 replicates using a parametric boostrap approach in Arelquin v.2.000 (Schneider *et al.*, 2000).

4.4 Results

4.4.1 Haplotype distribution and diversity

Sequence analysis revealed 22 haplotypes for *A. hortensis*, 10 for *A. distinctus* and 3 for *A. owenii*, containing 27, 26 and 4 polymorphic sites for each species respectively (Table 4.1). The most common haplotype for *A. hortensis* was AH1, occurring in 20.29 % of samples. Thirteen haplotypes were represented by a single individual (Table 4.2a). In *A. distinctus* the most common haplotype was AD1 and was present in 56.1 % of individuals, with six haplotypes represented by a single individual (Table 4.2b). The most common haplotype for *A. owenii* was AO1, occurring in 77.8 % of individuals (Table 4.2c).

The minimum spanning networks for *A. hortensis* and *A. owenii* (Figure 4.2a and c, respectively) showed that for the majority of haplotypes were removed only by a single substitution from their nearest neighbour. Haplotype AH4 represents a divergent branch separated by five substitutions from its nearest neighbour in *A. hortensis* (Figure 4.2a) and there was an unresolved relationship between haplotypes AH12, AH16, AH4 and AH20, and between haplotypes AH2, AH3 and AH5. The level of divergence between neighbouring haplotypes was greater for *A. distinctus* (Figure 4.2b) than either *A. hortensis* or *A. owenii*. An unresolved relationship between haplotypes AD3, AD2, AD1 and AD6 was found in *A. distinctus*. Unique haplotypes occurred in 18/22, 6/10 and 2/3 haplotypes for *A. hortensis*, *A. distinctus* and *A. owenii* respectively.

Haplotype diversity (Table 4.3a-c) was greatest for *A. hortensis*, but similar for *A. distinctus* and *A. owenii*. However, the greater haplotype diversity for *A. hortensis* may have been a result of the greater number of individuals sampled for this species (N=69) compared with *A. distinctus* (N=41) and *A. owenii* (N=11). Nucleotide diversity (Table 4.3a-c) was equivalent for *A. hortensis* and *A. distinctus*, but less for *A. owenii*, again probably reflecting the small number of samples obtained for *A. owenii*. The overall levels of haplotype diversity (0.6389-0.9143) within the *A. hortensis* aggregate were comparable to those for *D. reticulatum* 16S rRNA haplotypes (0.095-0.844) from across

Britain (Brookes, 2002). Nucleotide substitutions between haplotypes were represented by eight transversions, 19 transitions and two sites contained multiple substitutions for *A. hortensis*. In *A. distinctus* substitutions corresponded to six transitions, 14 transversions and six insertions or deletions. Only transitions were observed in *A. owenii*.

4.4.2 Nested Clade Analysis

Evaluation of the limits of parsimony indicated that mtDNA haplotypes separated by up to 13 mutational steps were connected in a parsimonious fashion ($P \geq 0.95$) for *A. hortensis* (n=22) and *A. distinctus* (n=10). Using maximum parsimony within these limits, one haplotype network was obtained for each species (Figure 4.3), with no ambiguity in the nested design. For *A. distinctus*, no clades showed significant geographical association of haplotypes based on permutational chi-square statistics. For *A. hortensis*, only 3-step clades (3-1 and 3-2) and the entire cladogram (4-1) showed a significant level of geographical subdivision. For 3-step clades, the inference key indicated that the 'geographical sampling scheme was insufficient to discriminate between fragmentation and isolation by distance.' For the 4-step clade, the sampling design was inadequate to discriminate between isolation by distance (short distance movements) vs. long-distance dispersal. For *A. distinctus*, no inference could be drawn about population structure and demography as the key in Templeton (1998) indicated that insufficient individuals or geographic locations were sampled to draw any conclusions.

4.4.3 Population structure

Differentiation among regions, among populations within each region and within populations was examined using analysis of molecular variance (Table 4.4). For *A. hortensis* approximately 50 % of the variation was accounted for among populations within groups (both north vs. south and east vs. west) and approximately 50 % within populations. In *A. distinctus* the majority of the variation lay within the populations. Estimated gene flow for *A. hortensis* between North and South ($F_{st} = 0.4832$), and East and West ($F_{st} = 0.4977$) was around 0.5 individuals per generation reflecting the high degree of genetic differentiation based on Fst between these regions. For *A. distinctus*

gene flow was estimated to be approximately 3 individuals per generation between north and south (Fst = 0.158) and 9 between east and west (Fst = 0.053). However, estimates of gene flow based on *Nm* may not accurately reflect genetic structure between populations since it is not able to differentiate between ongoing and historical processes and makes assumptions on population structure and diversity which may be violated (Hartl, 2000).

Pairwise comparisons between populations showed significant Fst values ranging between 0.1158 and 0.7048 for *A. hortensis*, and 0.0704 and 0.5446 for *A. distinctus* (Table 4.5 a-b respectively). Significant differentiation was shown between all pairs of populations in *A. hortensis* except for Blackpool vs. Gamlingay and Mold, Long Ashton vs. Gamlingay, Mold, and Cardiff, Cardiff vs. Gamlingay and Lissington, Gamlingay vs. Glyn Ceiriog, Mold and Hull. For *A. distinctus* most population comparisons showed no significant differentiation, except for Cardiff vs. Long Ashton, Glyn Ceiriog, Lissington and Mold, and Long Ashton vs. Cowbridge and Mold. A Mantel Test revealed no significant relationship between genetic distance (Fst) and geographic distance (*d*) for *A. hortensis* (P = 0.525), *A. distinctus* (P = 0.374) or *A. owenii* (P = 0.354).

4.4.4 Mismatch distribution

Mismatch distributions for each species were consistent with a unimodal model of distribution of a rapidly expanding population with P>0.05 for each SSD test (Figure 4.4a-c). *A. distinctus* was calculated to have the oldest expansion time (t = 43,000-154,000 years ago) and *A. hortensis* the most recent (t = 15,000-56,000 years ago), with *A. owenii* being intermediate between the two (t = 22,000-80,000 years ago). The effective population sizes before expansion were estimated for *A. hortensis* (1926-6895), *A. distinctus* (21-76) and *A. owenii* (5-19).

4.5 Discussion

4.5.1 Genetic diversity

Previous studies with allozymes have shown limited genetic diversity between populations of slug within the *A. hortensis* aggregate (Foltz *et al.*, 1982, 1984; Backeljau, 1985a), although it has been suggested that geographically isolated populations of *A. owenii* should be considered as distinct evolutionary significant units (Backeljau *et al.*, 1988). However, in this study, relatively high levels of genetic diversity were revealed for *A. hortensis* and *A. distinctus* both within and between populations using mtDNA. Although some degree of differentiation between populations of *A. owenii* was observed, many more samples need to be analysed before a true picture of genetic diversity and population structure can be determined for this species. Disparity between the levels of diversity indicated by enzyme electrophoresis and DNA analysis is expected, since polymorphism in electrophoretic 'alleles' requires amino acid replacement and hence variation due to silent nucleotide substitutions will not be revealed through these methods (Hartl & Clarke, 1997). Therefore, electrophoretic analysis will, by definition, underestimate the actual level of genetic variation present (Backeljau *et al.*, 2001). In addition, the degree of variation can be influenced by the techniques used (Backeljau, 1989).

The level of haplotype diversity identified for species within the *A. hortensis* aggregate in the British Isles is comparable with that found in British populations of *D. reticulatum* (Brookes, 2002). However, mtDNA haplotype diversity was considerably lower for other terrestrial pulmonates. In *Cepaea nemoralis* only two mtDNA haplotypes were common in British populations (Davison, 2000). The number of electrophoretic 'strains' found in other species of Arionid slug was variable. *A. intermedius* was found to have at least four strains (Backeljau & De Bruyn, 1991; Backeljau *et al.*, 1992), *A. fasciatus* more than 20 (Jordaens *et al.*, 1996) and in *A. silvaticus* and *A. fasciatus*, 64 and 33% of populations consisted of more than one strain (Backeljau *et al.*, 1997). However the number of strains detected is likely to increase with greater geographic and population sampling.

The disparity between the overall haplotype diversity for *A. hortensis* (0.9143) compared with *A. distinctus* (0.6646) and *A. owenii* (0.6389) may partly be explained by differences in sample number and geographic spread of samples between these species. Both *A. hortensis* and *A. distinctus* had one common haplotype occurring at relatively high frequency across the majority of populations sampled (Figure 4.2). For *A. hortensis*, haplotypes are grouped around the two or three most common haplotypes, which are likely to have given rise to the majority of other haplotypes by single base pair mutations and probably represent the ancestral haplotypes for this species. For *A. distinctus* there is one main haplotype, distinguished from most of its closest relatives by up to seven substitutions. In this case, the divergent branches may be a consequence of insufficient sampling to allow the extent of variation in this species to be disclosed. Again, the main haplotypes occurring in geographically distant populations are likely to represent the ancestral haplotypes and the rarer variants represent mutations of this haplotype and are probably more recent (Excoffier & Smouse, 1994). Insufficient individuals or geographic locations have been sampled to draw any conclusions regarding *A. owenii*.

No apparent geographic patterns were revealed for populations of *A. hortensis* or *A. distinctus*, either in a north-south or east-west direction. No geographic clines were found for populations of *D. reticulatum* across the British Isles (Brookes 2002). However, for *C. nemoralis* and *C. hortensis*, a north-south cline was observed in enzyme allele frequency changes (Ochman *et al.*, 1987) and an east-west cline was found in *C. nemoralis* mtDNA haplotypes (Davison, 2000). The most likely explanation for these clines is that the current distribution reflects two routes of colonisation after the last ice age and the limited gene flow between populations may have retained the original patterns of diversity (Davison, 2000). Arionid slugs are particularly prone to cyclic population explosions and regressions (Kerney, 1999), which may maintain genetic diversity through gene flow (Slatkin, 1987). Slugs, particularly agriculturally important species, live in heterogeneous frequently disturbed habitats which are insufficiently stable in time to allow selection to produce a gradient in allele frequency.

4.5.2 Population structure

The results of AMOVA indicated that for *A. distinctus* and *A. owenii* the majority of variation was within populations, whereas for *A. hortensis* variation was more or less equally partitioned among regions and within populations. However, high levels of within population diversity are not expected in species with a reportedly low dispersal ability and hence limited gene flow, but high levels of diversity were also found for *D. reticulatum* using microsatellites and mtDNA SSCP haplotypes (Brookes, 2002) and allozymes (Foltz *et al.*, 1984; Fleming, 1989a, b). In the latter studies, the results were explained by microhabitat variation so that local populations were as distinct from each other as those at greater geographic distances. Here, moderate levels of genetic differentiation were shown between populations of *A. distinctus* (Fst = 0.1093) and *A. owenii* (Fst = 0.1964), and a high level of differentiation was seen for *A. hortensis* (Fst = 0.4971). For *A. hortensis* the closest (Glen Ceiriog – Mold) and furthest (Cardiff – Hull) populations are equally highly differentiated, with Fst values of 0.6783 and 0.6664 respectively. For *A. distinctus* the closest populations with significant Fst values (Cardiff – Long Ashton) were highly differentiated (Fst = 0.4331) and the most distant (Cardiff – Lissington) were moderately differentiated (Fst = 0.2081). Moderate to low levels of differentiation among British populations of *D. reticulatum* were revealed through microsatellite analysis with overall Fst values of 0.062 (Brookes, 2002). In contrast to our results, in pairwise comparisons of populations with moderate levels of differentiation, the populations of *D. reticulatum* that were the closest geographically were shown to be the least differentiated (Brookes, 2002).

Estimates of gene flow between populations were determined by calculating *Nm* values. Varying degrees of gene flow between populations were implied, ranging between 0.21 – 23.21 individuals per generation for *A. hortensis* and 0.46 – 85.71 per generation for *A. distinctus*. However, no spatial structure was revealed using a Mantel Test to compare gene flow estimates against geographic distance and hence no genetic arrangement based on an isolation-by-distance model could be inferred. The results of nested clade analysis revealed no underlying population substructure for *A. distinctus* as more intermediate

population needed to be sampled. For *A. hortensis*, the pattern of geographic samples was insufficient to distinguish fragmentation or isolation by distance. In contrast, several studies on terrestrial snails clearly indicated a significant relationship between genetic differentiation and geographic distance (Pfenninger *et al.*, 1996; Arnaud *et al.*, 1999). However, the geographic scale over which we sampled was not sufficient to reveal such underlying patterns of population structure. It is also likely that individuals were sampled from more than one subpopulation/deme within an area. Samples formed by an admixture of local populations will hide underlying patterns of local population structure and only by sampling on the micro-geographic scale will the degree and frequency of dispersal, migration and gene flow between demes be determined. Our samples show differentiation at the macro-geographic scale and as such are likely to indicate low dispersal ability over the distances sampled (average interpopulation distance is 190 km). The minimum inter-population distance was 17 km and if it is assumed that dispersal is limited to that in the Wrightian neighbourhood (Wright, 1969) of 20 m per generation (for *C. nemoralis* (Wright, 1969; Davison, 2000)), then it would require 850 generations to disperse this distance which roughly equates to 850 years with a generation time of 1 year. It is possible that the speed of dispersal could be accelerated in a passive sense by human activity and the ranges of other species of slug have been expended in this manner such as *A. hortensis, A.distinctus* and *Lehmannia valentiana* (Férrusac, 1821) amongst others (Kerney, 1999). In the case of *Boetguerilla pallens*, a species unknown in the British Isles before 1972 when it was first recorded in Windermere (Colville *et al.*, 1974), it now has a distribution that extends from Scotland to Cornwall and Ireland, and it still spreading (Kernery, 1999).

4.5.3 Demographic history

In each of the three species, geographically isolated locations share some common haplotypes. The presence of the same common haplotype at high frequency across many of the populations could indicate that colonising individuals after the last glaciation were predominantly of the same lineage. In the case of *C. nemoralis*, only two haplotypes are common in the British Isles and it has been suggested that these represent recolonisation

by two invading lineages from mainland Europe following the last glaciation (Würm II) (Thomaz *et al.*, 1996; Davison, 2000). The population structure of molluscs, which is usually in a stepping-stone arrangement with limited migration between demes, allows the persistence of co-existing ancient mtDNA haplotypes in the very long term (Thomaz *et al.*, 1996). This structure is implied in *A. hortensis* through nested clade analysis, but more geographic locations need to be sampled before any firm conclusions can be drawn. It is therefore possible that the common widespread haplotypes in *A. hortensis* and *A. distinctus* represent ancient haplotypes. Frequent extinctions and recolonisations can be a source of gene flow between populations even without exchange of individuals (Slatkin, 1987). This should be particularly relevant to a species that is frequently associated with human activity and unstable habitats such as agricultural areas, gardens and waste-ground. However, in order to gain a complete picture of the demographic history of these species, it will also be necessary to analyse nuclear loci. Behavioural changes during founder events may facilitate hybridisation, as it has been suggested that sexual interactions may lose specificity subsequent to a founder event (Kaneshiro, 1989). Hybrids are well documented for certain arionid slugs, particularly within aggregates. In the case of closely related heterospecifics hybridisation and/or introgression subsequent to colonization may occur but to a different extent between different regions of the genome (DeSalle & Giddings, 1986). Since mtDNA is thought to be selectively neutral it may flow relatively easily through a hybrid zone and therefore mtDNA haplotype patterns could mask any hybrid zones between species.

Mismatch distributions within stable populations are expected to be multimodal. We found that within our species, they were skewed or unimodal indicating rapid expansion following bottlenecks or founder events. The steep concave curve for *A. distinctus* (and possibly for *A. owenii*) is consistent with a sudden reduction in population size following rapid expansion. Two small secondary expansion peaks are suspected for *A. distinctus* until convergence. The small value for θ_1 (2.261) for this species is consistent with a rapid reduction in population size (Rogers & Harpending, 1992). The mismatch distribution for *A. hortensis* indicates that the population expanded to several times it

original size and that further increases in population size have only had a minor effect (Rogers & Harpending, 1992). Mismatch distributions indicate that *A. distinctus* has the longest history within the British Isles, colonising between 43-154,000 years ago. Of the three species within the aggregate, this one has the widest recorded distribution. The rapid decrease in population size may have been a result of cooling periods and the secondary expansion peaks due to re-colonisation from refugia in warmer interglacial periods. In terms of the glacial history of the British Isles, it is debateable whether molluscs present in this region in the last interglacial period (Ipswichian) survived through the last glacial period (Devensian) 70,000 BP. Kerney (1966) suggests that the majority of the rich molluscan fauna of the last interglacial were exterminated at this time. Even though it is possible that a small number of hardy species survived in isolated refugia in the peri-glacial zone in the south, there is little direct evidence to support this hypothesis (Kerney, 1966, 1999). Species with Lusitanian distributions in Europe may represent such *in situ* survivals and may include the slugs *Testacella maugei* Férussac, and *Geomaculus maculosus* Allman (Kerney, 1966). As these are some of the least cold tolerant slug species and if they were able to survive in refugia it is more than likely that more cold tolerant species such as *A. hortensis* aggregate were also able to survive in these areas.

It is generally accepted that climate warming following deglaciation resulted in re-immigration of species from mainland Europe. By 15,000 – 10,000 BP it is estimated that around 27 species of land snails had re-established themselves (Kerney, 1999) and by 7,500 – 5,000 BP it is likely that 18 of the 29 extant species of slugs had arrived in Britain through passive dispersal (Kerney, 1999). The internal shells of *Agriolimax* (*Deroceras*) sp. and *Milax* sp. are known from deposits of the last interglacial (Sparks, 1964) making post-glacial re-immigration probable. In addition, *Agriolimax* (*Deroceras*) sp. remains are common in Holocene deposits and probably attained their present wide range as a result of human activity (Kerney, 1966).

4.6 References

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4.7 Figure Legends

Figure 4.1: Location, numbers of specimens and coordinates (latitude and longitude) for sample sites within the Britain Isles.

Figure 4.2: Minimum spanning networks of haplotypes for a) *Arion hortensis*, b) *A. distinctus*, c) *A. owenii*. The size of haplotypes is approximately proportional to their frequency. The relationship between haplotype and location is represented by pie charts. Dashed lines correspond to ambiguous connections between haplotypes.

Figure 4.3: Nested cladograms for a) *Arion hortensis* and b) *A. distinctus*. Each line in the network represents a single mutational change. Roman numerals represent haplotype numbers and are equivalent to those given throughout for each species. 0 indicates an interior node in the network that was not present in the sample and is therefore an inferred intermediate haplotype between the two nearest haplotypes in the network that differed by two or more mutations. Haplotypes are grouped into 1-step clades, 1-step nested into 2-step clades and 2-step nested into 3-step clades.

Figure 4.4: Mismatch distributions of populations (including upper and lower 95% confidence intervals for τ): *Arion hortensis* (τ = 2.873, 1.502-4.902; θ_0 = 0.353; θ_1 = 12.891; SSD P = 0.27), *Arion distinctus* (τ = 8.078, 3.073-13.774; θ_0 = 0.004; θ_1 = 2.261; SSD P = 0.52), *Arion owenii* (τ = 4.141, 1.094-11.891; θ_0 = 0.001; θ_1 = 3.438; SSD P = 0.07) The solid black line with ■ is the observed distribution and with □ is the simulated distribution based on the sudden expansion model. Dashed lines represent upper and lower confidence intervals at α = 0.05 calculated using 100 replicates.

4.8 Tables

Table 4.1: Polymorphic sites defining haplotypes of a) *Arion hortensis*, b) *A. distinctus* and c) *A. owenii* within in Britain. The first haplotype for each species is taken as the reference sequence; dots (.) indicate identical nucleotides, dashes indicate indels. Substitutions representing transversions are underlined.

a	Base Position																											
Haplotype	0 7 3	0 9 1	1 2 8	1 5 1	1 7 6	1 9 1	2 6 1	2 6 8	3 6 4	3 8 5	4 1 8	4 8 4	5 1 4	5 1 7	6 2 5	6 3 7	6 4 6	6 6 4	7 0 6	7 0 9	7 1 2	7 7 2	8 0 2	8 2 2	8 5 9	8 6 8	8 8 3
AH1	T	A	A	A	T	G	G	A	A	G	C	A	T	G	G	T	A	G	G	A	G	A	C	T	A	G	T
AH2	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.
AH3	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
AH4	.	.	.	T	A	.	.	.	.	.	.	G	.	T	.	.	.	A	.	.	.	.	T	.	.	.	.
AH5	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.
AH6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	T	.	.	.	.
AH7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.
AH8	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.
AH9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.
AH10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	C	.
AH11	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AH12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
AH14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
AH16	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
AH17	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	A	.	.	.	.	T	.	.	.	.
AH18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	.
AH20	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
AH21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
AH23	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
AH25	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	C
AH26	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
AH27	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.

b	Base position																										
Haplotype	013	021	038	121	137	190	200	264	269	282	324	345	360	363	381	439	536	673	679	682	712	740	818	830	837	858
AD1	A	A	A	T	-	-	G	-	-	A	A	G	G	G	G	A	C	C	G	A	T	G	G	T	T	T
AD2	.	.	.	C	-	-	G	-	T	.	-	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.
AD3	C	T	.	.	T	-	G	-	-	.	-	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.
AD4	.	.	.	.	-	-	G	-	-	.	A	.	.	.	.	.	.	.	.	.	.	A	A	G	C	G
AD5	.	.	.	.	T	-	G	-	-	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AD6	.	.	.	C	-	T	-	T	-	G	-	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.
AD8	.	.	.	C	-	-	G	-	T	.	-	.	.	A	.	G	T	.	A	.	.	.	.	.	.	.
AD9	.	.	.	.	-	-	G	-	-	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
AD10	.	.	-	C	-	T	-	T	-	G	-	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.
AD11	.	.	.	.	T	-	G	-	-	.	A	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.

c	Base Position			
Haplotype	276	317	506	532
A01	T	C	C	T
A02	C	T	.	C
A04	C	T	T	C

Table 4.2: Haplotype distribution by population, available sample sizes and number of haplotypes for a) *Arion hortensis*, b) *A. distinctus* and c) *A. owenii*.

a)

Population / Haplotype	Blackpool	Long Ashton	Cardiff	Lissington	Glyn Ceiriog	Gamlingay	Mold	Hull	N
AH1	1	3	4	3	1	1	1		14
AH2					8				8
AH3			1	8					9
AH4								6	6
AH5					4				4
AH6	1							1	4
AH7	1								1
AH8					1				1
AH9			1						1
AH10		1							1
AH11		2							2
AH12	3	2		1					6
AH14			1						1
AH16				1					1
AH17				1					1
AH18	3								3
AH20								1	1
AH21		1							1
AH23		1							1
AH25				1					1
AH26				1					1
AH27						1			1
N	**9**	**10**	**7**	**16**	**14**	**1**	**4**	**8**	**69**
Haplotypes	**5**	**6**	**4**	**7**	**4**	**1**	**3**	**3**	

b)

Population / Haplotype	Cardiff	Long Ashton	Cowbridge	Glyn Ceiriog	Gamlingay	Lissington	Mold	N
AD1	4	6	1	5	2	5		23
AD2				2		1	1	4
AD3				1				1
AD4			1					1
AD5	5		1					6
AD6						1	1	2
AD8							1	1
AD9							1	1
AD10			1					1
AD11			1					1
N	**9**	**6**	**5**	**8**	**2**	**7**	**4**	**41**
Haplotypes	**2**	**1**	**5**	**3**	**1**	**3**	**4**	

c)

Population / Haplotype	Bodmin	Glyn Ceiriog	Gamlingay	N
AO1	5			5
AO2	1	1	1	3
AO4	1			1
N	**7**	**1**	**1**	**9**
Haplotypes	**3**	**1**	**1**	

Table 4.3: Haplotype and nucleotide diversities for all populations ± standard deviations for a) *Arion hortensis*, b) *A. distinctus*, c) *A. owenii*. Estimates are based on pairwise differences.

a Population	Haplotype Diversity	Nucleotide Diversity
Blackpool	0.8333 ± 0.0980	0.0013 ± 0.0011
Long Ashton	0.8889 ± 0.0754	0.0018 ± 0.0013
Cardiff	0.7143 ± 0.1809	0.0016 ± 0.0012
Lissington	0.7417 ± 0.1053	0.0024 ± 0.0016
Glyn Ceiriog	0.6264 ± 0.1098	0.0011 ± 0.0008
Gamlingay	n/a	n/a
Mold	0.8333 ± 0.2224	0.0018 ± 0.0016
Hull	0.4643 ± 0.2000	0.0030 ± 0.0020
Overall	**0.9143 ± 0.0167**	**0.0034 ± 0.0020**

b Population	Haplotype Diversity	Nucleotide Diversity
Cardiff	0.5556 ± 0.0902	0.0006 ± 0.0007
Long Ashton	0.0000 ± 0.0000	0.0000 ± 0.0000
Cowbridge	1.0000 ± 0.1265	0.0071 ± 0.0047
Glyn Ceiriog	0.6071 ± 0.1640	0.0038 ± 0.0024
Gamlingay	0.0000 ± 0.0000	0.0000 ± 0.0000
Lissington	0.5238 ± 0.2086	0.0037 ± 0.0024
Mold	1.0000 ± 0.1768	0.0068 ± 0.0049
Overall	**0.6646 ± 0.0756**	**0.0033 ± 0.0019**

c Population	Haplotype Diversity	Nucleotide Diversity
Bodmin	0.5238 ± 0.2086	0.0019 ± 0.0014
Glyn Ceiriog	n/a	n/a
Gamlingay	n/a	n/a
Overall	**0.6389 ± 0.1258**	**0.0021 ± 0.0015**

Table 4.4: AMOVA based on pairwise difference between haplotypes for a) *Arion hortensis* and b) *Arion distinctus.* Locations are partitioned by north-south and east-west.

a		
		% Variance
(E/W)	**Among regions**	-6.17
	Among populations within regions	54.48
	Within populations	51.68
	Fst	0.4832
	Nm	0.53
(N/S)	**Among regions**	0.23
	Among populations within regions	49.54
	Within populations	50.23
	Fst	0.4977
	Nm	0.50

b		
		% Variance
(E/W)	**Among regions**	-10.65
	Among populations within regions	15.98
	Within populations	94.67
	Fst	0.0533
	Nm	8.88
(N/S)	**Among regions**	13.43
	Among populations within regions	2.35
	Within populations	84.22
	Fst	0.1578
	Nm	2.67

Table 4.5: Pairwise Fst comparisons (below diagonal) and Nm (above diagonal) between populations of a) *A. hortensis*, b) *A. distinctus*, c) *A. owenii*. Significance values: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant.

a	Blackpool	Long Ashton	Cardiff	Lissington	Glyn Ceiriog	Gamlingay	Mold	Hull
Blackpool	-	22.44	0.94	1.24	0.32	1.83	3.35	0.27
Long Ashton	0.2081 **	-	22.44	3.82	0.31	-1.23	3.82	0.27
Cardiff	0.3461 ***	0.021 ns	-	12.75	0.25	-1.00	1.62	0.25
Lissington	0.2873 ***	0.1158 *	0.0377 ns	-	0.47	0.45	2.23	0.30
Glyn Ceiriog	0.6130 ***	0.6152 ***	0.6636 ***	0.5163 ***	-	0.24	0.54	0.21
Gamlingay	0.2142 ns	-0.6888 ns	-1.0000 ns	-0.5246 ***	0.6783 ***	-	4.00	0.43
Mold	0.1297 ns	0.1157 ns	0.2362 *	0.1832 *	0.4795 ns	-0.1111 ns	-	0.42
Hull	0.6531 ***	0.6481 ***	0.6664 ***	0.6287 ***	0.7048 ***	0.5379 ns	0.5412 **	-

b	Cardiff	Long Ashton	Cowbridge	Glyn Ceiriog	Gamlingay	Lissington	Mold
Cardiff	-	0.65	5.73	2.35	6.26	1.90	0.42
Long Ashton	0.4331 **	-	6.60	5.97	nc	9.5	0.56
Cowbridge	0.0803 ns	0.0704 *	-	85.71	-2.43	-8.20	6.17
Glyn Ceiriog	0.1755 **	0.0773 ns	0.0058 ns	-	-3.14	-6.92	4.67
Gamlingay	0.2421 ns	0.0000 ns	-0.2595 ns	-0.1897 ns	-	-2.64	2.82
Lissington	0.2081 **	0.0500 ns	-0.0649 ns	-0.0779 ns	-0.2338 ns	-	9.96
Mold	0.5446 ***	0.4728 **	0.0750 ns	0.0967 ns	0.1508 ns	0.0478 ns	-

c	Bodmin	Glyn Ceiriog	Gamlingay
Bodmin	-	1.5	1.5
Glyn Ceiriog	0.2500 ***	-	nc
Gamlingay	0.2500 ***	0.0000 ns	-

Table 4.6: Great circle distances (km) between two points calculated from latitude and longitude coordinates using the programme Earth (Byers, 1999).

a	Blackpool	Long Ashton	Cardiff	Lissington	Glyn Ceiriog	Gamlingay	Mold	Hull	Cowbridge
Blackpool	-								
Long Ashton	267	-							
Cardiff	259	38	-						
Lissington	186	252	283	-					
Glyn Ceiriog	99	172	161	195	-				
Gamlingay	265	189	220	131	220	-			
Mold	73	196	186	188	25	228	-		
Hull	172	309	323	57	210	189	196	-	
Cowbridge	267	53	17	299	169	237	194	338	-

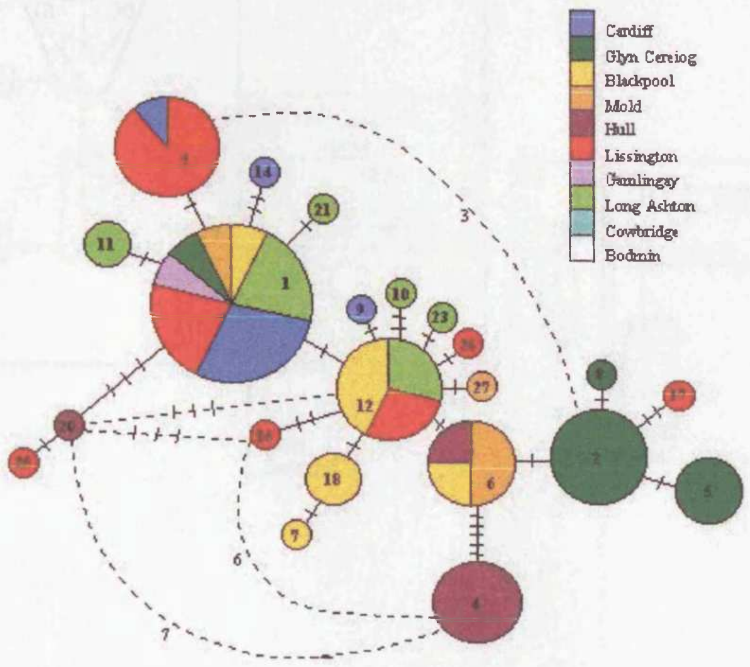
4.9 Figures

Figure 4.1

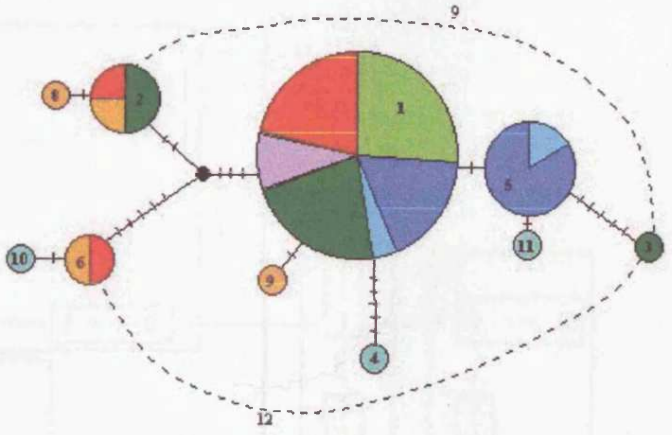
	Location	Grid reference (Latitude/Longitude)	*A. hortensis*	*A. distinctus*	*A. owenii*
1	Blackpool	53 49 14 N 3 2 29 W	9	-	-
2	Hull	53 51 1 N 0 24 46 W	8	-	-
3	Lissington	53 20 10 N 0 20 16 W	16	7	-
4	Gamlingay	52 9 25 N 0 11 46 W	1	2	1
5	Mold	53 9 48 N 3 8 47 W	4	4	-
6	Glyn Ceiriog	52 56 17 N 3 11 7 W	14	8	1
7	Cowbridge	51 25 32 N 3 26 2 W	-	5	-
8	Cardiff	51 29 18 N 3 13 3 W	7	9	-
9	Long Ashton	51 25 33 N 2 40 17 W	10	6	-
10	Bodmin	50 26 39 N 4 42 16 W	-	-	7

Figure 4.2

a)



b)



c)

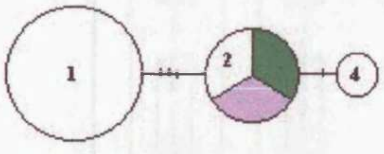
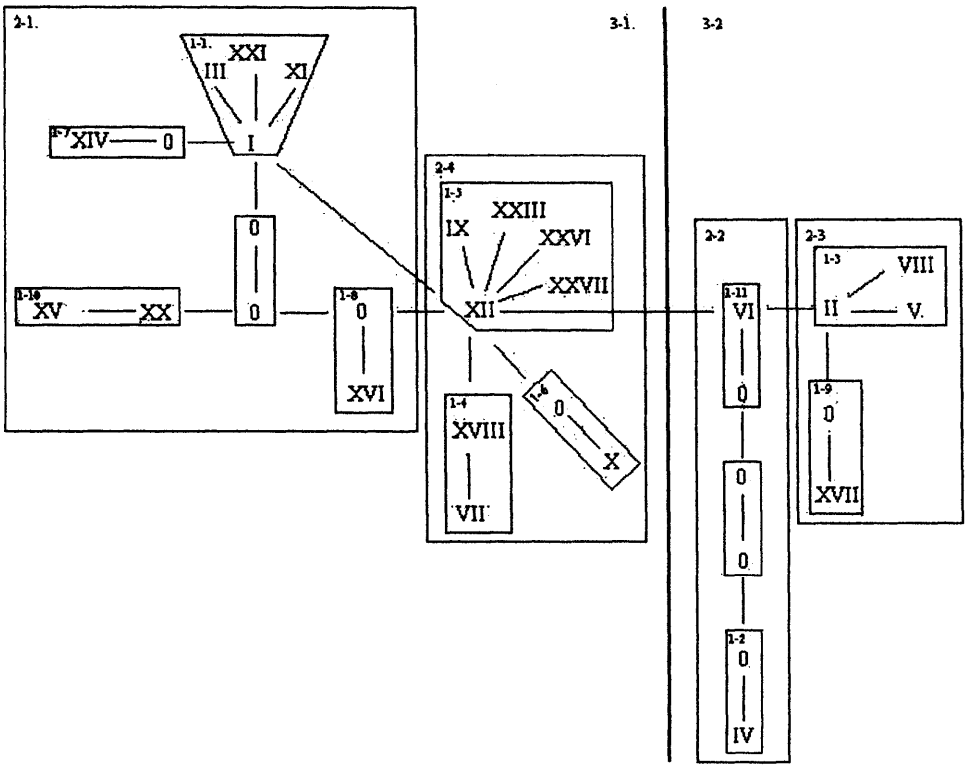
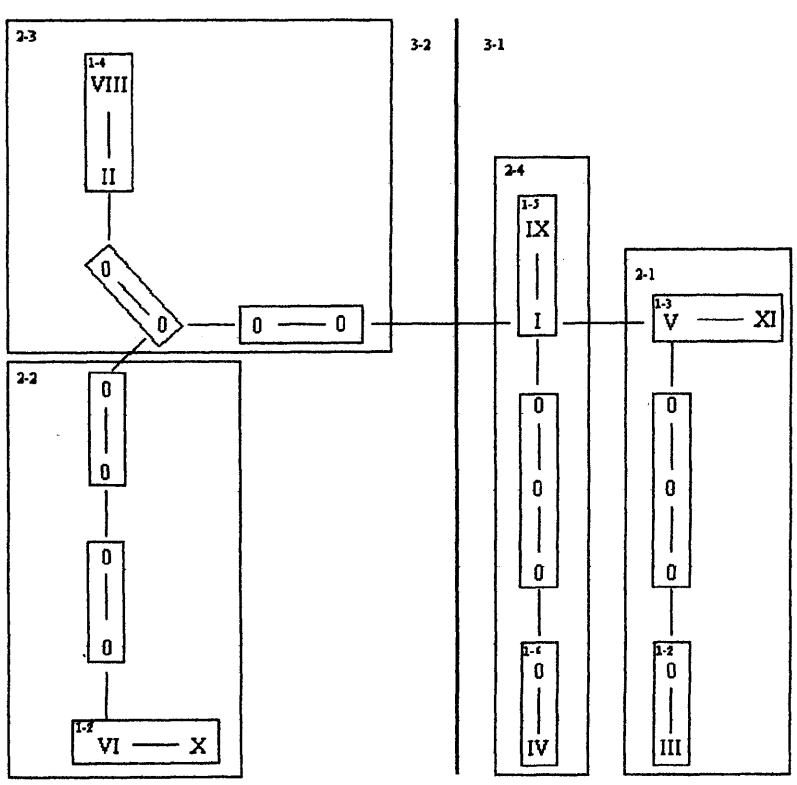


Figure 4.3

a)


2-1.
3-1.
3-2
1-1.
III
XXI
XI
I
XIV
0
2-4
1-3
IX
XXIII
XXVI
XXVII
XII
0
0
1-10
XV
XX
1-8
0
XVI
1-4
XVIII
VII
1-6
0
X
2-2
1-11
VI
0
0
0
1-2
0
IV
2-3
1-3
VIII
II
V.
1-9
0
XVII

b)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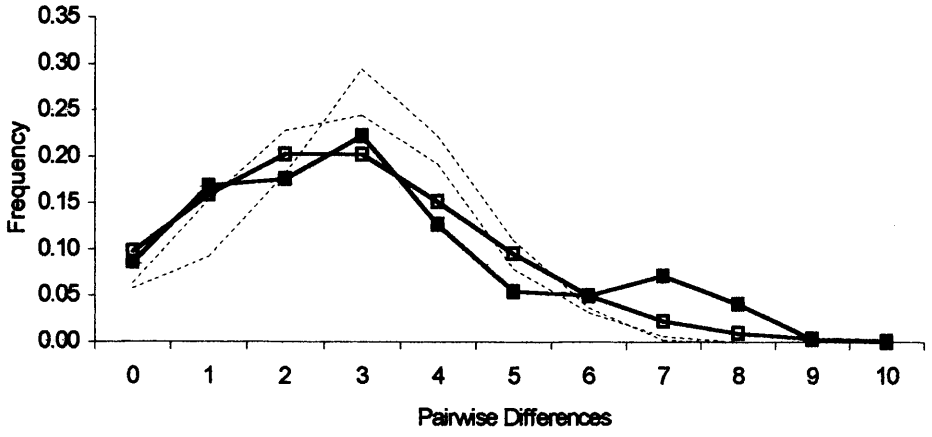
3-2
3-1
1-4
VIII
II
0
0
0
0
2-4
1-5
IX
I
0
0
0
1-1
0
IV
2-1
1-3
V
XI
0
0
0
1-2
0
III
2-2
0
0
0
0
1-2
VI
X

Figure 4.4

a)



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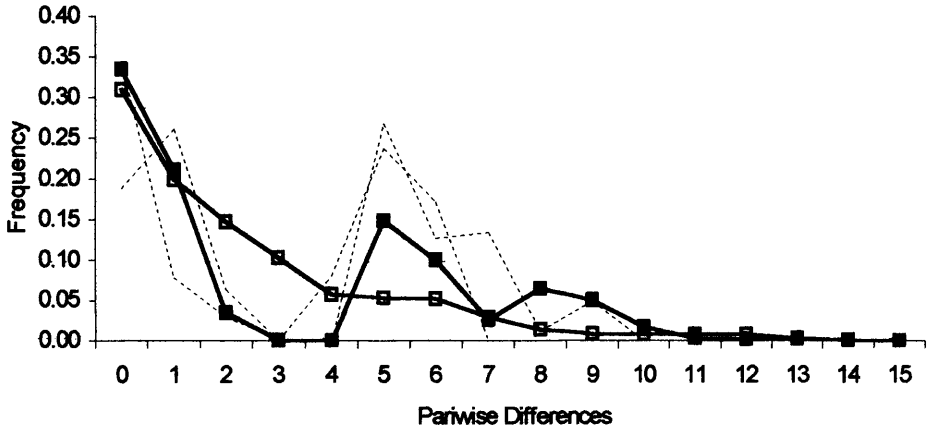
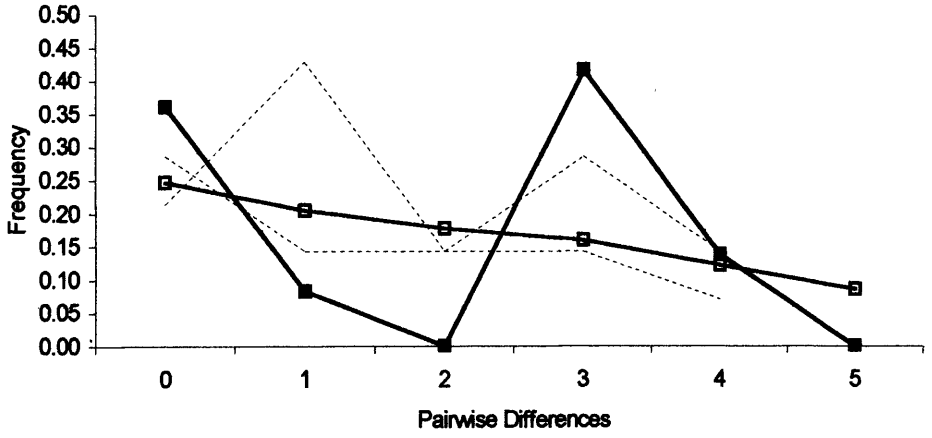


c)



Chapter 5

Modified version to be submitted for publication in Molecular Ecology

(format according to journal specifications)

Comparative sensitivity of biochemical and molecular approaches for predator gut content analysis in laboratory based feeding trials.

Ciara S Dodd, Rhian S Thomas, Michael W Bruford, David M Glen* and William OC Symondson.

Cardiff School of Biosciences, Main Building, Park Place, Cardiff University, Cardiff, CF10 3TL, UK

* Styloma Research Ltd., Phoebe, The Lippiatt, Cheddar, BS27 3QP, UK

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Corresponding Author: Ciara S Dodd
Cardiff School of Biosciences,
Main Building,
Park Place,
Cardiff University,
Cardiff,
CF10 3TL,
UK

Tel: 029 20875776
Fax: 029 20874305

DoddCS@cf.ac.uk

Running Title: Techniques for detection of predation on slugs

5.1 Abstract

Carabid beetles are important predators of slugs in arable crops and can significantly reduce the size and distribution of pest populations in the field. Conventionally, predator gut samples are analysed using prey specific monoclonal antibodies (MAbs) and ELISA. A recent alternative approach is to amplify the DNA of the target prey from within the predator's gut with specific primers and the polymerase chain reaction (PCR). In this study, the beetle *Pterostichus melanarius* (Illiger) was used to investigate predation on the slugs *Deroceras reticulatum* Müller and *Arion hortensis* Férrusac using molecular techniques. Robust species- and genus-specific primers were designed that amplified small fragments of slug mitochondrial DNA. The results of cross reactivity tests confirmed primer specificity. Timed laboratory-based DNA decay rate experiments show that slug remains can be detected following ingestion by the beetle for up to 49 hours following ingestion. The sensitivity of established MAb ELISA and the DNA-based method are compared. These results are discussed in the context of future developments of predator gut content analysis.

5.2 Introduction

Recent advances in techniques for predator gut content analysis have demonstrated that the use of DNA-based methods are becoming a viable alternative to MAb ELISA, with potentially a degree of sensitivity equivalent to immunological based approaches (Symondson, 2002a, b). Therefore, as predation on a target species can now be detected for at least a 24-hour period, the system is essentially suitable for analysis of predation in the field.

Until now, the use of prey-specific monoclonal antibodies (MAbs), coupled with enzyme linked immunosorbent assay (ELISA), has been the method of choice for predator gut content analysis (Symondson, 2002a, b). Early DNA based studies proved that only limited detection was possible if single copy nuclear DNA was targeted (Agusti *et al.*, 1999, 2000), and with these, the greatest success was achieved by targeting fragments smaller than 300bp in length, although with these shorter fragments the maximum detection period was still only 4 hours. Targeting amplified esterase genes (Zaidi *et al.*, 1999) or multiple copy nuclear genes such as ITS ribosomal gene clusters (Hoogendoorn & Heimpel, 2001) has resulted in much improved detection times of 28 hours and 10 hours, respectively, and again has confirmed that shorter fragments can be detected for longer periods of time. However, it has become clear that targeting mitochondrial (mt) genes (Chen *et al.*, 2000; Agusti & Symondson, 2001; Agusti *et al.*, 2003), which have a naturally high copy number within cells (>300), gives the most promising results. To date the longest reported detection period has been achieved using specific primers designed to target short fragments of the mitochondrial COI gene (Agusti & Symondson, 2001), with which prey DNA could be detected within the gut of the predator 32 hours following ingestion. From these results, it seems likely that mtDNA is an ideal candidate for the molecular detection of predation when short fragments of DNA are amplified using prey specific primers and polymerase chain reaction (PCR).

In this study, we used two model systems employing common prey and predator species within UK arable crops, to determine the comparative sensitivity of immunological (MAb ELISA) and molecular (DNA) approaches. The rate of decay of slug (*Deroceras reticulatum* Müller and *Arion hortensis* Férrusac) protein and mtDNA were determined following ingestion by a generalist carabid predator (*Pterostichus melanarius* (Illiger)). Immunological studies have shown that slug material is often found in the guts of *P. melanarius* collected from arable fields (Symondson *et al.*, 1996; Bohan *et al.*, 2000).

In Britain and Europe, *D. reticulatum* is usually considered to be the most abundant and damaging species, and is the commonest slug species on arable land (South, 1992), causing millions of pounds worth of damage to arable crops annually. This species is a particular problem because of its method of foraging close to the surface of the soil near germinating seeds (Martin & Kelly, 1986). Providing the conditions are suitable, it is able to feed and reproduce all year round (Hunter & Symonds, 1971) leading to high population densities, which in arable crops can exceed 200 per m^2 (Glen *et al.*, 1989). As *D. reticulatum* is usually sympatric with one or more species of Arionidae in arable fields (Glen & Wiltshire, 1988), the presence of both may lead to significant economic losses. In total, twelve species of *Arion* occur in the UK, most of which are able to attain pest status in arable crops (Symondson *et al.*, 1999). Members of the *A. hortensis* aggregate can become serious agricultural pests under the right conditions. The aggregate comprises three species, but the pest status of each is difficult to determine, since prior to the 1970s they were considered one species and, as separate species, remain under-recorded (Kerney, 1999). *A. hortensis* and *Arion distinctus* are relatively common and often co-occur, whereas *Arion owenii* Davies is generally considered to be rarer, and due to its restricted distribution will not be considered further here. Members of this aggregate have been introduced to many parts of the world, are widely spread by human activity (Kerney, 1999) and have been reported as pests of a wide range of surface and root crops (South, 1992). In laboratory experiments, member species of the *A. hortensis* aggregate were more likely to damage seeds than seedlings, whereas other species such as *D. reticulatum*, *Arion ater* (Linneus) and *Arion fasciatus* (Nilsson) caused equal

damage to both (Duthoit, 1964). In crops such as oil seed rape, other arionids including *Arion lusitanicus* Mabille can become pests, where they have been shown to forage at night from adjacent wild flower strips (Frank, 1998a, b), although *A. lusitanicus* is not considered one of the major pest species in Great Britain, unlike in continental Europe. However, this species can still form a part of the diet of *P. melanarius*, predation by which has been demonstrated on this species (Paill *et al.*, 2002) and its eggs (Symondson *pers. comms.*).

Traditionally slug control has been achieved by the use of molluscicides based on the carbamate methiocarb and the acetaldehyde metaldehyde (Port & Port, 1986), the estimated annual use of which is 4800 tonnes (£10million) in Great Britain alone (Garthwaite & Thomas, 1996), with 99% of this usage occurring in arable crops. However, methiocarb is toxic to carabids (Kennedy, 1990) and can severely reduce the populations of these potentially beneficial non-target predatory arthropods, and both compounds have undesirable side-effects on non-target wildlife. Therefore, there is an increasing need to investigate alternative ecological approaches to controlling prevalent agricultural pests such as slugs.

Carabid beetles are important predators of slugs and have been shown to play a significant role in controlling these pests. In particular, the distributions of *P. melanarius* and slugs are dynamically associated, with beetles aggregating to areas of high slug biomass. Bohan *et al.* (2000) found that in June beetles and slugs formed positive aggregations, but by July, there was a reduction in the rate of slug population growth in areas of high beetle density, which remained at a constantly high level. The distribution of beetles testing positive for slug protein using MAb-ELISA was significantly associated with the distribution of large slugs in June, but in July, few large slugs were found where there were many slug positive beetles (Bohan *et al.*, 2000). Growth of the slug population declined as the numbers of beetles increased, suggesting that predation was not opportunistic, but direct and dynamic and that predation elicited changes in the spatial distribution and local density of the slugs.

Symondson *et al.* (2002) also presented evidence for a dynamic interaction between *P. melanarius* and its slug prey. Over a five-year period, a strong relationship between the crop mass of beetles and slug numbers in the soil was demonstrated, indicating that the slugs constituted a major part of the beetles' diet. The change in the beetle population each year was strongly related to slug numbers in the soil during the previous year and to the crop mass of the beetles suggesting that the nutritional status and reproductive success of the beetles was influenced by the slug population.

In order to investigate such trophic interactions, it is necessary to develop sensitive assays that will allow the determination of prey choice by a predator post-mortem. Currently the assay method of choice for predator gut content analysis is MAb-ELISA, but more recently DNA-based approaches are also being explored. In this paper, we report the first fully replicated comparative study to assess the sensitivity of protein- and DNA-based approaches for predator gut content analysis using timed laboratory-feeding trials. In order to test the hypothesis that the sensitivity of MAbs and DNA are equivalent, the rates of decay of slug protein and DNA following ingestion by the predator were determined using gut extracts from the same individuals. A series of mtDNA PCR primers were developed for *D. reticulatum* and *A. hortensis* and amplification success was compared directly with protein concentration obtained using a mollusc-specific MAb and ELISA.

5.3 Materials and methods

5.3.1 DNA samples, extraction, amplification and sequencing

Beetles (*P. melanarius*) and slugs of agricultural importance (*D. reticulatum, A. hortensis, A. distinctus,* and *Arion intermedius* Normand) were collected from an established field site at Long Ashton, Bristol, UK (Kendall *et al.*, 1995; Symondson *et al.*, 1996, 2002) for DNA extraction and sequencing. Additional specimens were also collected from sites in Lincolnshire and Wales so that genetic variation in these species could be assessed before primers were designed. Total genomic DNA was isolated from the head and tentacles of slugs and from thoracic muscle tissue of beetles, using a high salt extraction method modified by Collins *et al.* (1987) from Livak (1984). The third domain of the mitochondrial 12S rRNA gene (400bp) was amplified by PCR using the general invertebrate primers SRN14588 and SRJ14233 (Simon *et al.*, 1994) in a Perkin-Elmer 9700 Automated Thermocycler. These primers were also used for sequencing.

Amplification of the 12S rRNA gene sequence was carried out in a 25 µl reaction containing approximately 200 ng total genomic DNA, 1X PCR reaction buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl (InvitrogenGibco, Life Technologies®), 4 mM $MgCl_2$, 0.025 mM each dNTP, 0.5 µM each primer and 0.625 units of Taq (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 45 s, 72 °C for 1 min 15 s, then a final extension step of 72 °C for 10 min.

PCR products were purified for sequencing using the GeneClean Turbo for PCR Kit (Bio101) following manufacturers instructions. Each sample was sequenced in both forward and reverse directions using a ABI Prism 377 semi-automated DNA Analyser using the Prism Big Dye Terminator v2 Ready Reaction Kit (Perkin Elmer Applied Biosystems). The sequencing reaction contained 2 µl DNA, 1 µl of either forward or reverse primer (1.6 pmol/µl), 1 µl Big Dye and 1 µl sterile water (Sigma-Aldrich). The

thermocycling reaction consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min.

GenBank Accession numbers for the 12S rRNA sequences are for *D. reticulatum* (AY423668), *A. distinctus* (AY423654-63), *A. hortensis* (AY423632-53), *A. owenii* (AY423664-66), *A. intermedius* (AY423667), and *P. melanarius* (AY423669).

5.3.2 Sequence alignment and primer design

Following a species-specific secondary structure analysis for each ribosomal gene (see Dodd *et al.*, this thesis (Chapter 3)), predator (beetle) and prey (slug) sequences were aligned according to a secondary structure template (Hickson *et al.*, 1996). Potential primer sites were first identified by eye in regions where the predator and prey sequences differed. Primer pairs were then tested for primer-primer binding and secondary structure formation using Omiga v2 (Accelerys Inc.). Four primers were designed for *D. reticulatum* (one forward and three reverse), which in combination gave rise to three primer pairs (A, C, E) and one primer pair for *A. hortensis* (F), as well as one pair specific for the genus *Arion* (I) (Table 5.1). Large DNA fragments have been found to digest more quickly than small ones in predator guts (Agusti *et al.*, 1999; 2000; Zaidi *et al.*, 1999; Hoogendoorn & Heimpel, 2001), therefore primer pairs were chosen that generated small amplicons ranging in size from 99-294 bp. The forward primer of pairs A, C and E was fluoro-labelled with HEX and the forward primer of pairs F and I was fluoro-labelled with FAM. The PCR products were separated on a 6 % acrylamide gel, run on the ABI Prism 377 semi-automated DNA analyser. PCR products were multi-loaded on the gel so that two or three amplicons were run in each lane, together with TAMRA 350 size standard (Applied Biosystems). Each gel was run with a positive and negative control and results were analysed using Genescan Analysis and Genotyper v2 (Applied Biosystems). Samples were considered positive if the electropherogram showed a peak of the target size amplifying with ≥100 fluorescence units.

The three *Deroceras* primers pairs were used to test if there was any difference in amplification success of a range of fragment sizes, below 300 bp, with increasing time since ingestion of the target DNA.

5.3.3 PCR amplification with slug primers and cross-reactivity testing

For each primer pair, the target species and a negative control were amplified in a 10 µl reaction volume containing approximately 200 ng total genomic DNA, 1X buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl (InvitrogenGibco, Life Technologies®)), 2 mM $MgCl_2$, 0.025 mM each dNTP, 1.0 µM each primer and 0.04 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 44-56 °C for 45 s, 72 °C for 1min, then a final extension step of 72 °C for 10 min.

Once optimised for the target species, all primer pairs were tested for cross-reaction against at least three individuals of each of the predator, other related species and potential prey species (Table 5.2). A PCR was considered positive by the presence of a band of the target size on the gel. For the general *Arion* primers (I), size differences in amplicons from different species of *Arion* were determined.

5.3.4 Laboratory-based feeding studies

A timed DNA decay rate experiment was conducted for *D. reticulatum* and for *A. hortensis*, the structures of which are outlined in Figures 3.2a and b, respectively. Single *P. melanarius* were placed in individual petri dishes (2 cm x 5 cm) containing moistened filter paper. The beetles were each fed a single blow fly larva (*Calliphora* sp.) and allowed to feed *ad lib* for 24 hours, after which time any beetles that had not fed were removed from the experiment. Approximately 400 beetles were selected for the *D. reticulatum* and 200 for the *A. hortensis* experiment. The beetles were then denied further prey and maintained in a controlled temperature room at 16 °C under a L16h:D8h regime for 14 days. Ten of these beetles (five males and five females) were killed and frozen as

negative controls. The remaining beetles were then transferred to clean petri dishes containing moist filter paper and allowed to feed on an immobilized whole wounded *D. reticulatum* or *A. hortensis* for two hours. Only beetles that had been observed feeding during that time were retained. The selected beetles were then divided into two groups each containing an equal number of males and females. The beetles in Group I (refed) were each provided with half a house fly larva (*Musca domestica* Linneus) every 24 hours, to mimic the availability of alternative prey that would be accessible in the field; beetles in Group II (not refed) were not provided with any alternative prey. Samples of 10 beetles from each group (five males and five females) were killed and frozen at 0, 2, 4, 8, 12, 18, 24, 36, and 48 hours after removal from the slug meals. As the beetles could have ingested the slug meals at any point during the two-hour feeding period, the mid-point (after 1 hour) was considered as the time of consumption. Therefore, one hour has been added to each time interval in the subsequent analyses (Harwood *et al.*, 2001). The *Arion* decay rate experiment was carried out as described above, except that only female beetles were used as insufficient male beetles were caught for them to be used in the experiment, all beetles were provided with alternative prey, five female beetles were killed at each time period and the experiment was conducted for 72 hours. Timed decay rate experiments for *A. distinctus* or *A. intermedius* were not conducted, although primers were developed for these species as it was not possible to collect enough slugs for such an experiment.

5.3.5 Extraction of predator gut contents

Beetles were thawed at room temperature and the foregut was removed as described in (Symondson *et al.*, 2000). The foregut was weighed and homogenized in a 1:19 w:v ratio with 1X phosphate buffered saline (PBS). The homogenates were centrifuged at 8,000 rpm for 15 min at room temperature. The majority of the supernatant was transferred to a clean 1.5 ml Eppendorf tube and stored at –20 °C for subsequent analysis using MAb ELISA. The particulate remains and 80 µl of the supernatant were retained for DNA extraction using the QIAamp® DNA Mini Kit (Qiagen Ltd.) in accordance with manufacture's instructions (section 2.1.2.2). Extracted DNA was stored at –20 °C.

5.3.6 DNA analysis

The DNA extractions from the *Deroceras* decay rate experiment were screened by PCR with the specific *D.reticulatum* primers (A, C, E) and from the *Arion* decay rate experiment with the *A. hortensis* (F) and general *Arion* (I) primers using the reaction conditions described above (Section 3.2.2.3).

5.3.7 ELISA

The samples were screened by indirect ELISA on a Pro-Bind™ micro-titre plate (Beckton Dickinson Labware, USA) at room temperature using the general anti-mollusc monoclonal antibody (MAb), AiW-1C9 (RS Thomas, A McKemmy, DM Glen & WOC Symondson, unpublished). This antibody was used at a concentration of 1:250 diluted in PBS-Tween. Goat antimouse IgM (μ-chain specific) horseradish peroxidase enzyme conjugate (Sigma-Aldrich) was diluted 1:2,000 for use with AiW-1C9 with PBS-Tween. Each ELISA plate included a X1.5 dilution series of *D. reticulatum* standards, stabilized with heterologous protein also diluted 1:20,000 (w/v) in 1X PBS, that provided slug protein concentrations between 315 and 9 ng/200 μl. Heterologous protein consisted of starved beetle gut diluted to a concentration of 1:20,000 (w/v). Slug protein concentrations were calculated following a protein assay using the BioRad Protein Assay System (Bio-Rad Laboratories GmbH, Munich, Germany). The significance level for this antibody was 3.2 ng/200 μl, based on the highest cross-reaction, ± 2.5 standard deviations, with a non-target species, which in this case was the earthworm *Lumbricus casteneus* Linneus.

5.3.8 Statistical analysis

Linear regression analysis was used to determine the rates of decay of the different amplicons. Rate of DNA decay was determined using percent PCR success and mean intensity of fluorescence. The sensitivity of DNA and ELISA results were compared using mean intensity of fluorescence and protein concentration equivalents, both of which are quantitative measures of foregut DNA and protein content respectively. Analysis of covariance (ANCOVA) was used to ascertain the relationship between the decay rates

within each experiment according to i) beetle sex, ii) provision of alternative prey and iii) amplicon size (i & ii refer to the *Deroceras* experiment only). Detection half-life was calculated for each DNA amplicon and MAb according to the regression equation, $y = mx+c$, which is rearranged so that $x = y-c/m$, where $y = 50\%$, half the initial mean intensity of fluorescence or half the initial protein concentration. For MAb AiW-1C9, the time at which all protein was considered to have left the system and the significance level were calculated by rearranging the equation as above. The interaction between beetle foregut weight with beetle sex and provision of alternative prey was examined using two-way ANOVA.

5.4 Results

5.4.1 Primer specificity

Sequences, optimal annealing temperatures and fragment sizes for the specific primers are shown in Table 5.1. The results of the cross-reactivity tests (Table 5.2) showed that two pairs of primers (A & C) designed for *D. reticulatum* were specific for *D. reticulatum* with one pair (E) also amplifying a congener, *D. panormitanum* (Lessonna & Pollonera). Primers designed for *A. hortensis* (F) were specific and did not co-amplify other closely related species and the Genus level primer pair (I) amplified all members of the genus *Arion* that were tested, and these different species could be potentially identified by amplicon size (Table 5.2). None of the primers amplified the predator or alternative prey.

5.4.2 Rate of decay of slug DNA within the gut of Pterostichus melanarius

5.4.2.1 Deroceras reticulatum

Using the primer pairs A, C and E, we were able to detect *D. reticulatum* DNA from within the guts of carabid predators. The percentage PCR success and mean intensity of fluorescence (MFU) for each amplicon are summarised in Table 5.3. It was possible to detect slug remains in 10 % of the beetles for all amplicons at 37 hours following ingestion when the beetles were provided with alternative prey. However, when no alternative prey was provided, slug DNA could only be detected in one amplicon (C) at this time period. When the data for male and female beetles were combined, slug remains could be detected in 5 % of beetles for amplicons A and E and in 20 % for amplicon C. Slug DNA could only be detected in beetle guts for 49 hours following ingestion in one amplicon (C). For this amplicon, using the primers (DR11F + DR16MR), it was possible to detect slug remains in 0 % of beetles provided with alternative prey, in 20 % that were not provided with alternative prey and in 10 % when data for the two groups were combined.

The rate of decay of slug DNA was determined using percent PCR success against time. When the rate of decay of slug DNA was compared in male and female beetles for each amplicon, there was no significant difference between the slopes or the y-axis intercepts within Groups I (alternative prey) and II (no alternative prey) respectively (Table 5.4a). Therefore, for subsequent analyses, the data for male and female beetles were combined. When the rates of decay of each amplicon were compared between Group I and II using ANCOVA, no significant difference was found between the slopes or y-axis intercepts with either group for each amplicon (Table 5.4b). In order to obtain an overall decay rate for each amplicon, and to enable the decay rates of the different amplicons to be compared, the data for Group I and Group II were combined to provide a single regression for each amplicon (Figure 5.3a-c). In all analyses, the slope of each regression line was significantly different from zero at $P<0.001$. When these were compared using the combined data in a single ANCOVA, no significant difference was found between the rates at which the slopes declined ($F_{2,21} = 0.17$; $P>0.05$) or the y-axis intercepts ($F_{2,21} = 2.19$; $P>0.05$). The ability to detect slug DNA within the gut of the predator decreased in a linear manner with time since ingestion (Figure 5.3a-c).

Mean intensity of fluorescence decreased with time since ingestion of the target DNA (Figure 5.3d-f), but this was not a linear relationship, as $\log_{10}$ transformation of MFU provided the best R^2 value against time. When the rates of decay of the three fragments were compared, no significant difference was found between the slopes ($F_{2,21} = 0.09$; $P>0.05$), but the y-axis intercepts ($F_{2,21} = 4.01$; $P<0.05$) were significantly different. This was probably because the greatest MFU for amplicons A and E was not obtained immediately following ingestion of prey, but after 3 hours. However, for amplicon C the greatest mean intensity was seen immediately following ingestion of prey. This was confirmed by pairwise comparisons using ANCOVA which showed that the y-axis intercept of amplicon C was significantly different from that of amplicons A ($F_{1,14} = 7.08$; $P<0.05$) and E ($F_{1,14} = 4.73$; $P<0.05$), but verified that the slopes were not significantly different ($F_{1,14} = 0.02$; $P>0.05$ and $F_{1,14} = 0.08$; $P>0.05$, respectively). There was no significant difference between the slopes ($F_{1,14} = 0.15$; $P>0.05$), or the y-axis intercepts

($F_{1,14}$ = 0.44; P>0.05) of amplicons A and E. As no difference was found between the slopes of any of the amplicons, their rates of decay were equivalent. A similar initial increase in detection has been shown in MAbs (Symondson *et al.*, 2000; Harwood *et al.*, 2001), which is explained by digestion revealing internal binding sites within target molecules. However, this is unlikely to be the case with DNA, as digestion should have the opposite effect and reduce the number of available targets due to degradative processes.

The detection half-life of amplicons A, C and E was calculated using the regression equation for each fragment, for percent PCR success (Figure 5.3a-c) and mean intensity of florescence (Figure 5.3d-f). Detection half-lives ranged between 19.18 and 29.01 hours (Table 5.3).

The foregut weights of the two sexes fed *ad lib* on slugs were significantly greater for females compared with males within Group I (mean ± SE males 0.0168 ± 0.0020 g; females 0.0321 ± 0.0032 g; P<0.001) and Group II (mean ± SE males 0.0164 ± 0.0023 g; females 0.0248 ± 0.0026 g; P<0.001), indicating that male and female beetles did not consume equivalent amounts of slug and/or alternative prey. Foregut weights of male beetles were not significantly different between groups (P>0.05), but the foregut weights of female beetles provided with alternative prey were significantly heavier than females that received no alternative prey (P<0.01). In all comparisons, beetle foregut weights decreased significantly with time (P<0.001). There was no interaction between beetle sex and time (P>0.05) or the provision of alternative prey with time (P>0.05).

5.4.2.2 Arion hortensis

Slug DNA was detected in 80 % and 60 % of the beetles for amplicons F & I respectively, at 37 hours following ingestion, and in 20 % of the beetles at 49 hours following ingestion. Prey remains could not be detected in predators 73 hours following ingestion with either set of primers.

In all analyses, the slope of each regression line was significantly different from zero at $P<0.001$. The best R^2 values were obtained when percent PCR success and MFU were square root transformed and plotted against time (Figure 5.4 a-d). Therefore, the rate of decay of *Arion* DNA was not linear. When percent PCR success was used to compare the rate of decay of amplicons F and I, no significant difference was found between the slope declines ($F_{1,16} = 0.36$; $P>0.05$) or the y-axis intercepts ($F_{1,16} = 0.08$; $P>0.05$). Similarly, when the rates of decay of the two fragments were compared using mean intensity of fluorescence, no significant difference was found between the slopes ($F_{1,16} = 0.07$; $P>0.05$), or y-axis intercepts ($F_{1,16} = 0.11$; $P>0.05$).

The half-lives of each amplicon was calculated using the regression equation for each fragment (Figure 5.4a-d). The half-life for the *A. hortensis* specific amplicon (F) was 29.7 hours using percent PCR success and 19.65 hours using mean intensity of fluorescence and for the general *Arion* amplicon (I) 27.68 hours using percent PCR success and 24.37 hours using mean intensity of fluorescence (Table 5.5).

5.4.3 Rate of decay of slug protein within the gut of Pterostichus melanarius

The protein concentration of the *D. reticulatum* antigen used to calculate the *D. reticulatum* protein concentration equivalents in the subsequent analyses was 1.577 mg/ml. The rate of decay of slug protein in the gut of *P. melanarius* for the *Deroceras* specific MAb AiW-1C9 was determined in this study. In order to be able to compare directly the sensitivity of antibody and DNA technologies the decay rate of this antibody was determined using the same individuals as used for the DNA analysis for *D. reticulatum*.

The rate of decay of slug protein within the guts of male and female beetles for Groups I and II using AiW-1C9 showed no significant difference between the decline of the slopes (Group I $F_{1,18} = 0.33$, $P>0.05$; Group II $F_{1,18} = 0.62$, $P>0.05$) or the y-axis intercepts (Group I $F_{1,18} = 0.07$, $P>0.05$; Group II $F_{1,18} = 0.02$, $P>0.05$) for either group using ANCOVA. Therefore, for subsequent analyses, the data for male and female beetles were

combined. Similarly, no significant difference was found between the slopes ($F_{1,18}$ = 1.20; P>0.05) or y-axis intercepts ($F_{1,18}$ = 0.56; P>0.05) of the regression lines between Group I and Group II when they were compared using ANCOVA. The overall rate at which slug protein decayed in the guts of all beetles, determined using AiW-1C9, showed a significant relationship (R^2 = 95.2 %; $F_{1,9}$ = 176.81, P<0.001) (Figure 5.5). The half-life of the antigen using this antibody was reached after 71.38 hours and the significance level (the level above which samples may be considered positive for mollusc remains) of 3.20 ng/200 µl was reached after 143.76 hours. Individual samples could be identified as containing *D. reticulatum* remains at any value above the *Lumbricus casteneus* significance level of 3.20 ng/200 µl, which accounted for the highest cross reaction (McKemmy unpublished). All slug protein was considered to have disappeared from the system after 257.52 hours.

5.4.4 Antibody vs. DNA methodology

Even though mean intensity of fluorescence and protein concentration equivalents are both quantitative measures of foregut DNA and protein content, respectively, they cannot be compared directly using ANCOVA. Therefore, the rate of decline of the slopes, as a percent of initial fluorescence intensity or protein concentration, was compared directly using ANCOVA. In all analyses, the slopes were significantly different from zero at P<0.001. The comparison of AiW-1C9 with amplicons A and E using ANCOVA, showed a significant difference between the slopes ($F_{1,14}$ = 17.53; P<0.01 and $F_{1,14}$=28.34; P<0.001 respectively), but no significant difference between the y-axis intercepts ($F_{1,14}$ = 0.01; P>0.05 and $F_{1,14}$ = 1.00; P>0.05 respectively). However, there was a significant difference between the slopes ($F_{1,14}$ = 26.22; P<0.001), and the y-axis intercepts ($F_{1,14}$ = 14.49; P<0.01) for AiW-1C9 and amplicon C (226 bp).

5.5 Discussion

This is, to our knowledge, the first study to use fluorescent PCR for predator gut content analysis and an attempt to quantify predation by PCR. Using this technique, we have identified the remains of slug DNA in the guts of carabid predators in timed laboratory feeding trials. Robust, species-specific primers were designed for *D. reticulatum* and *A. hortensis*, to amplify small fragments of mitochondrial DNA (109-294 bp) because these are more likely to be detected in the gut of the beetle following degradative digestive processes that break down the prey DNA into small pieces. Essentially, this digested DNA poses similar challenges to ancient DNA (aDNA), as digestive processes cause breakage of the DNA strand due to loss of bases and crosslinks (Herrmann & Hummel, 1994), which means that aDNA is always fragmented with a typical size range of 100-500 bp and in low copy number (Hoss, 1995). Inhibitors that are co-purified with the DNA may impede PCR amplification of the target sequence (Hermann & Hummel, 1994). As only small amounts of DNA are usually extracted in these circumstances, there is a greater chance of amplifying the target sequence if multiple copy genes, such as those in mtDNA, are chosen rather than singly copy nuclear genes, because the sequence produced by the PCR will be derived from a larger number of templates.

Although previous studies (Agusti *et al.*, 1999, 2000; Zaidi *et al.*, 1999; Chen *et al.*, 2000; Hoogendoorn & Heimpel, 2001) have demonstrated that short fragments can be amplified with greater success than longer ones, we found no significant difference in the rates of decay of our different sized amplicons. Therefore, it is possible that a size threshold exists, below which amplification should be successful. In this study, slug remains were successfully detected from within carabid guts 37 hours following ingestion with all primer pairs, and at 49 hours using three sets of primers. These results show the longest detection periods reported for prey DNA from predator guts to date. A similar study (Agusti *et al.*, 2003) targeting the mitochondrial COI gene of a pear psyllid in a heteropteran predator system, achieved 5-15 % amplification success after 32 hours with 271 bp and 188 bp fragments respectively, whereas we achieved up to 80 % success at 37

hours. The only other study to date, in which mtDNA was targeted (Chen *et al.*, 2000), resulted in relatively short detection times. Chen *et al.* (2000) detected aphid DNA from within the gut of predators for a maximum detection period not exceeding 9 hours. Other multiple copy genes have also provided useful targets for detecting predation.

A study of a model system, in which insecticide resistant mosquito larvae were used as the prey and a carabid beetle as the predator, demonstrated a detection limit of 28 hours following ingestion of the prey (Zaidi *et al.*, 1999), but in this case multiple copy nuclear esterase genes responsible for insecticide resistance, rather than mitochondrial genes were targeted. Hoogendoorn & Heimpel (2001) found that they were able to detect lepidopteran prey DNA for 12 hours following ingestion by a coccinellid predator. In this case, nuclear ribosomal gene clusters and ITS were targeted. Amplification of single copy nuclear (SCN) genes was less reliable though, at best resulting in detection of a 310 bp fragment in 60 % of predators 4 hours following ingestion (Agusti *et al.*, 1999).

In carabids, the minimum requirement is for the probe to recognize target prey for long enough to detect predation during the previous night, normally from beetles caught in pitfall traps (Symondson *et al.*, 1996). The results of this study clearly show that the *Deroceras* and *Arion* primers tested were capable of reliably amplifying the target species within this time period as the half lives, or more accurately the median detection limit at least for decay based on percent PCR success, of the amplicons produced in this study ranged between 19-29 hours. Therefore, this should be a successful system for the analysis of predation in the field. To date there have been no studies in which predation by arthropods in the field has been investigated using DNA-based methods. Antibody technology has long been established as the method of choice for predator gut content analysis and it frequently facilitates long detection periods for prey remains within the predator's gut. By testing the same samples with antibody and DNA methodologies, we have directly compared these two techniques in order to determine the relative sensitivity of each. When the sensitivities of the antibody and DNA techniques were compared, it was apparent that the detection times achieved using antibodies were far longer than

those achieved using DNA based methods. The detection period recorded for the decay rate of antibody recognisable proteins within *P. melanarius* using the MAb AiW-1C9, indicates that this is a very effective antibody for the detection of predation on molluscs in this carabid species. In comparison to the half-lives of the DNA amplicons, the half life of AiW-1C9 was much longer at 71.38 hours and considerably longer than for other anti-mollusc antibodies such as DrW-2D11 (Symondson & Liddell, 1995) or DrW-1G4 (Symondson & Liddell, 1996). The long detection times achieved for AiW-1C9 can be accounted for by the very low degree of cross reactivity that this antibody shows with non-mollusc species. However, it is not always advantageous to be able to detect prey within the predator for a long period of time as this may make it more difficult to interpret the behaviour of the predator in the period immediately before its capture. Longer detection times are likely to result in the disclosure of more predation events over an increased period of time, which will correspondingly make the results more difficult to interpret. Therefore, shorter periods are more likely to accurately delineate the period within which the predation event took place. Usually in this type of experiment, traps are set and left in place overnight and then collected the next morning, often in conjunction with the placing of e.g. stick traps to monitor prey availability within the crop. If a probe has a relatively short detection period, then it will be easier to relate positive samples to the availability of prey at the time of trapping.

Using the MAb AiW-1C9 and the *Deroceras* primers, we did not observe a statistically significant difference in detection of prey remains within the guts of male and female beetles, for either DNA or protein, although this phenomenon was reported previously by Symondson *et al.* (1999) who found that the detection period for males was approximately 30 % longer than for females. However the overall regression slopes and hence rates of decay for male and female beetles were not significantly different. Although there was no significant difference in detection between males and females or in the presence of alternative prey, the gut weights of females were consistently heavier then for males. Other studies (Symondson & Liddell, 1993, 1995; Symondson *et al.*, 1999) have also shown female foregut weight to be greater than males. This was true

whether individuals were starved or provided with alternative prey (Symondson *et al.*, 1999), indicating that females eat more than males. However, we did not find a significant difference in the rate of decay of the target species in the presence or absence of an alternative prey. Symondson *et al.* (1999) suggested that feeding on an alternative prey slows down the movement of the antigen out of the foregut into the rest of the digestive system so that movement of antigen out of the gut is faster than the rate of antigen digestion within the foregut. This would lead to significantly extended detection periods for the prey when the predator was fed an alternative prey species in addition to the target species (Symondson & Liddell, 1995). This does not seem to be the case with our results, or previous findings for carabid and staphylinid predators in which there was no significant difference between the rate of decay in beetles fed an alternative prey (Lovei *et al.*, 1990).

The use of fluorescent PCR still needs further optimisation and development before it is comparable with the degree of sensitivity and quantification that can be achieved by the use of MAb ELISA and even with additional optimisation, it may never achieve comparability. The use of standard PCR and agarose based screening has less potential for development towards quantifiable analysis of predation although will be of great value if rapid screening is required to show purely the proportion of positive and negative samples in a data set. Even so, the use of basic fluorescent PCR, as used in this investigation, has a number of advantages over standard PCR methods that have been used until now. One major advantage is that a semi-quantitative measure of amplification can be attained by observing the intensity of fluorescence on the electropherogram for each product. In addition, the sensitivity of the detection on acrylamide gel in an automated sequencer is such that it is possible to accurately separate bands that are only 1bp different in size, a facility that is not available when running PCR products on an agarose. Interpretation of a photograph of an agarose gel is subjective as only a visual measure of amplification can be attained, which may result in faint products being scored as false negatives.

The general *Arion* primers were specifically designed for a region of the mitochondrial 12s rRNA gene in which amplicon size variation between species was expected. The target gene was chosen as rRNA genes commonly contain insertions and deletions (Kjer, 1995) and general primers are available which anneal to conserved flanking regions on either side of the third domain of this gene, allowing easy amplification across wide taxonomic groups (Simon *et al.*, 1994). In molluscs, it appears that 12s rRNA is very variable with insertions and deletions occurring between species and genera (Dodd *et al.*, this thesis (Chapter 6)), although this is not observed in many arthropods, such as aphids, which exhibit little variation in this gene (*pers obs*; Simon *et al.*, 1994).

By using a suite of primers working at different taxonomic levels, i.e. genus and species, it is possible to identify the range of prey within that taxonomic group consumed by a predator and then target the positive samples with specific primers. This approach minimises cost and maximises screening efficiency when processing large numbers of samples. Species-specific primers were designed with different fluorescent labels and amplicon sizes allowing multiple loading of the different amplicons on the gel, which can then be accurately sized and identified. If only an estimate of the range of species consumed by the predator is required, then the general primers could be used alone, as long as the different species had different amplicon sizes. However, comprehensive cross-reactivity testing of the primers is necessary otherwise, misidentification of positive samples could occur. The primary advantage of using fluorescent methods is that it facilitates the approach described above, using general primers with which a range of species can be amplified in a single PCR reaction, and then be identified by amplicon size. This procedure is not possible with an agarose based screening method, as it is not sufficiently sensitive to distinguish amplicons differing in only a few base pairs.

The use of fluorescent PCR for predator gut content analysis, is a further step towards the development of quantitative PCR approaches such as TaqMan (Haugland *et al.*, 1999) or molecular beacons (Tyagi *et al.*, 1998; Poddar & Le, 2001), in which it is possible to target a large number of potential prey species at the same time by using multiple

fluorescent dyes. However, the system that we have developed will allow rapid and accurate screening of predators trapped from the field and offers a system that is sufficiently sensitive to rival that provided by MAb ELISA.

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5.7 Author information box

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5.8 Figure legends

Figure 5.1: Structure of DNA and antigen decay rate experiments: a) Beetles in Group I were firstly fed *ad lib* with *Deroceras reticulatum* for 2 hours and then refed with an alternative prey (*Musca domestica*), and in Group II were fed *ad lib* with *Deroceras reticulatum* for two hours, but were not provided with alternative prey, before being killed at 0-48 hours post-feeding; b) Beetles were fed *ad lib* with *Arion hortensis* for two hours and then refed an alternative prey (*Musca domestica*), before being killed at 0-72 hours post-feeding.

Figure 5.2: Rate of decay of *Deroceras* DNA within the gut of *Pterostichus melanarius* for three *D. reticulatum* specific amplicons (A, C, E) labelled with HEX and separated by acrylamide gel electrophoresis using ABI377 automated sequencer. (a-c) Rate of decay expressed as percent PCR success; (d-f) rate of decay expressed as mean intensity of fluorescence (MFU).

Figure 5.3: Rate of decay of *Arion* DNA within the gut of *Pterostichus melanarius* for one *A. hortensis* specific (F) and on general *Arion* (I) amplicon labelled with FAM and separated by acrylamide gel electrophoresis using ABI377 automated sequencer. (a-b) Rate of decay expressed as percent PCR success; (c-d) rate of decay expressed as mean intensity of fluorescence (MFU).

Figure 5.4: Rate of decay of *Deroceras reticulatum* protein within the gut of *Pterostichus melanarius* using the monoclonal antibody AiW-1C9. *D. reticulatum* protein = -0.1536 time + 2.4649; R^2 = 95.2 %. Dotted line represents the significance level of 3.2 ng/200 µl relating to the highest cross-reaction with a non-target species.

5.9 Tables

Table 5.1: Primer sequences, optimal PCR annealing temperatures, and amplicon sizes for *Deroceras reticulatum* species specific primers (A-E), *Arion hortensis* (E), *A. distinctus* (F), *A. intermedius* (H) and *Arion* Genus (I) specific primers. Primers marked * were labelled at the 5'end with HEX; primers marked † were labelled at the 5'end with FAM, for use with fragment analysis software on the ABI 377 semi-automated DNA Analyser.

Name	Primers	Sequence 5'-3'	Annealing temperature (°C)	Amplicon size (bp)
A	DR11F*	CTATACACAATTTTTAAATAAGC	50.4	109
	DRF29RC	GTCTCTGGTTTATCTATTATTGGT		
C	DR11F*	CTATACACAATTTTTAAATAAGC	45.1	226
	DR16MR	GCCAGTAACTTATTTGGT		
E	DR11F*	CTATACACAATTTTTAAATAAGC	50.0	294
	DR50R	AAATTTACTTTCAAGTCCAGC		
F	AH1MF†	CACCAAGATACTCAACCCAC	55.0	130
	AH2R	CGAACGCCCACTTATAGG		
I	Ai1F†	CACATAAATGATAGTCACC	44.4	204-221
	AR2R	ATACTTACAAGTCCATCTTT		

Table 5.2: Cross-reactivity between slug specific primers and predator and alternative prey DNA, expressed as positive (✓) or negative (✗) for the target band as visualised on agarose gel. Where possible, three individual specimens were tested for each species with each primer pair. For the *Arion* genus primers (I), a diagnostic amplicon size is also given for the species that the primers amplify.

Invertebrates	Primer Pairs Size in bp				
	A 109	C 226	E 294	F 130	I 204-221
Mollusca					
Deroceras reticulatum (Müller) (Stylommatophora: Agriolimacidae)	✓	✓	✓	✗	✗
Deroceras panormitanum (Lessona & Pollonera) (Stylommatophora: Agriolimacidae)	✗	✓	✓	✗	✗
Arion hortensis Férussac (Stylommatophora: Arionidae)	✗	✗	✗	✓	207
Arion distinctus Mabille (Stylommatophora: Arionidae)	✗	✗	✗	✗	221
Arion owenii Davies (Stylommatophora: Arionidae)	✗	✗	✗	✗	210
Arion intermedius Normand (Stylommatophora: Arionidae)	✗	✗	✗	✗	215
Arion silvaticus Lohmander (Stylommatophora: Arionidae)	✗	✗	✗	✗	220
Arion circumscriptus Johnson (Stylommatophora: Arionidae)	✗	✗	✗	✗	217
Arion fasciatus (Nilsson) (Stylommatophora: Arionidae)	✗	✗	✗	✗	204
Arion subfuscus Draparnaud (Stylommatophora: Arionidae)	✗	✗	✗	✗	206
Arion flagellus Collinge (Stylommatophora: Arionidae)	✗	✗	✗	✗	214
Arion lusitanicus Mabille (Stylommatophora: Arionidae)	✗	✗	✗	✗	205
Arion ater (Linneus) (Stylommatophora: Arionidae)	✗	✗	✗	✗	209
Limax flavus Linnaeus (Stylommatophora: Limacidae)	✗	✗	✗	✗	✗
Limax maximus Linnaeus (Stylommatophora: Limacidae)	✗	✗	✗	✗	✗
Lehmannia marginata (Müller) (Stylommatophora: Limacidae)	✗	✗	✗	✗	✗
Tandonia sowerbii (Férussac) (Stylommatophora: Milacidae)	✗	✗	✗	✗	✗
Tandonia budapestensis (Hazay) (Stylommatophora: Milacidae)	✗	✗	✗	✗	✗
Milax gagetes (Draparnaud) (Stylommatophora: Milacidae)	✗	✗	✗	✗	✗
Boettgerilla pallens Simroth (Stylommatophora: Boettgerillidae)	✗	✗	✗	✗	✗
Vallonia pulcinella (Müller) (Stylommatophora: Valloniidae)	✗	✗	✗	✗	✗
Discus rotundatus (Müller) (Stylommatophora: Discidae)	✗	✗	✗	✗	✗
Cepaea nemoralis (Linnaeus) (Stylommatophora: Helicidae)	✗	✗	✗	✗	✗
Cepaea hortensis (Müller) (Stylommatophora: Helicidae)	✗	✗	✗	✗	✗
Monarcha cantiana (Montagu) (Stylommatophora: Helicidae)	✗	✗	✗	✗	✗
Helix aspersa Müller (Stylommatophora: Helicidae)	✗	✗	✗	✗	✗
Candidula intersecta (Poiret) (Stylommatophora: Helicidae)	✗	✗	✗	✗	✗
Annelida					
Lumbricus terrestris Linneus (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Lumbricus castaneus Linneus (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Lumbricus rubellus Linneus (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Allolobophora chlorotica (Savigny) (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Eisenia fetida Savigny (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Octolasion cynaeum Oerley (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Aporrectodea rosea (Savigny) (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Aporrectodea longa (Ude) (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗
Aporrectodea caliginosa (Savigny) (Haplotaxida: Lumbricidae)	✗	✗	✗	✗	✗

Insecta					
Pterostichus melanarius (Illiger) (Coleoptera: Carabidae)	x	x	x	x	x
Poecilus caruanae (Linneus) (Coleoptera: Carabidae)	x	x	x	x	x
Sitobion avenae (Fabricus) (Hemiptera: Aphididae)	x	x	x	x	x
Metopolophium dirhodium (Walker) (Hemiptera: Aphididae)	x	x	x	x	x
Myzus persicae (Sulzer) (Hemiptera: Aphididae)	x	x	x	x	x
Rhopalosiphum padi (Linneus) (Hemiptera: Aphididae)	x	x	x	x	x
Calliphora vicina Robineau-Desvoidy (Diptera: Calliphoridae)	x	x	x	x	x
Musca domestica Linneus (Diptera: Muscidae)	x	x	x	x	x

Table 5.3: Percentage PCR success and mean intensity of fluorescence (MFU) for *D. reticulatum* remains following ingestion by *Pterostichus melanarius* in the presence (Group I) and absence (Group II) of alternative prey, and in the two groups combined (Group I+II), for different mtDNA amplicons and DNA decay half-life.

Time since ingestion (hours)	Amplicon	A (109bp)						C (226bp)						E (294bp)					
	Group	I		II		I+II		I		II		I+II		I		II		I+II	
		%	MFU	%	MFU	%	MFU	%	MFU	%	MFU	%	MFU	%	MFU	%	MFU	%	MFU
1		90	1714	90	1714	95	1714	100	2808	100	2808	100	2808	90	1398	90	1398	90	1398
3		90	2128	100	2196	95	2162	100	1777	100	2391	100	2084	100	1572	100	1449	100	1511
5		90	1734	60	153	75	944	90	1610	70	719	80	1165	90	1338	60	307	75	822
9		90	961	40	331	65	646	100	1040	100	1002	100	1021	90	987	80	506	85	747
13		70	741	70	1047	70	894	100	1073	90	1353	95	1213	80	1120	70	1073	75	1097
19		50	228	60	771	55	500	80	948	80	1080	80	1014	70	682	40	544	55	613
25		20	121	10	52	15	86	50	544	80	498	65	521	30	393	30	55	30	224
37		10	33	0	13	5	23	10	71	30	56	20	63	10	58	0	0	5	29
49		0	0	0	0	0	0	0	0	20	107	10	53	0	0	0	0	0	0
DNA decay half-life (hrs)		-	-	-	-	19.18	22.09	-	-	-	-	29.01	26.17	-	-	-	-	21.19	22.91

Table 5.4: Rates of decay of amplicons of *Deroceras* DNA within the guts of *P. melanarius*, compared using ANCOVA: a) males vs. females in the presence (Group I) and absence (Group II) of alternative prey; and b) Group I vs. Group II in which data for male and females is combined within each group. In each case, percent mean intensity of fluorescence was used to determine the rate of decay of slug DNA.

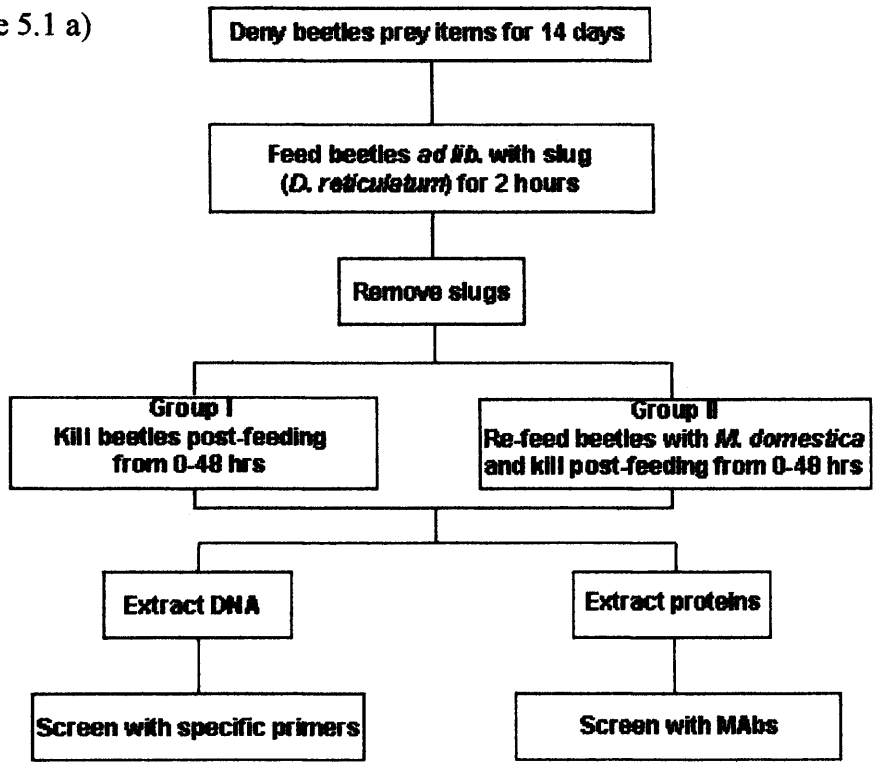
		% PCR Success				**Mean Intensity of Fluorescence**			
		Slope		y-axis intercept		Slope		y-axis intercept	
a) Male vs. female	Amplicon	$F_{1,14}$	P	$F_{1,14}$	P	$F_{1,14}$	P	$F_{1,14}$	P
Group I	A	2.12	>0.05	0.37	>0.05	0.03	>0.05	0.04	>0.05
	C	0.00	>0.05	0.17	>0.05	0.49	>0.05	0.00	>0.05
	E	0.48	>0.05	0.88	>0.05	0.00	>0.05	0.02	>0.05
Group II	A	0.01	>0.05	0.04	>0.05	0.03	>0.05	0.51	>0.05
	C	3.09	>0.05	0.05	>0.05	0.03	>0.05	0.43	>0.05
	E	0.00	>0.05	0.20	>0.05	0.23	>0.05	0.00	>0.05
b) Group I vs. Group II	A	0.00	>0.05	0.00	>0.05	0.65	>0.05	0.14	>0.05
	C	1.54	>0.05	0.01	>0.05	0.69	>0.05	0.76	>0.05
	E	0.17	>0.05	0.04	>0.05	0.68	>0.05	0.03	>0.05

Table 5.5: Percentage PCR success and mean intensity of fluorescence (MFU) for *Arion hortensis* remains following ingestion by *Pterostichus melanarius* determined using species specific and genus specific primers and DNA decay half-life.

Time since ingestion (hours)	Amplicon			
	F		I	
	%	MFU ± SE	%	MFU ± SE
1	100	8520 ± 0.61	100	6953 ± 12.71
3	60	4574 ± 21.41	100	7259 ± 3.78
5	80	4939 ± 17.7	100	4818 ± 12.12
9	80	6267 ± 17.74	80	5064 ± 16.55
13	100	6363 ± 13.95	100	4482 ± 12.57
19	80	2638 ± 13.82	80	671 ± 8.12
25	100	1165 ± 5.95	40	629 ± 9.15
37	80	3564 ± 18.29	60	1992 ± 15.86
49	20	237 ± 6.89	20	484 ± 9.84
73	0	0 ± 0.00	0	0 ± 0.00
DNA decay half-life (hrs)	29.70	19.65	27.68	24.37

5.10 Figures

Figure 5.1 a)



b)

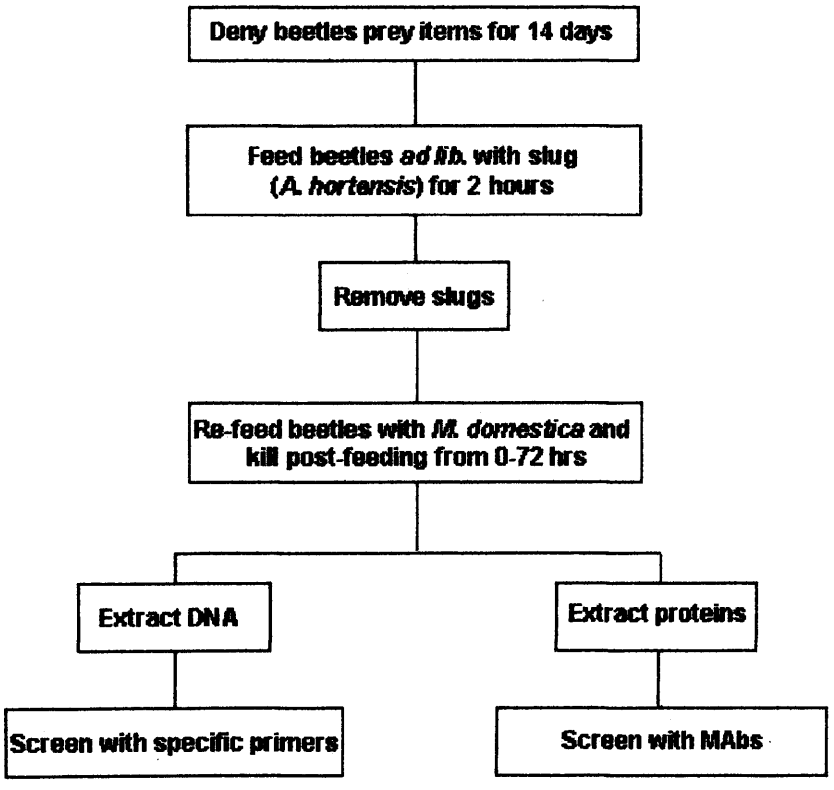
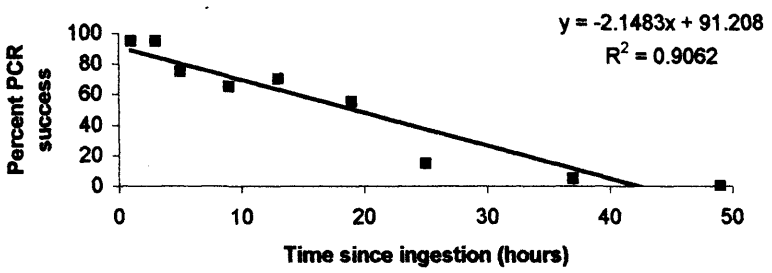
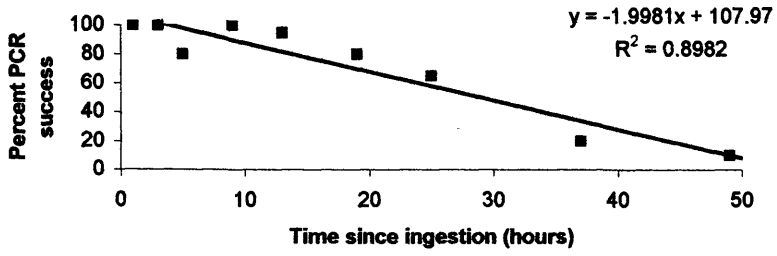


Figure 5.2

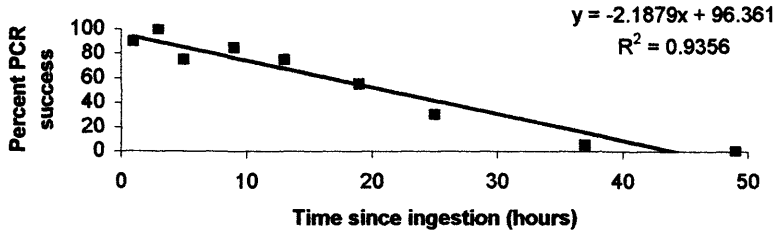
a) Rate of decay of *D. reticulatum* DNA determined by percent PCR success for amplicon A



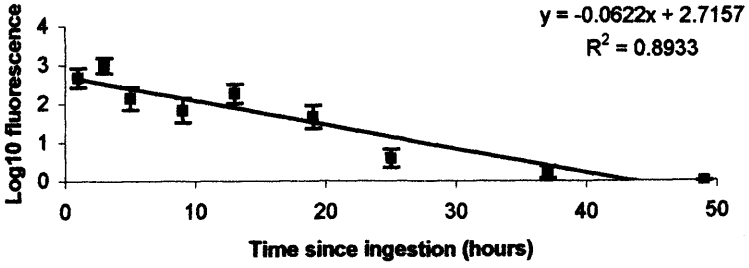
b) Rate of decay of D. reticulatum DNA determined by percent PCR success for amplicon C



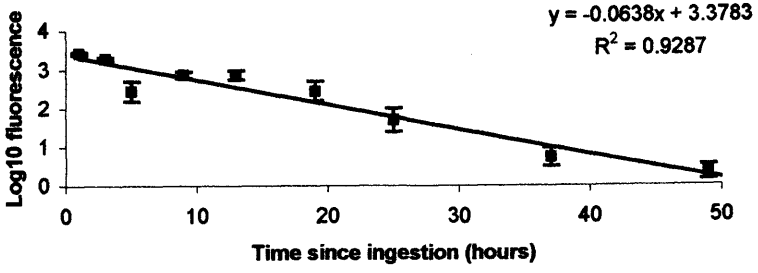
c) Rate of decay of D. reticulatum DNA determined by percent PCR success for amplicon E



d) Rate of decay of D. reticulatum DNA determined by mean intensity of fluorescence for amplicon A



e) Rate of decay of D. reticulatum DNA determined by mean intensity of fluorescence for amplicon C



f) Rate of decay of D. reticulatum DNA determined by mean intensity of fluorescence for amplicon E

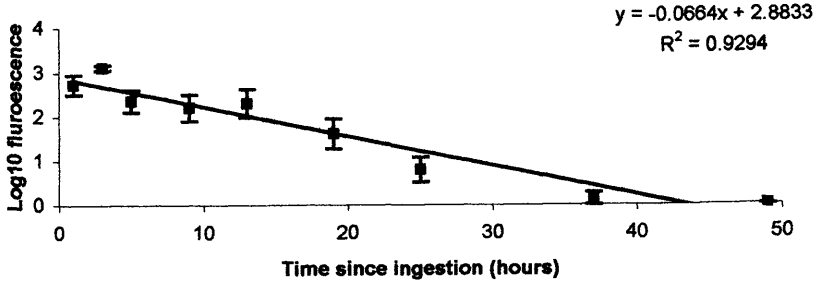
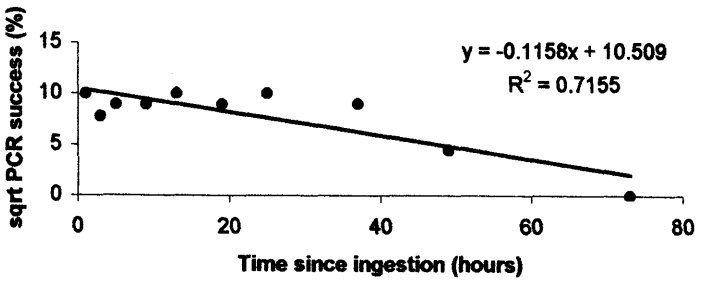
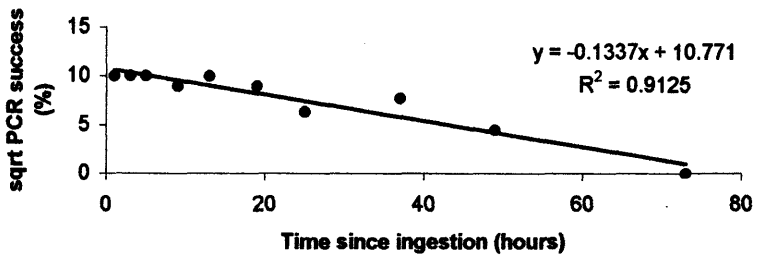


Figure 5.3

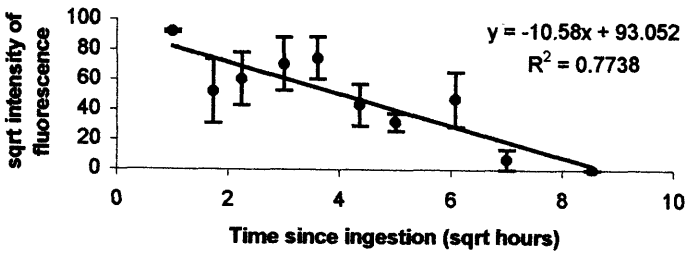
a) Rate of decay of *A.hortensis* DNA determined by percent PCR success for amplicon F



b) Rate of decay of *A.hortensis* DNA determined by percent PCR success for amplicon I



c) Rate of decay of *A.hortensis* DNA determined by mean intencity of fluorescence for amplicon F



d) Rate of decay of A.hortensis DNA determined by mean intensity of fluorescence for amplicon I

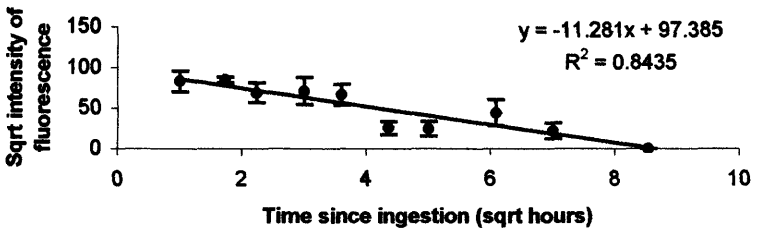
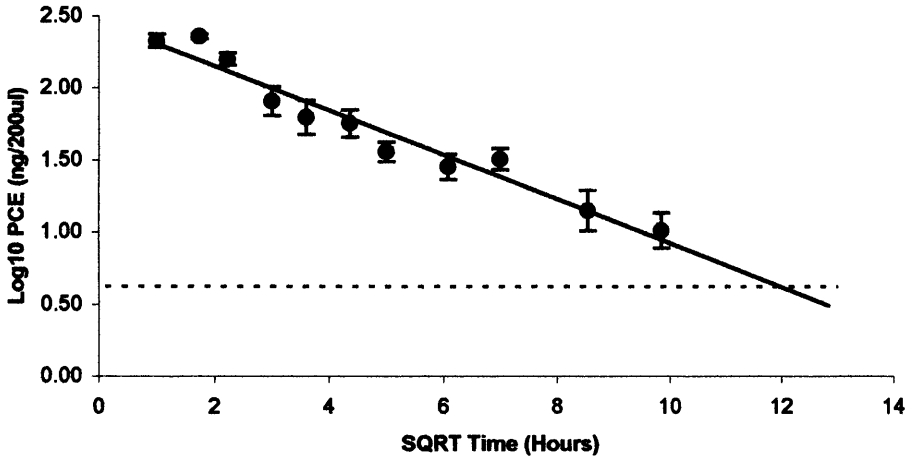


Figure 5.4


2.50
2.00
1.50
1.00
0.50
0.00
Log10 PCE (ng/200ul)
0
2
4
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8
10
12
14
SQRT Time (Hours)

Chapter 6

Modified version to be submitted for publication in Molecular Ecology

(format according to journal specifications)

Detecting predation on slugs by *Pterostichus melanarius* (Illiger) in the field using fluorescent-based PCR and MAb ELISA.

Ciara S Dodd, James D Harwood*, Sue K Coe, Georgina L Harper, David M Glen**
Michael W Bruford and William OC Symondson

Cardiff School of Biosciences, Main Building, Park Place, Cardiff University, Cardiff, CF10 3TL, UK

* Present address: Department of Entomology, University of Kentucky, S-225 Agric. Science Center – North, Lexington, KY 40546-0091, USA.

** Styloma Research Ltd., Phoebe, The Lippiatt, Cheddar, BS27 3QP, UK

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Corresponding Author: Ciara S Dodd
Cardiff School of Biosciences,
Main Building,
Park Place,
Cardiff University,
Cardiff, CF10 3TL,
UK

Tel: 029 20875776
Fax: 029 20874305

DoddCS@cf.ac.uk

Running Title: Detection of predation on slugs in the field

6.1 Abstract

Pterostichus melanarius (Illiger) is a well known predator of slugs in agroecosystems. Conventionally, predation by arthropods in the field is determined through extracting prey proteins from the predators gut and analysing these using prey specific monoclonal antibodies and ELISA. Species- and genus-specific primers were designed that amplify small fragments of mitochondrial DNA to investigate predation on the slugs *Deroceras reticulatum* (Müller) and *Arion hortensis* aggregate by the carabid beetle *P. melanarius*. Primers were developed to amplify fragments of the mitochondrial 12S rRNA gene ranging in size from 226 bp and 208-221 bp for *D. reticulatum* and *Arion* sp. respectively. Beetle gut samples were also analysed for prey specific proteins using a *D. reticulatum* specific and a general mollusc antibody. We report the first results for the detection of prey remains in carabid beetles caught from the field using DNA-based techniques. Overall, 3.8 % and 0.7 % of beetles contained *D. reticulatum* or *Arion* sp. DNA respectively, and 8 % and 43 % contained *D. reticulatum* or mollusc protein. Comparison of these results with the availability of slugs in the field and with beetle foregut weight indicated that as slug density increased so did beetle foregut weight and mollusc protein concentration, suggesting that the beetles were preying upon slugs.

6.2 Introduction

Slugs are one of the most important pests in European agriculture, responsible for millions of pounds worth of damage to arable crops annually. Changes in farming practices appear to have exacerbated the problem, stimulating a 70-fold increase in the use of molluscicides since the early 1970s (Garthwaite & Thomas, 1996). However, these compounds have unwanted side effects on non-target species and can severely reduce the populations of potentially beneficial non-target predatory arthropods such as carabids (Purvis & Bannon, 1992; Kendall *et al.*, 1995).

Recent studies have established the role of carabids as predators of slugs within agroecosystems (Symondson & Liddell, 1993a; Symondson *et al.*, 1996; Bohan *et al.*, 2000; McKemey *et al.*, 2001). In particular, the distributions of the carabid beetle *Pterostichus melanarius* (Illiger) and slugs were dynamically associated, as initially areas of high slug and beetle density coincided, but later, where there were high densities of beetles there were only a few slugs (Bohan *et al.*, 2000). The distribution of slug-positive beetles was significantly associated with the distribution of slugs greater than 25 mg in June, but in July, few such slugs were found where there were many slug positive beetles (Bohan *et al.*, 2000). Growth of the slug population declined as the numbers of beetles increased, suggesting that predation was not opportunistic, but direct and dynamic, and that predation elicited changes in the spatial distribution and local density of the slugs. Symondson *et al.* (2002) presented further evidence for a dynamic interaction between *P. melanarius* and its slug prey. Over a five-year period, there was a strong relationship between the crop mass of the beetles and slug numbers in the soil, indicating that the slugs were a major part of the beetles' diet. The change in the beetle population from year to year was strongly related to slug numbers in the soil and crop mass of the beetles in the previous year, indicating that slugs had an influence on the nutritional status, and reproductive success of the beetles. It is well established from other studies that *P. melanarius* can significantly reduce slug populations in the field, has great importance for slug population dynamics and that activity density of *P. melanarius* is dynamically

associated with slug biomass (Symondson *et al.*, 1996; Bohan *et al.*, 2000; McKemey, 2000; Symondson *et al.*, 2002). These factors are particularly important if *P. melanarius* is to be utilised as an *in situ* biocontrol agent within agroecosystems.

The method of choice for studying beetle-slug interactions is currently prey-specific monoclonal antibodies (MAbs) coupled with enzyme linked immunosorbent assay (ELISA) (Symondson, 2002a, b). Recent development and refinement of DNA-based methods for predator gut content analysis have demonstrated that such techniques are now an alternative option for this area of research. Predation on a target species can be detected for at least the 24 hour period prior to the capture of the predator using DNA-based detection methods (Zaidi *et al.*, 1999; Agustí *et al.*, 2003, in press; Dodd *et al.*, this thesis Chapter 5), which is sufficient to be able to detect predation during the previous night (Symondson *et al.*, 1996). However, direct comparison of biochemical and molecular techniques (Dodd *et al.*, this thesis Chapter 5) suggested that MAb ELISA is a much more sensitive assay than DNA-based approaches, in cases where antibodies have extended detection limits (Harwood *et al.*, 2001). In such circumstances, it is unlikely that they will be comparable with DNA-based studies.

Only two studies have used PCR-based DNA techniques to detect predation by invertebrates in the field, neither of which has investigated predation by carabids. Both studies targeted mtDNA using PCR-based methods. Hoogendoorn & Heimpel (2003) successfully identified *Ostrinia nubialis* Hübner DNA from the guts 1 % of coccinellid predators tested. They suggested that the low number of positive results were due to low densities of egg masses and low encounter rates by predators. Agusti *et al.* (2003) amplified Collembola DNA from the guts of spiders and was able to demonstrate that the spiders were exercising prey choice since the collembolan most frequently identified in spider guts was the least numerous of the three target species of Collembola identified from the field.

In this paper, we report the first fully replicated study to assess predation by the carabid *P. melanarius* in the field using both DNA- and protein-based detection methods and we compare the sensitivity of these techniques for beetles collected in the field. In addition, we investigate the relationships between predation, the availability of prey in the field and cultivation practice.

6.3 Materials and methods

4.3.1 Field samples

The dynamics of predation by beetles on slugs were studied within a large-scale field experiment (Field 75, Long Ashton Research Station, Bristol), as described by Kendall *et al.* (1995) and Symondson *et al.* (1996, 2002). The field site was subdivided according to a randomised block design into 25 experimental plots (12 x 30 m), in five blocks (60 x 30 m) and within each block, treated according to five different cultivation practices (Figure 6.1). In Spring 2001, the year of study, the field was planted with broad beans (*Vicia faba*), which was preceded in rotation by a cereal crop.

Beetles were sampled once per week from 1st June - 9th September 2001 inclusive. Beetles were captured using pitfall traps, positioned 5 m from the centre of each plot in the direction of each corner of the plot. A wire mesh platform positioned within the trap prevented the beetles from feeding on smaller potential prey items whilst they were in the trap. The inside walls of the trap were coated with Fluon® (polytetrefluoroethylene) (Whitford Plastics) (a non-stick coating) to prevent the beetles escaping. Each trap was protected from flooding by a plastic rain cover, which did not impede beetle entry into the trap. The traps were placed in the field at approximately 1600 hours and were collected the next morning. Following collection, *P. melanarius* were sexed, placed in individual Eppendorf tubes and stored at –80 °C until processing. Where available, two female beetles were randomly chosen per plot per sampling date for use in the analysis.

The pitfall trapping as described above provided estimates of the activity-density of *P. melanarius* in each plot each week and the mollusc population in each plot was monitored by sampling a 25 cm x 25 cm x 10 cm area of soil once per month throughout the year. The invertebrates within the soil samples were collected by slowly flooding the soil sample over a period of several days, using a standard soil flooding procedure (Glen *et al.*, 1992). The earthworm population was evaluated by flooding a 50 cm x 50 cm area within each plot once per month with 5 % formaldehyde and collecting any worms

that came to the surface. In total 10 litres of 5 % formaldehyde solution was applied. The area was enclosed within a metal frame (50 x 50 cm) and five litres of formaldehyde was added. After approximately 10 min had elapsed, all worms that appeared on the surface were collected. A further five litres was added and left as before, and all worms were collected.

6.3.2 Extraction of predator gut contents

DNA and protein were extracted from the guts of the beetles simultaneously. Beetles were thawed to room temperature and the foregut was removed as described in Symondson *et al.* (2000). The foregut was weighed and homogenized in a 19:1 w:v ratio with phosphate buffered saline (PBS). The homogenates were centrifuged at 8,000 rpm for 15 min at room temperature. The majority of the supernatant was transferred to a clean eppendorf and stored at –20 °C for subsequent analysis using ELISA with a general mollusc (AiW-1C9) (Thomas, 2002; RS Thomas, A McKemey, DM Glen & WOC Symondson, unpublished) and *Deroceras reticulatum* Müller -specific (DrW-1G4) (Symondson & Liddell, 1996) monoclonal antibody. The particulate remains and 80 µl of the supernatant were retained for DNA extraction using the Qiagen DNA Mini Kit (Qiagen) in accordance with manufacturer's instructions. Extracted DNA was stored at –20 °C.

6.3.3 PCR amplification and MAb ELISA

The DNA extractions were screened via PCR with the *D. reticulatum* specific primers DR11F (CTA TAC ACA ATT TTT AAA TAA GC) and DR16MR (GCC AGT AAC TTA TTT GGT) and general *Arion* primers Ai1F (CAC ATA AAT GAT AGT CAC C) and AR2R (ATA CTT ACA AGT CCA TCT TT) (Dodd *et al.*, this thesis Chapter 5). DR11F was 5' fluorolabelled with HEX and Ai1F 5' fluorolabelled with FAM. Samples were amplified in a 10 µl reaction volume containing approximately 200 ng total genomic DNA, 1× buffer (20 mM Tris-HCl (pH 8.4); 50 mM KCl), 2 mM $MgCl_2$, 0.025 mM each of dATP, dGTP, dCTP and dTTP, 1.0 µM each primer and 0.04 units of Taq polymerase (InvitrogenGibco, Life Technologies®) and made up to volume with sterile

water (Sigma-Aldrich). Thermocycling conditions were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 44.4 or 45.5 °C for 45 s, 72 °C for 1 min, then a final extension step of 72 °C for 10 min. The PCR products were multi-loaded on a 6 % acrylamide gel, so that two amplicons were run in each lane, together with TAMRA 350 size standard (Applied Biosystems) on an ABI Prism 377 semi-automated DNA analyser. Each gel was run with a positive (*D. reticulatum* or *Arion* sp.) and negative (water) control and results were analysed using Genescan Analysis and Genotyper v2 (Applied Biosystems). Samples were considered positive if the electropherogram showed a peak of the target size amplifying with ≥100 fluorescence units.

The protein extracts were screened by indirect ELISA (described in detail in Harwood *et al.*, 2001) on a Pro-Bind™ micro-titre plate (Beckton Dickinson Labware) at room temperature using the general anti-mollusc monoclonal antibodies (MAb), AiW-1C9 (Thomas, 2002) and DrW-1G4 (Symondson & Liddell, 1996). AiW-1C9 was used at a concentration of 1:250 and DrW-1G4 at 1:5,000 diluted in PBS-Tween. Goat antimouse IgM (γ-chain specific) horseradish peroxidase enzyme conjugate (Sigma-Aldrich) was diluted 1:2,000 for use with AiW-1C9 and at 1:2,500 for DrW-1G4 with PBS-Tween following optimisation. Each ELISA plate included a x1.5 dilution series of *D. reticulatum* standards, stabilized with heterologous protein also diluted 1:20,000 (w/v) in PBS, that provided slug protein concentrations between 315 and 9 ng/200 µl. Heterologous protein consisted of starved beetle gut diluted to a concentration of 1:20,000 (w/v) (Symondson & Liddell, 1995). Slug protein concentrations were calculated following a protein assay using the BioRad Protein Assay System (Bio-Rad Laboratories).

6.3.4 Statistical analysis

Time since ingestion was calculated in DNA-MAb comparisons by rearranging the regression equation from the decay rate experiments, so that $x = (y-c)/m$, where x = time, y = protein concentration equivalent or fluorescence units and c and m are constants.

Although beetles and arthropod prey were trapped from the field once per week throughout the study in all plots, in order to analyse the results in conjunction with slug and earthworm population data, collected at monthly intervals, numbers of beetles and arthropod prey were pooled within each month. The interacting effects of cultivation practice, date and prey population on beetle foregut protein concentration and gut weight were examined in pairwise comparisons by two-way analysis of variance (ANOVA) and linear regression using Minitab 13. The proportion of positive samples with both antibodies and DNA were compared using a paired sample t-test after arcsine transformation.

6.4 Results

6.4.1 Detection of slug DNA within the guts of field caught Pterostichus melanarius

D. reticulatum and *Arion* sp. DNA could be detected from the guts of field-collected beetles. In total 600 individual beetles were analysed, of which 23 (3.8 %) were positive for *D. reticulatum* remains and 0.7 % (6 individuals) were positive in the expected size range (204-218 bp) (Dodd *et al.*, this thesis Chapter 5) for *Arion* sp., amplifying with ≥100 fluorescent units (FU). Based on the size of the PCR products obtained, two beetles had consumed *Arion hortensis* Férrusac, two *Arion intermedius* Normand and two *Arion distinctus* Mabille. These results were verified by screening these samples with specific primers designed to amplify the individual species (*A. hortensis, A. intermedius* and *A. distinctus*). Samples were collected from the field between 1st June and 11th September 2001, but *D. reticulatum* positive samples were only detected between 18th July and 25th July 2001 and *Arion* remains between 14th June 2001 and 5th September 2001. No significant relationships were revealed between the number of DNA-positive beetles and the availability of slugs ($F_{1,18} = 1.44$; $P>0.05$), cultivation practice ($F_{1,18} = 0.03$; $P>0.05$) or month ($F_{1,18} = 0.81$; $P>0.05$). Details of the numbers of slugs, snails and earthworms captured in the field during the sampling period are given in Table 6.1 and Figure 6.2.

6.4.2 Detection of slug protein within the guts of field-caught Pterostichus melanarius

Of the 600 *P. melanarius* tested, 43.3 % were positive for mollusc protein using AiW-1C9 and 8.0 % had consumed *D. reticulatum* using DrW-1G4 respectively. Slug-positive beetles were identified as those individuals with protein concentration equivalents (PCE's) over the significance level for the highest non-target cross-reaction for each antibody. This was determined as 2.5 standard deviations above the highest non-target cross-reaction. For AiW-1C9 the significance level was 3.20 ng/200 µl and for DrW-1G4 it was 52.11 ng/200 µl.

6.4.3 Prey-specific mitochondrial DNA primers vs. MAbs

When the DNA-positive samples were compared with the MAb results, we found differences between them. These are summarised in Tables 6.2 and 6.3 for *D. reticulatum* and *Arion* sp., respectively. Of the beetles positive for *Deroceras* DNA, no individuals were considered positive with both DrW-1G4 and the *Deroceras* specific primers, although 12 out of 22 were also positive for mollusc protein with AiW-1C9. From the beetles that were positive for *Arion* DNA, three beetles were also positive for mollusc protein with AiW-1C9, but no beetles were positive for DNA and DrW-1G4. Based on the decay rates of slug DNA and protein within the guts of beetles (Dodd *et al.*, this thesis Chapter 5), the slug-positive beetles were considered to have consumed the slugs between 5-157 hours before capture for AiW-1c9, between 21-54 hours before capture for the *Arion* primers and 14-21 hours before capture for the *Deroceras* primers. The proportion of beetles positive with both antibodies was significantly different ($t = -8.86$; $P<0.001$), as were the numbers of DNA-positive and AiW-1C9-positive beetles ($t = -8.37$; $P<0.001$). There was no significant difference between the proportion of DNA-positive and DrW-1G4-positive beetles ($t = -0.63$; $P>0.05$).

6.4.4 Interaction between predators and prey

Linear regression analysis revealed some interesting interactions between beetle foregut slug/mollusc protein content and the availability of potential prey within the field. Significant relationships were observed when mollusc protein concentration equivalents (PCE) for AiW-1C9 (Figure 6.3a-e) were regressed against numbers of slugs, Acari, Diptera larvae, and *Aporrectodea* sp., and against beetle foregut weight. Of these the relationships were significantly positive for PCE against slug abundance and beetle foregut weight, and significantly negatively correlated for PCE against the abundance of *Aporrectodea* sp., Acari and Diptera larvae. Significant relationships were observed when mollusc protein concentration equivalents for DrW-1G4 (Figure 6.4a-d) were regressed against numbers of Hymenoptera, Aphididae, Araneae and against month. The relationship between PCE and the abundance of Aphididae, Araneae and Hymenoptera was significantly negative, where as the relationship between PCE and month was

significantly positive. Beetle foregut weight (Figure 6.5a-e) was significantly and positively correlated with slug and Collembola abundance and negatively correlated with the abundance of *Aporrectodea* sp., earthworms and month. No other comparisons were significant.

6.4.5 The effect of cultivation practice

No significant interactions were observed between either beetle foregut weight (F4,10 = 1.17; P>0.05) or beetle abundance ($F_{4,1520}$ = 1.25; P>0.05) or prey abundance (earthworms ($F_{4,10}$ = 0.40; P>0.05); slugs ($F_{4,10}$ = 0.99; P>0.05); Aphididae ($F_{4,10}$ = 0.23; P>0.05); Diptera larvae ($F_{4,10}$ = 0.09; P>0.05); Hymenoptera ($F_{4,10}$ = 0.01; P>0.05); Acari ($F_{4,10}$ = 0.21; P>0.05); Araneae ($F_{4,10}$ = 0.16; P>0.05); *Aporrectodea* sp. ($F_{4,10}$ = 0.15; P>0.05) with the five different cultivation treatments within the field.

6.5 Discussion

6.5.1 Prey-specific mtDNA primers or MAbs for use in predation field studies

For the first time, we have demonstrated that it is possible to identify predation by carabids in the field using DNA-based detection methods. Two previous studies have demonstrated that this is a suitable technique for identifying predation by invertebrates in the field (Agusti *et al.*, 2003; Hoogendoorn & Heimpel, 2003). In these studies, the proportion of predators testing positive for prey DNA ranged between 1 % (Hoogendoorn & Heimple, 2003) and 20-38 % (Agusti *et al.*, 2003).

In our analysis, a high percentage (43.3 %) of beetles tested positive for mollusc remains with both antibodies and 8 % were considered to have specifically consumed *D. reticulatum*. Only half the number of beetles tested positive for *D. reticulatum* DNA compared with the *D. reticulatum*–specific MAb, but this difference was not significant. These results are most probably a reflection of the low density of *D. reticulatum* in the field during the study period. By analysing the same samples with both MAbs and prey-specific primers, we can gain an understanding of the relative sensitivity of each method. We have previously demonstrated that MAbs are more sensitive than DNA based methods for detecting predation by carabids (Dodd *et al.*, this thesis Chapter 5). The difference between the proportion of slug DNA-positive beetles compared with the number of beetles positive for general mollusc remains is highly significant. MAbs are well known for their sensitivity and long detection periods (e.g. Symondson & Liddell, 1993b; Symondson & Liddell, 1995; Symondson *et al.*, 1999; Harwood *et al.*, 2001), but so long as predation can be detected in the 12 hour period prior to the capture of the predator, then longer detection is not necessarily an advantage. We suggest that the differences in the DNA results and those obtained with AiW-1C9 may be partially explained by the difference in sensitivity of the two techniques. Since AiW-1C9 is a general mollusc antibody, reacting equally well with slugs and snails (RS Thomas, A McKemy, WOC Symondson & DM Glen, unpublished) we suggest that some of the beetles testing positive with this antibody may have consumed snails, or species of slugs

other than *D. reticulatum* or *Arion* sp. No interaction was found between protein concentration in the beetle gut and snail abundance, but it is possible that only small juvenile snails are susceptible to predation by *P. melanarius.* In addition, flooding of soil samples may possibly not be an optimal method for assessing snail populations and therefore the availability of snails in the field may have been different to that indicated by this method of monitoring, particularly if snails were prevalent in the above ground vegetation. It is arguable that if snails were abundant in the vegetation then they would be unavailable to a ground dwelling predator such as *P. melanarius* until they descended the plants to the soil surface. However, monitoring the snail population in the crop canopy and in the marginal vegetation would have provided more accurate information of the abundance of snails at the field site.

The results obtained with the MAbs are consistent with those in previous studies (Symondson & Liddell, 1993a; Symondson *et al.*, 1996, 2002a; Bohan *et al.*, 2000). A previous study at the same field site found that 83 % of beetles tested positive for slug protein and that the quantity of slug antigen in the beetle's gut was correlated with the biomass of slugs in the soil (Symondson *et al.*, 1996). In addition, Bohan *et al.* (2000) found that 11 % of beetles trapped in an arable field during June and July tested positive for slug protein. These results (Symondson *et al.*, 1996; Bohan *et al.*, 2000) suggest that *P. melanarius* alters its activity-density in response to slug biomass and that this is to some degree related to cultivation practice, with beetles favouring plots that are themselves favourable to increased slug biomass (Symondson *et al.*, 1996). However, we found no relationship between cultivation practice and beetle or slug abundance.

6.5.2 Beetle-slug interactions

A significant positive relationship was observed between mollusc protein concentration equivalents and the availability of slugs (Figure 6.2a), which suggests that the availability of slugs is correlated with the availability of all molluscs in the diet of *P. melanarius.* Our results also show a significant increase in beetle foregut weight with increasing slug numbers (Figure 6.4a), which suggests that as the availability of slugs increases in the

field so does the level of predation on them by the beetles and this is correlated with better nutritional status of the beetles. However, at our site (Field 75), the numbers of *D. reticulatum* (n=10) obtained from the field by soil flooding was extremely low in 2001, compared with the same period in 2002 when 356 *D. reticulatum* were recorded. This difference could be accounted for, at least in part, by the climatic conditions experienced during the summer of 2001. The mean daily temperature, relative humidity and rainfall for June-September 2000-2002 are summarised in Table 6.2. The weather during the early part of the summer in 2001 was particularly hot and dry, becoming wetter, but not cooler in mid-summer then becoming drier again by September. In contrast, the average summer temperatures were cooler in 2002 and the early part of the summer was wetter. In hot dry conditions slugs retreat into deep crevices within the soil (Glen & Moens, 2002), where the level of ground moisture is higher, resulting in a corresponding decrease in surface activity (Port & Port, 1986). This is a particular problem in clay soil, as it retains moisture and contributes to slug survival, but in dry sandy soil, which has low moisture retention, slugs are unable to survive dry periods (Hunter, 1966). The soil at the field site is a sandy-clay loam and we expect that slugs survive dryness by moving into deep soil as it dries out. Soil samples taken throughout the study only examined the top 10 cm of soil, and Glen *et al.* (1992) found that slugs were virtually absent from the top 10 cm of arable soil in the dry summers of 1983 and 1984, but the numbers rapidly recovered in the autumn when the soil became moister. As the soil samples taken throughout the study only examined the top 10cm of soil, it is probable that most molluscs sheltering deeper in the soil were missed and therefore the numbers trapped are not necessarily indicative of the total population and hence potential for predation on these species in the field.

Another factor that could affect the degree of predation by the beetles on slugs is the size of slug available in the field. The mean size class of *D. reticulatum* found at the field site between June and September 2001 was 73.1 mg (range = 7.1-257.9 mg) and for *A. intermedius* was 71.8 mg (range = 37.4-211.6 mg). McKemey *et al.* (2001) demonstrated in laboratory arena trials that *P. melanarius* preferred slugs that were <40 mg, although

the beetles were able to kill slugs >40 mg after prolonged exposure. However, beetles recently collected from the field have been observed to attack slugs that are much larger than this (McKemey and Thomas *pers obs*). Bohan *et al.* (2000) reported a significant association of beetles to the distribution of slugs >25 mg in the field and suggested that the smallest slugs were unavailable to the beetles as they remained under the soil surface. We would suggest that this observation is supported by our results, as under the dry conditions experienced in the field in 2001, the smallest individuals would be able to shelter in the deepest crevices out of range of the beetles.

Alternatively, our results, showing a high percentage of slug positive beetles, may be partly accounted for by secondary predation, as generalist predators are known to consume other predators or their remains in the field (Chiverton, 1984). It has been frequently suggested that analysis with antibodies may detect secondary predation where the antibody reacts to the proteins in the guts of the initial rather than final predator (Sunderland, 1996). This was found in a polyclonal antiserum against *D. reticulatum* (Tod, 1973), but recent evidence (Harwood *et al.*, 2001), shows that such food chain errors are unlikely to play a significant role in predation as prey from the initial predator was only detected using MAb ELISA immediately after it was fed to the second predator. Therefore, it is highly unlikely that the confounding effects of secondary predation influenced the results presented here.

6.5.3 The role of alternative prey in the diet of P. melanarius

P. melanarius is a highly generalist species reportedly feeding on almost any invertebrate prey with no clear preferences (Symondson *et al.*, 2002a). Investigations with carabids indicate that they feed on a range of prey including Annelida, Arachnida, Diptera, Hemiptera, Hymenoptera, Coleoptera, Araneae and Mollusca (Pollet & Desender, 1987; Dixon & McKinlay, 1992; Symondson *et al.*, 2000; Snyder & Ives, 2001).

There was a positive correlation between beetle foregut weight and the concentration of slug protein within the beetle's gut (Figure 6.3e) and this positive relationship was most

probably the result of predation by beetles on slugs, since gut weight (Figure 6.5a) and the concentration of slug protein in the beetle gut (Figure 6.3a) increased with increasing slug abundance. Significant negative relationships were observed between the availability of earthworms and mean PCE in the beetle gut (Figure 6.3b) and between beetle foregut weight and the availability of *Aporrectodea* sp. and earthworms (Figures 6.5b and c, respectively). This suggests that, when predation on slugs by beetles is high, the availability of earthworms is low, but as the number of slugs decreases, predation on earthworms probably increases (Figures 6.3a and 6.5b-c). During the sampling period, the number of earthworms caught was much greater then slugs (Figure 6.2) and therefore the potential for predation on worms was greater. Symondson *et al.* (2000) found that >36 % of *P. melanarius* trapped from the field had consumed earthworm. Their results showed that as beetle foregut weight and total food availability declined, the earthworm content of the beetles guts progressively increased. This suggested that *P. melanarius* was feeding increasingly on earthworms at times when alternative or preferred prey were less available. Harwood *et al.* (unpublished) have shown in laboratory feeding trials that slugs are poor quality prey for *P. melanarius* and that earthworms, either alone or as part of a mixed diet, produced heavier beetles. In addition, when one or more further prey items were added to poor quality slug or aphid diets, there was a corresponding increase in beetle weight gain (Harwood *et al.*, unpublished). This suggests that earthworms are nutritionally preferred prey items in the diet of *P. melanarius*. Therefore, in the field it is possible that beetles either switch to eating earthworms in preference to slugs when they are available or that earthworms are more accessible to beetles when unfavourable conditions drive slug deep into soil crevices where the beetles are unable to reach them.

A similar negative relationship was observed between beetle foregut slug protein concentration and the availability of Diptera larvae (Figure 6.3d). Harwood *et al.* (unpublished) showed that *P. melanarius* will eat Diptera larvae in the laboratory, but they are like slugs and aphids, poor quality prey items as a single species diet. However, as part of a mixed diet they increase beetle fitness and fecundity (Harwood *et al.*, unpublished). It is possible that in the absence of slug prey, beetles will also consume

Diptera larvae under circumstances similar to those described above for earthworms. It is thought that a varied diet increases the chance of obtaining all the necessary amino acids necessary for increased fecundity and survival (Greenstone, 1979; Wallin *et al.*, 1992; Toft, 1996). The ability to utilise alternative prey when preferred prey densities are low places generalist predators at an advantage over those that are more specialised, particularly in a disturbed agricultural environment since it enables them to retain reasonable population densities (Doutt & DeBach, 1964; Murdoch *et al.*, 1985).

A significant negative relationship was found between slug protein concentration in beetle foreguts and the density of Aphididae (Figure 6.4a). The beetles are known to consume aphids (Winder, 1990), even though they are a poor quality prey item (Harwood *et al.*, unpublished). Slug protein concentration in the beetle's gut was low when there were few slugs available. This decrease in slug number coincided with the time when the abundance of aphids in the field increased in July/August. Therefore the fact that weather conditions suitable for aphid increase are unsuitable for slug activity on the soil surface may explain the negative correlation between aphid abundance and predation on slugs by beetles. This is also the most likely explanation for the negative relationship between spider abundance and slug protein concentration in beetle foreguts. Beetles have been observed to pick lyniphiid spiders from their webs (Harwood, *pers. obs.*) and feed readily on these prey items in the laboratory (Harwood *et al.*, 2001), but it is not a common event in the field and it would not account for the relationship observed here. As with the aphids, spider abundance and activity peaked in mid-summer, coinciding with the decrease in the slug population/available slug biomass during the hottest part of the year. The interaction between Hymenoptera and slug protein concentration in beetle guts is probably coincidental and caused by a secondary unknown factor. However, it is also possible that an interaction between aphids and their hymenopteran parasitoids is responsible for the relationship between protein concentration and hymenopteran abundance (Figure 6.4b). The most abundant family of Hymenoptera caught on sticky traps were Braconidae some of which are parasitoids of aphids. Only a few aphid mummies were found on the sticky traps and these were insufficient to account for a high

level of parasitism. However, some braconid species cement their aphid hosts down before pupating in them (Chinery, 1993) so the majority of mummies are likely to remain attached to the plants rather than fall to the ground.

A significant relationship between beetle foregut weight and the density of Collembola is suggested (Figure 6.5b), which would indicate that Collembola contribute significantly to the diets of beetles. However this is unlikely as morphological constraints such as mandible size mean that beetles like *P. melanarius* with large mandibles are incapable of handling very small prey (Finch, 1996). Therefore, due to their size, it is unlikely that the beetles will be capable of feeding on most Collembola (or Acari). We suggest that this relationship is coincidental as both slugs and Collembola are attracted to areas of high moisture content. The beetles would be attracted to these areas to prey on the slugs and hence a positive correlation would be seen between beetle gut weight (primarily caused by predation on slugs) and a high abundance of Collembola. Humidity is therefore the secondary factor causing the relationship rather than predation. This may also explain the relationship between Acari and slug protein content in the beetle guts (Figure 6.3c).

6.5.4 The effect of cultivation practice on predators and prey

In this study it appears that cultivation practices have had no effect on predation by beetles as no significant interactions were observed between cultivation treatment and a) beetle abundance, b) beetle foregut weight or c) beetle foregut slug protein concentration equivalents. Positive relationships have been previously shown to exist between slug and beetle populations and cultivation practice (Symondson *et al.*, 1996, 2002b). Studies (Bohan *et al.*, 2000; Symondson *et al.*, 2002b) suggested that the distribution and activity density of beetles is related to the abundance and distribution of slugs, and that slug distribution can be influenced by cultivation treatment (Symondson *et al.*, 1996). The number of slugs that were trapped during the period of this study was exceptionally low, and there was no relationship between the distribution of the slugs and cultivation treatment (data not shown). It is likely therefore that there was simply not a sufficiently large slug population during the period of the study to reveal any such relationships.

6.5.5 Conclusions

We have been able to demonstrate for the first time that it is possible to detect predation by carabids on slugs in the field. Although our results show that MAb ELISA detects a greater number of predation events, the strength of DNA-based methods relates to the identification of several different prey species in the same assay. By developing genus level primers (i.e. general *Arion* primers – amplicon I) that amplify a section of DNA that varies in length between species, it is possible to identify species by amplicon size. Although primers were not multiplexed in these PCR reactions this would indeed be possible and would further improve the cost effectiveness and efficiency of the assay.

6.6 References

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6.7 Author information box

This study was conducted as part of a PhD studentship funded by Cardiff University and BBSRC. Ciara Dodd was a PhD student working with Bill Symondson and Mike Bruford's groups at Cardiff University. Bill Symondson has worked for many years on the development of novel methods of detecting and quantifying prey remains in predators. The field study was part of a long-term collaboration between Bill Symondson and David Glen at IACR-Long Ashton, studying predator-prey interactions in agroecosystems. The field sampling was conducted by technician Sue Coe and by postdoc James Harwood, and specimens were identified by James Harwood.

6.8 Figure legends

Figure 6.1: Field 75 IACR-Long Ashton, showing plot number and treatment allocation.

Figure 6.2: The number/treatment/month of all species of earthworm, the most common species of earthworm (*Aporrectodea* sp.) and slugs trapped between June-September 2001 by formaldehyde extraction and soil flooding respectively.

Figure 6.3: Relationships between beetle foregut slug protein concentration, identified using the MAb AiW-1C9, and the number/treatment/month of a) slugs, b) *Aporrectodea* sp., c) Acari, d) Diptera larvae, and e) beetle foregut weight.

Figure 6.4: Relationships between beetle foregut slug protein concentration identified using the MAb DrW-1G4 and the number/treatment/month of a) Aphididae, b) Hymenoptera, c) Araneae, and d) time.

Figure 6.5: Relationships between beetle foregut weight and the number/treatment/month of a) slugs, b) *Aporrectodea* sp., c) earthworms, d) Collembola, and e) time.

6.9 Tables

Table 6.1: Number of slugs, snails (*Candidula intersecta (Ci), Vallonia pulchella (Vp), Vertigo pygmaea (Vpy), Cochlicopa lubrica (Cl)*), earthworms and the most common genus of earthworms *Aporrectodea* (*Ap.* sp), caught by gradual soil flooding (slugs and snails) and by formaldehyde extraction (earthworms) between June-September 2001. Treatment refers to type of cultivation practice: 1 = straw incorporated; non-inversion tillage, 2 = straw incorporated; plough, 3 = stubble incorporated; non-inversion tillage, 4 = stubble incorporated; plough, 5 = stubble; non-till direct drilling.

Month	Treatment	Slugs	*Ci*	*Vp*	*Vpy*	*Cl*	Earthworms	*Ap.* sp
June	**1**	2	1	1			16	
	2						1	
	3		3				4	
	4						3	
	5	3	2	4			9	
July	**1**	3		1			93	51
	2	1					75	49
	3	1					62	30
	4	1		2			69	36
	5	1	1				51	16
Aug	**1**	2	1	8			146	40
	2						162	60
	3		3	1			99	38
	4						69	47
	5	2	1	12	7	6	137	58
Sept	**1**	1					76	36
	2						108	62
	3						122	44
	4						72	41
	5	1					138	32

Table 6.2: Identification of *Arion*-positive field-caught beetles using general *Arion* primers, showing estimated time since ingestion and comparison with ELISA results for the same individuals. The detection limit for DrW-1C9 is 3.20ng/200ul; for DrW-1G4 is 52.11 ng/200ul; for mtDNA primers is 100 fluorescence units (FU). Monoclonal antibody positive samples are highlighted in **bold** type. Time since ingestion was calculated using the regression equations $\log_{10}(y+1) = -0.1536\text{sqrt}(x) + 2.4649$ for DrW-1C9, $\log_{10}(y+1) = -0.1249\text{sqrt}(x) + 2.8435$ for DrW-1G4 and $\text{sqrt}(y) = -11.281\text{sqrt}(x) + 97.395$ for the mtDNA *Arion* primers.

Sampling Date	Gut weight (g)	Amplicon size (bp)	Species	DNA (FU)	Time since ingestion (h)	1C9 PCE (µg/200µl)	Time since ingestion (h)	1G4 PCE (µg/200µl)	Time since ingestion
14 June	0.0049	218.9	AD	2000	21.79	0.00	-	14.91	178.78
21 June	0.0078	209.11	AH	200	54.45	**357.22**	0.33	45.87	89.55
25 July	0.0045	209.36	AH	400	47.06	0.00	-	10.06	217.24
02 Aug	0.0121	215.94	AI	1500	27.03	**104.92**	8.36	19.17	156.18
05 Sept	0.0096	216.16	AI	2000	21.79	**710.33**	6.33*	14.37	182.23
05 Sept	0.0033	218.35	AD	300	50.37	0.00	-	46.06	89.28

* Anomalous result beyond the upper limit of the assay. Very high and very low protein concentration equivalent values are often beyond the sensitivity of the spectrophotometer used to read ELISA plates, and therefore an inaccurate reading is obtained. In this case, a PCE of 710 ng/200µl should have a calculated time since ingestion which is more recent than for the other samples. However, an intermediate value is obtained indicating that this reading is beyond the scope of the assay.

Table 6.3: Identification of *Deroceras* positive field caught beetles using general *Arion* primers, showing estimated time since ingestion and comparison with ELISA results for the same individuals. The detection limit for AiW-1C9 is 3.20ng/200µl; for DrW-1G4 is 52.11 ng/200µl; for mtDNA primers is 100 fluorescence units (FU). Monoclonal antibody positive samples are highlighted in **bold** type. Time since ingestion was calculated using the regression equations $\log_{10}(y) = -0.1536\text{sqrt}(x) + 2.4649$ for DrW-1C9, $\log_{10}(y) = -0.1249\text{sqrt}(x) + 2.8435$ for DrW-1G4 and $\log_{10}(y) = -0.0638x + 3.3783$ for the mtDNA *Deroceras* primers.

Sampling Date	Gut weight (g)	DNA (FU)	Time since ingestion (h)	1C9 PCE (µg/200 µl)	Time since ingestion (h)	1G4 PCE (µg/200 µl)	Time since ingestion (h)
18 July	0.0071	110	20.95	1.72	210.66	0.00	-
18 July	0.0045	247	15.45	0.00	-	12.21	197.84
18 July	0.0062	200	16.89	**79.61**	13.48	7.79	244.24
18 July	0.0036	150	18.84	**6.03**	120.28	2.92	362.53
18 July	0.0082	110	20.95	1.72	210.66	36.34	105.54
18 July	0.0089	100	21.06	**326.46**	0.10	3.12	353.81
18 July	0.0077	209	16.59	**6.89**	112.16	5.19	290.37
18 July	0.0088	217	16.33	0.00	-	5.52	283.11
18 July	0.0033	175	17.79	0.00	-	9.74	220.56
18 July	0.0041	276	14.69	**11.09**	85.46	17.4	164.71
18 July	0.0078	111	20.89	**8.61**	99.21	17.85	162.44
18 July	0.0075	150	18.84	**3.44**	157.61	9.41	224.14
18 July	0.0061	148	18.93	**24.98**	48.28	5.84	276.56
25 July	0.0109	100	21.06	0.00	-	15.58	174.72
25 July	0.003	124	20.14	**25.74**	47.11	32.12	114.54
25 July	0.0125	100	21.60	1.43	226.09	4.87	297.96
25 July	0.0061	156	18.58	0.00	-	19.79	153.42
25 July	0.008	137	19.46	**30.02**	41.33	10.06	217.24
25 July	0.0051	144	19.12	0.96	-	17.99	161.75
25 July	0.007	194	17.09	**17.63**	62.95	14.28	182.82
02 Aug	0.0087	241	15.62	0.00	-	11.79	201.28
02 Aug	0.0097	294	14.26	**127.69**	5.45	11.36	204.96
02 Aug	0.0056	142	19.22	0.00	-	26.04	130.69

Table 6.4 : Mean daily temperature (°C), mean relative humidity (%), total rainfall (mm) and number of wet days per month from June-September 2000-2002 at Totterdown, Bristol, UK weather centre. Source: www.afour.demon.co.uk

Month	Mean daily temperature °C	Mean Relative Humidity (%)	Total rainfall (mm)	Number of wet days	Comments
2000					
June	17.2	76	27.9	12	Warmest June since 1976. Rainfall only 45.5% of 30 year average.
July	17.9	78	56.5	11	Warmer than 30 year average. Rainfall 108% of 30 year average.
August	18.5	83	133.9	10	Rainfall 198% of 30 year average.
September	15.7	93	137.2	19	Wettest September since 1976. Rainfall 187% of 30 year average.
2001					
June	16.5	80	35.6	7	Exceptionally hot dry month. Lowest monthly rainfall total since June 2000.
July	18.9	84	73.9	9	Rainfall 157% of the 30 year average for July
August	22.6	87	120.9	17	Rainfall 163% of the 30 year average for August
September	15.1	83	26.6	15	Warmer and drier than average month with 1/3 of the usual September rainfall
2002					
June	15.6	83	63.7	15	Rainfall 102% of 30 year average.
July	17.5	86	72.9	8	Coolest July since 1993 and wettest since 1992. Rainfall 146% of 30 year average.
August	18.6	84	48.2	6	Rainfall only 65% of 30 year average.
September	16.0	86	40.9	6	Higher than average temperature. Rainfall only 52% of 30 year average.

6.10 Figures

Figure 6.1

Treatment	Straw disposal	Cultivation
A	Straw incorporated	non-inversion tillage
B	Straw incorporated	plough
C	Stubble incorporated	non-inversion tillage
D	Stubble incorporated	plough
E	Stubble	no-till, direct drilling

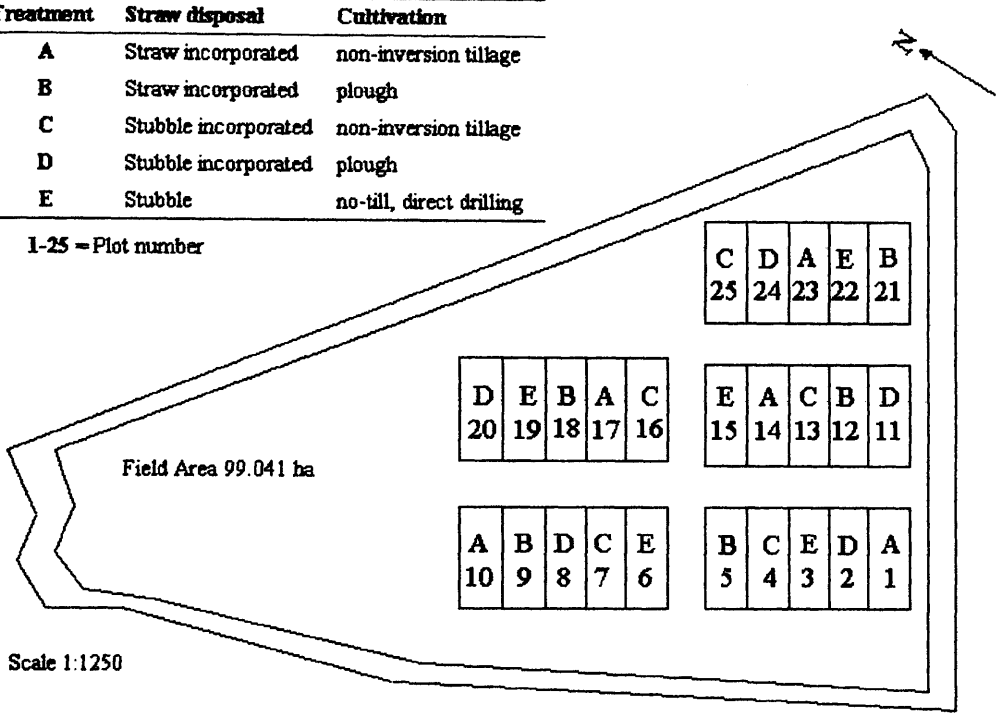


Figure 6.2

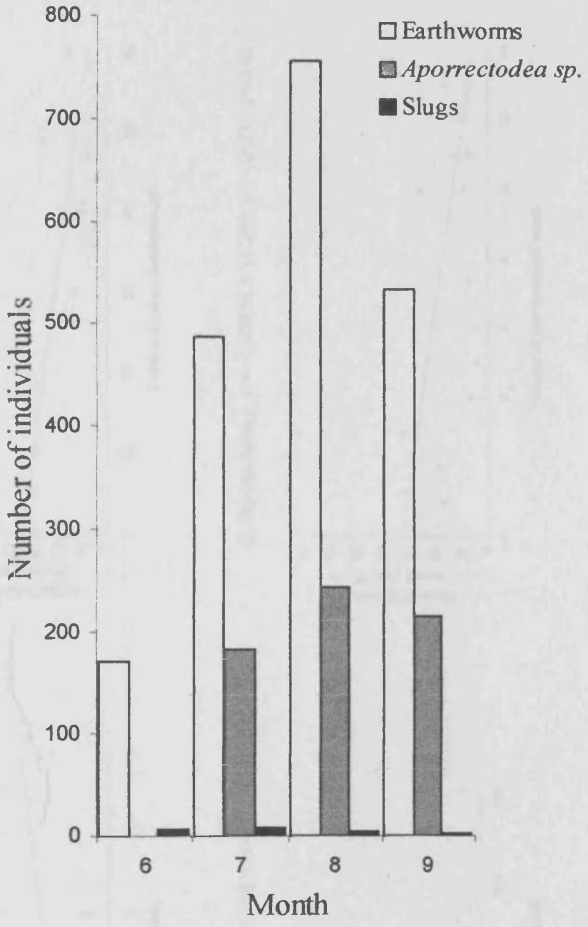
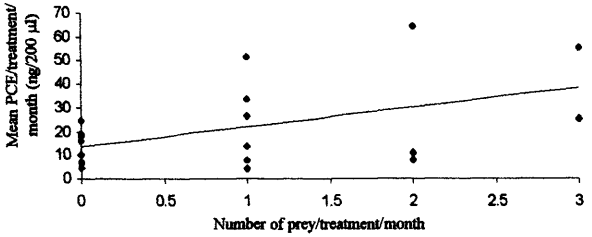
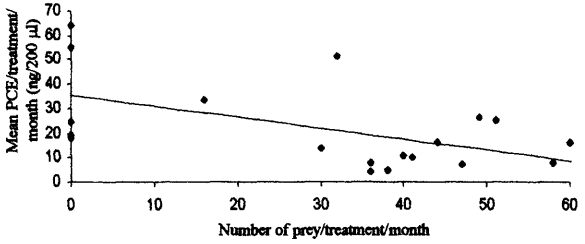

800
700
600
500
400
300
200
100
0
Number of individuals
6
7
8
9
Month
Earthworms
Aporrectodea sp.
Slugs

Figure 6.3

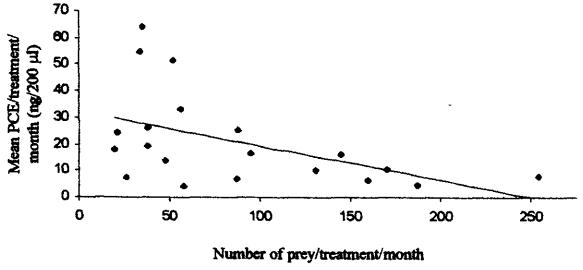
a) Slugs; $y = 8.2321x + 13.669$; $R^2 = 0.2302$; $P<0.05$



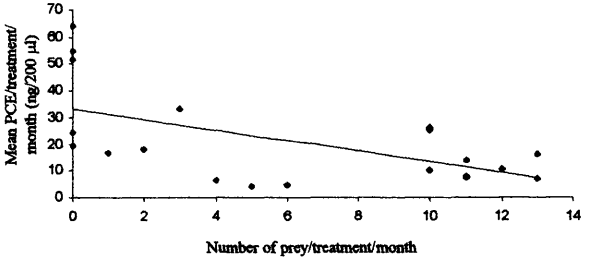
b) *Aporrectodea* sp.; $y = -0.4486x + 35.434$; $R^2 = 0.3098$; $P<0.05$



c) Acari; $y = -0.1293x + 32.351$; $R^2 = 0.238$; $P<0.05$



d) Diptera larvae; $y = -1.9985x + 33.269$; $R^2 = 0.337$; $P<0.01$



e) Beetle foregut weight; $y = 6452.6x + 27.836$; $R^2 = 0.3226$; $P<0.01$

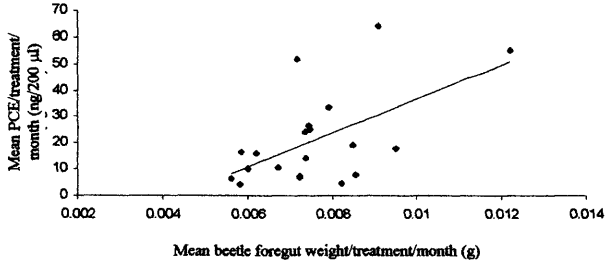
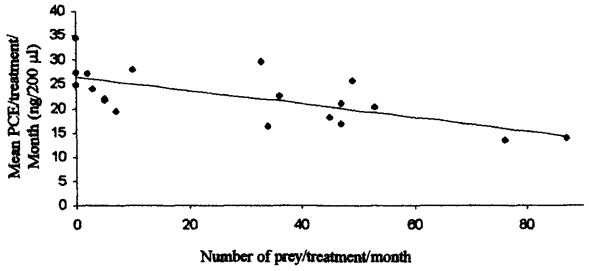
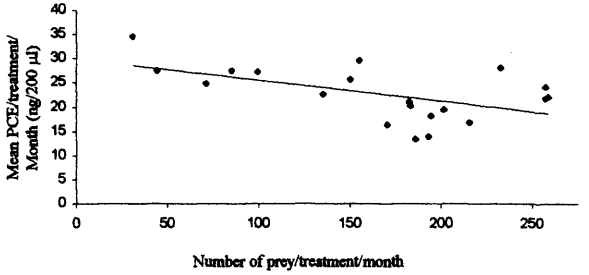


Figure 6.4

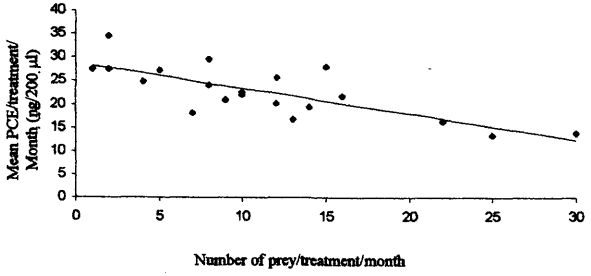
a) Aphididae; $y = -0.1408x + 26.466$; $R^2 = 0.485$; $P=0.001$



b) Hymenoptera; $y = -0.0436x + 29.868$; $R^2 = 0.2948$; $P<0.05$



c) Araneae; $y = -0.549x + 28.848$; $R^2 = 0.5805$; $P<0.001$



d) Month; $y = 2.2263x + 5.9748$; $R^2 = 0.2132$; $P<0.05$

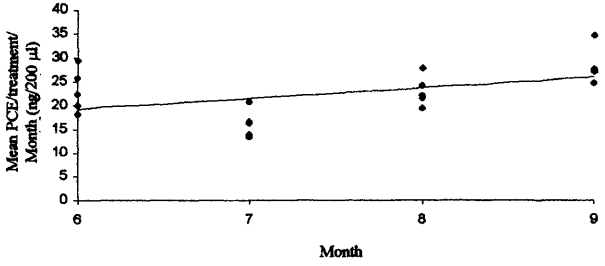
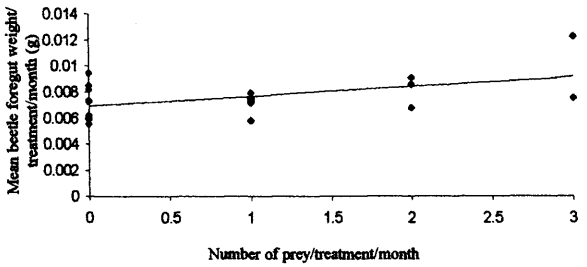
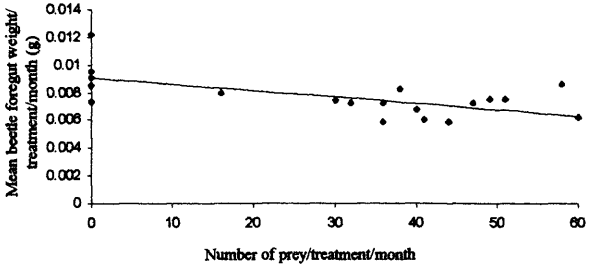


Figure 6.5

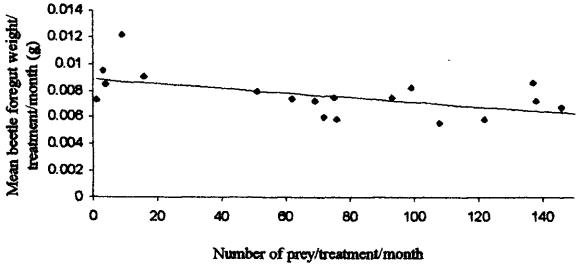
a) Slug density; $y = 0.0007x + 0.0069$; $R^2 = 0.2338$; $P<0.05$



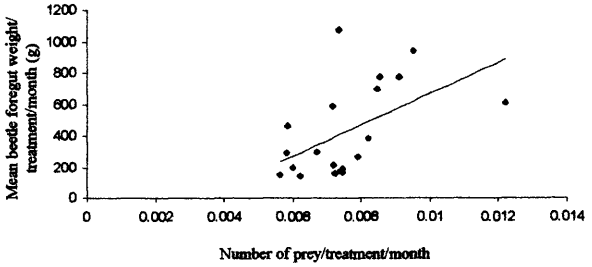
b) *Aporrectodea* sp.; $y = -5E\text{-}05x + 0.0009$; $R^2 = 0.4185$; $P<0.01$



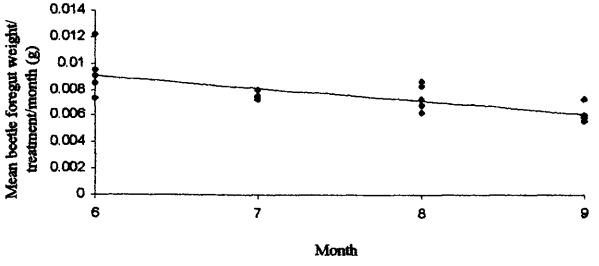
c) Earthworms; $y = -2E\text{-}05x + 0.0089$; $R^2 = 0.33$; $P<0.01$



d) Collembola; $y = 100076x - 330.58$; $R^2 = 0.2755$; $P<0.05$



e) Month; $y = -0.001x + 0.0149$; $R^2 = 0.5317$; $P<0.001$



Chapter 7

General Discussion

7.1 Discussion

The results reported in this thesis provide details of techniques that may become essential in the future for investigating predator-prey interactions. The study of predation by arthropods especially on soft-bodied prey such as slugs is difficult as few solid remains can be detected in the gut. Indigestible remains, such as the radula, may not always be consumed by the predator (Davies 1953; Luff 1974). The use of biochemical techniques for investigating predation under these circumstances has an established history (Symondson, 2002a, b) and the use of DNA-based methods have been shown to have potential in this area of research (Agusti *et al.*, 1999, 2000, 2003a, b; Zaidi *et al.*, 1999; Chen *et al.*, 2000; Hoogendoorn & Heimpel, 2001). However, until now there have been no published studies using DNA-based techniques for investigating predation on slugs (Chapter 2 and 5), no comparison of the sensitivities of biochemical and molecular technologies (Chapter 5), and no application of DNA-based methods for detecting predation by carabids in the field (Dodd *et al.*, 2003; Chapter 6).

This study is the first to investigate predation on slugs by carabids using DNA-based methods. Earlier studies using DNA techniques showed limited success when long fragments of single copy nuclear DNA were amplified (Agusti *et al.*, 1999, 2000). The detection times achieved, improved when shorter fragments and multiple copy genes such as nuclear genes conferring insecticide resistance (Zaidi *et al.*, 1999) or ribosomal gene clusters (Hoogendoorn & Heimpel, 2001) were targeted. Only two published studies have utilised multiple copy mitochondrial DNA (mtDNA) (Chen *et al.*, 2000; Agustí *et al.*, 2003), targeting the mitochondrial cytochrome oxidase subunits II and I genes respectively. The results reported in this thesis have shown that the mitochondrial 12S rRNA gene is also a useful target for detecting predation on slugs, providing sufficient variability to allow differentiation at the species and genus levels. Primers were designed that amplified fragments of mtDNA, ranging in size from 99-294 bp, which successfully detected slug remains from within the guts of the carabid beetle *Pterostichus melanarius* (Illiger). Five primer pairs were designed that specifically amplified *Deroceras*

reticulatum (Müller), one pair each were designed that specifically amplify *Arion hortensis* (Férussac), *Arion distinctus* Mabille and *Arion intermedius* Normand, and one pair amplified all members of the genus *Arion* that were tested.

Laboratory based feeding trials were conducted to allow determination of the rate of decay of slug DNA and protein within the gut of the beetle. Although previous studies have shown shorter detection periods for larger DNA amplicons (Agusti *et al.*, 1999, 2000; Chen *et al.*, 2000; Hoogendoorn & Heimpel, 2001), no significant difference was found between the rates of decay of the different sized amplicons produced by the species-specific or general *Arion* primers used in these investigations. Slug DNA was detected for a maximum of 49 hours following consumption by the beetle, compared with a maximum period of 32 hours for pear psyllid mtDNA within the gut of a heteropteran predator (Agustí *et al.*, 2003). Therefore, the results of these experiments have provided the longest detection limits of digested prey DNA to date.

To date no studies have attempted to quantify predation using DNA-based methods. In order to address this issue, we analysed the beetle gut samples with both unlabelled and fluoro-labelled mtDNA primers. The amplicons produced using unlabelled primers were screened on agarose gel stained with ethidium bromide (Chapter 2), but the amplicons produced with the fluoro-labelled primers were run on acrylamide gel and analysed using ABI Genescan™ and Genotyper™ software (Chapter 5). The intensity of fluorescence of each PCR in the decay rate experiment was used to provide a semi-quantitative measure of the rate of DNA decay since ingestion by the beetle. These results were compared with the rate of decay of slug protein within the beetle gut to ascertain the relative sensitivity of the two techniques.

Previous studies have determined the rate of decay of slug protein within in beetle guts using monoclonal antibody (MAb) techniques (e.g. Symondson & Liddell, 1993a, b, 1996; Symondson *et al.*, 1995, 1997, 1999). In general, the use of MAbs coupled with enzyme linked immunosorbent assay (ELISA) is a very sensitive assay system, resulting

in extended detection limits for mollusc remains within beetle guts. In fact, this is generally considered the method of choice for arthropod predator gut content analysis (Symondson, 2002a, b). However, since the advent of reliable DNA-based detection methods (e.g. Zaidi *et al.*, 1999; Agustí *et al.*, 2003), there have been no publications directly comparing the sensitivity of these two methodologies. In order to address this issue, we extracted both slug protein and DNA from beetle guts in timed laboratory feeding trials (Chapter 5) and from field caught specimens (Chapter 6). In terms of sensitivity and detection limit, MAb ELISA was shown to be a much more sensitive technique. In all comparisons, the gradient of the slope was steeper for the rate of decay of DNA compared with protein, reflecting the shorter detection limit of DNA. However, extended detection limits are not always advantageous as the critical time for identifying predation events is the 12 hour period prior to the capture of the predator. To be able to gain an insight into predator behaviour in the field, it is necessary to relate predator gut contents with the availability of prey at the time of capture. If an assay system identifies the remains of prey consumed by the predator several days prior to capture then this will make interpretation of the degree of predation on the availability of prey difficult. All the primer pairs used here were able to reliably amplify prey remains for 24 hours following consumption.

A number of studies have applied antibody technology to the analysis of predation by carabids in the field (Symondson *et al.*, 1996, 2002; Bohan *et al.*, 2000), but none have utilised DNA-based methods. Only two studies have investigated predation in the field by invertebrates other than carabids. In both cases, prey remains were successfully amplified from field caught predators using prey-specific ITS (Hoogendoorn & Heimpel, 2003) and mtDNA (Agusti *et al.*, 2003b) primers. The results presented in Chapter 6 demonstrate that mtDNA primers are suitable tools for detecting predation on slugs by beetles in the field. Analysis of the same samples with both mtDNA primers and MAbs demonstrated that MAb ELISA reveals a greater proportion of predation events than PCR. This is unsurprising as we demonstrated in laboratory decay rate experiments that the rate of decay of DNA was faster than for protein. Comparison of results suggests that

beetles could have consumed slug, but at a time beyond the detection limit of the primers and sensitivity of the assay. An alternative explanation is that the larger proportion of positive samples observed from the general mollusc antibody result from predation on other species of slugs and/or snails. Two-way ANOVA and regression analysis revealed that beetle foregut slug protein content increased with increasing slug abundance, and that beetle foregut weight increased with increasing slug abundance. These results indicate that the results obtained with the antibodies were in fact due to predation on slugs by the beetles. However, we found no interaction between these variables and cultivation practise. This was surprising, as previous studies of predation by carabids on slugs at the same field site revealed interactions between the slug and beetle population with cultivation treatment (Symondson *et al.*, 1996, 2002). We suggest that the extremely low slug population during the period of our investigation may account for this difference.

Interactions between beetles and the availability of alternative prey species were also observed. In particular, a negative correlation between slug protein concentration in the beetle gut and the abundance of earthworms suggested that when the availability of slugs was low, earthworms became an important item in the beetle's diet. Symondson *et al.* (2000) suggested that earthworms played an important role in the diet of *P. melanarius*, particularly when the availability of preferred prey items such as slugs was limited. Our results would also suggest that this is the case.

In order to design robust species-specific mtDNA primers for closely related species, it was necessary to sequence individuals from a wide geographic range to accommodate intra-species genetic variability. Analysis of these sequences revealed considerable intra- and inter-specific variation within members of the *Arion hortensis* aggregate, which may provide useful phylogenetic (Chapter 3) and phylogeographic (Chapter 4) information. Therefore, this work was expanded to maximise the geographic spread and number of individuals sampled per population. To the best of our knowledge, there have been no publications relating to the molecular evolution or phylogeography of the *Arion hortensis*

aggregate within the British Isles. Previous taxonomic separation of these species has concentrated on morphological (Davies 1977, 1979) and biochemical data, primarily at the species rather than the population level. The results of phylogenetic analysis (Chapter 3) of the *A. hortensis* aggregate using mtDNA sequence data confirms the results of earlier studies using enzyme electrophoresis (Backeljau, 1985a, b, 1987) showing that these are three distance species. Trees derived from the 12S rRNA gene indicate a close relationship between *A. intermedius* and *A. owenii*, indicating that *A. intermedius* is part of the same subgenus, *Kobelita* as the *A. hortensis* aggregate. This was suggested from morphological (Davies, 1979) and enzyme electrophoresis (Backeljau, 1985a) and our results add validity to this proposition. In general; we found the 12S rRNA gene to be much more informative than COI for determining species relationships.

It has been suggested that the mitochondrial genomes of at least some species of mollusc are evolving rapidly (Chiba, 1999). The data presented in Chapter 3 of this thesis indicate that this is also the case for the *A. hortensis* aggregate which shows levels of intra- and inter-specific divergence that are greater than would normally be expected for closely related species. Investigation of sequence characteristics such as nucleotide composition revealed patterns comparable with other molluscs (Medina & Walsh, 2000) and across wider taxonomic groups (Vawter & Brown, 1993), particularly for the 12S rRNA gene. In terms of 12S rRNA secondary structure slug sequences adhered well to the structural template (Hickson *et al.*, 1996), showing patterns of variation in regions of the template that were also variable in other taxa (Hickson *et al.*, 1996; Medina & Walsh, 2000).

At the population level, high levels of polymorphism were observed within and between populations of these slugs. Similar levels of heterogeneity have been recorded in *D. reticulatum* mtDNA (Brookes, 2002), and snails e.g. *Cepaea nemoralis* (Thomaz *et al.*, 1996), *Helix aspersa* (Guiller *et al.*, 2001) and *Mandarina* (Chiba, 1999), although other species appear to be much less divergent, e.g. limpets of the genus *Patella* (Koufopanou *et al.*, 1999). Both *A. hortensis and A. distinctus* have common widespread haplotypes,

occurring in the majority of populations sampled and these probably represent ancestral haplotypes. Mismatch distribution indicates founder events occurred between 154,000-15,000 BP followed by rapid expansion for all three aggregate species. It is probable, particularly for *A. distinctus* that the populations have gone through bottlenecks caused by environmental fluctuations during glacial oscillations. However, it is debatable whether these species would have been able to survive in refugia in the peri-glacial zone in the south during the last glacial maximum, although at least two species of slugs, *Testacella maugei* Férussac and *Geomaculus maculosus* Allman may represent such *in situ* survivals during this period (Kerney, 1966). If these species, which are some of the least cold tolerant slugs that occur in the UK were able to survive then it is likely that hardier species such as the arionids were also able to do so. However, analysis of mtDNA alone will only provide a partial gene genealogy, which may present an incomplete history of the organisms if hybridization has taken place. The neutrality of mtDNA means that it may flow relatively easily through a hybrid zone and therefore mtDNA haplotype patterns could mask any hybrid zones between species. If we are to gain a complete picture of the demographic history of these species, it will be necessary to extend the analysis to include nuclear markers. Since hybridisation between several species of closely related Arionid slugs is well documented, it is also possible that members of the *A. hortensis* aggregate may form hybrid species under certain circumstances, such as founder events (DeSalle & Giddings, 1986; Kaneshiro, 1989).

7.2 Future Research

Although the confounding effects of secondary predation have been shown to be minimal with monoclonal antibodies and ELISA, this issue has not so far been addressed using DNA-based detection methods. Personal observation would suggest that food chain errors would not be elucidated using PCR, as reliable amplification of digested slug was only possible when the beetles had consumed fresh material. Similarly, the effects of scavenging have not been investigated. Currently the used of MAb ELISA in not able to distinguish predation from scavenging, but for the reasons stated above, we would speculate that this is unlikely to be a source of error with DNA-based detection methods.

One potential advantage of DNA-base detection methods over those using antibody technology is the possibility of screening a sample for the remains of multiple prey in the same reaction. The utility of multiplexed PCR reaction should be investigated as this would radically decrease screening cost and increase efficiency. This approach should be particularly applicable to the use of fluorescent PCR techniques.

Analysis of the foregut contents of beetles caught form the field showed a high proportion of individuals positive for mollusc remains. Only a fraction of these individuals had consumed *D. reticulatum*, the most prevalent pest species of slug in agroecosystems. It is possible that these beetles had consumed snails, therefore specific primers should be designed for the snail species found at the field site to determine whether the beetles preying on snails.

To gain a more accurate picture of the phylogeography of the *A. hortensis* aggregate in the British Isles and Europe, individuals from a wider geographic range, particularly from mainland Europe need to be analysed. In particular, these will be required to address population structure and past demographic history. Nested clade analysis was unable to differentiate between isolation by distance and past fragmentation in terms of population structure. European samples will be particularly valuable for determining the postglacial

history of the aggregate in the British Isles and may give a better indication of routes of colonisation from mainland Europe.

In order to accurately determine the phylogenetic relationships within the *A. hortensis* aggregate, and in particular to resolve the supposition that *A. intermedius* is indeed part of the same subgenus, other members of the genus need to be analysed.

7.3 Conclusions

This study has contributed to developing novel techniques for investigating invertebrate predator-prey interactions and it is a further step towards the use of fluorescent-based approaches for predator gut content analysis and quantification of predation using DNA. This is the first investigation to compare directly the sensitivity of protein and DNA based approaches, and although the greater sensitivity of antibodies compared with PCR has been revealed, the advantages and disadvantages of both techniques have been highlighted. These results demonstrate that DNA-based approaches are appropriate for investigating predation by carabids on slugs in the field.

Phylogenetic and phylogeographic analysis of the *A. hortensis* aggregate has revealed interesting relationships within the Arionidae at the subgeneric and population levels and has provided important information regarding the demographic history of these species in the British Isles. Of particular note, is the usefulness of the 12S rRNA gene in resolving taxonomic issues in slugs.

7.4 References

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Appendices

Appendix 1

Nucleotide alignment for COI sequences

Appendix 1: Alignment of 576 base pairs of COI sequences. Ingroup taxa are *Arion hortensis* (AH1-AH27), *A. disrinctus* (AD1-AD11), *A. owenii* (AO1-AO4). Outgroup taxa are *A. intermedius* (AI), *Deroceras reticulatum* (DR). Codon start position 1. Identical nucleotides are represented by (.).

```
#AH1  ACC GGC TTG TCC TTG TTA ATC CGA CTT GAG CTC GGT ACT ACC GGG GTT TTA ACT GAT GAT CAA TTT TTT AAC GTT ATC
#AH2  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T
#AH7  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH8  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ...
#AH9  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH10 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH12 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH14 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH17 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH20 ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH21 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH25 ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AD1  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ... ... ... ..T ..A ..T
#AD2  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ..G ... ... ..T ..A ..T
#AD3  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ... ... ... ..T ..A ..T
#AD4  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ... ... ... ..T ..A ..T
#AD6  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ... ... ... ..T ..A ..T
#AD7  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ..G ... ... ..T ..A ..T
#AD8  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ..G ... ... ..T ..A ..T
#AD9  ..T ..G ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ... ... ... ..T ..A ..T
#AD11 ..T .AG ..A ..T C.T C.. ..T ..G T.A ..A T.G ..A ... ..T ..A ... C.. ..A ... ... ... ... ... ..T ..A ..T
#AO1  ..A ..T ..A ... ..A C.. ..T ..T T.G ..A ..T ..A ... ..G ..A A.. ... ... ... ... ..G ... ... ..T ... ..T
#AO2  ..A ..T ..A ... ..A C.. ..T ..T T.G ..A ..T ..A ... ..G ..A A.. ... ... ... ... ..G ... ... ..T ... ..T
#AO4  ..A ..T ..A ... ..A C.. ..T ..T T.G ..A ..T ..A ... ..G ..A A.. ... ... ... ... ..G ... ... ..T ... ..T
#AI   ..A ..A ..A ..T ..A C.T ..T ..G T.A ... T.A ..A ..A ..A ..A A.C C.G ..G ..C ... ... ..C ... ..T ..A G.T
#DR   ..G ..G C.T ..T C.T ... ..T ... T.A ..A T.A ..A ..A G.G ..A ... ... CTA ... A.. ..T ... ... ..T ..A G.A
```

	GTT	ACT	GCT	CAC	GCT	TTT	GTA	ATA	ATT	TTT	TTT	ATG	GTT	ATA	CCC	TTA	ATG	ATC	GGG	GGC	TTT	GGA	AAT	TGA	ATA	GTG
#AH1																										
#AH2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..G	...	...	...	...
#AH5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH10	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH12	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH14	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH17	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH18	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH21	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH25	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AD1	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD2	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD3	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD4	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD6	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD7	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD8	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD9	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AD11	..A	...	...	..T	..G	...	A.T	...	...	...	...	..A	...	...	..T	...	..A	..T	..T	..G	...	..C	...	...	...	A.T
#AO1	...	...	...	..T	..A	...	..T	...	...	...	...	..A	..C	...	..T	...	..A	..T	..T	..A	...	..T	...	...	...	A.T
#AO2	...	...	...	..T	..A	...	..T	...	...	...	...	..A	..C	...	..T	...	..A	..T	..T	..A	...	..T	...	...	...	A.T
#AO4	...	...	...	..T	..A	...	..T	...	...	...	...	..A	..C	...	..T	...	..A	..T	..T	..A	...	..T	...	...	...	A.T
#AI	..C	..A	...	..T	..A	...	..T	...	...	...	...	..A	...	...	..A	...	...	..T	...	..G	...	...	...	...	...	..T
#DR	..A	...	..A	..T	..A	...	..T	...	...	...	...	...	..G	...	..T	A.T	..A	..T	..A	...	...	..T	...	..G	..G	..A

	CCA	CTA	TTA	TTA	GGG	GCT	CCG	GAC	ATA	AGG	TTC	CCT	CGA	ATG	AAT	AAT	ATA	AGT	TTC	TGA	CTT	CTC	CCC	CCT	TCT	CTA
#AH1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH2	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH10	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH12	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH14	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH17	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH18	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH21	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AH25	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#AD1	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD2	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD3	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD4	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD6	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD7	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD8	..C	T..	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD9	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AD11	..C	...	...	C..	..A	..C	...	..T	...	..T	...	..A	...	..A	...	...	...	..G	..T	...	..A	..A	..G	...	...	..C
#AO1	..C	T..	C..	...	..A	...	...	..T	...	..A	..T	..G	...	..A	...	...	...	..G	..T	...	T.A	T.A	..A	..C	..A	..T
#AO2	..C	T..	C..	...	..A	...	...	..T	...	..A	...	..G	...	..A	...	...	...	..G	..T	...	T.A	T.A	..A	..C	..A	..T
#AO4	..C	T..	...	...	..A	...	...	..T	...	..A	...	..G	...	..A	...	...	...	..G	..T	...	T.A	T.A	..A	..C	..A	..T
#AI	...	...	...	A.T	..A	..A	..A	..T	...	..T	...	..C	..G	...	...	...	T..	..A	..T	...	...	..T	...	..A	...	T..
#DR	..C	T.G	...	A.T	..A	...	...	..T	...	..T	..T	...	...	..A	...	...	...	..G	..T	...	T.A	..T	...	...	..A	T.T

```
#AH1  CTT TTA CTT ATT GTA TCA AGT ATG GTG GAA GGT GGG GCC GGC ACG GGT TGG ACA GTT TAT CCT CCC TTA AGA AGA ATT
#AH2  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ...
#AH5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH7  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH8  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH9  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH10 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH12 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH14 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ...
#AH17 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ...
#AH18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH21 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH25 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AD1  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.G ... ..T TCA
#AD2  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.. ... ..T TCA
#AD3  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ... ..T C.G ... ..T TCA
#AD4  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.G ... ..T TCA
#AD6  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.. ... ..T TCA
#AD7  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.. ... ..T TCA
#AD8  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.. ... ..T TCA
#AD9  T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.G ..T ..T TCA
#AD11 T.A C.. T.A ... TGT ..T ... ..A ... ... ..A ... ..A ... ..T ..C ..A ..T ... ..C ..C ..T C.G ... ..T TCA
#AO1  T.A C.T T.A ... TGC ..C ..A ..A ..A ... ... ..T ..A ..A ..T ..A ..A ..C ..G ... ..A ... ... ... ..T TCA
#AO2  T.A C.T T.A ... TGC ..C ..A ..A ..A ... ... ..T ..A ..A ..T ..A ..A ..C ..G ... ..A ... ... ... ..T TCA
#AO4  T.A C.T T.A ... TGC ..C ..A ..A ..A ... ... ..T ..A ..A ..T ..A ..A ..C ..G ... ..A ... ... ... ..T TCA
#AI   ..A ... T.A ... TGT ..T ..A ..A ..T ..G ..A ..T ..G ..A ..A ... ..A ... ... ..C ... ..G ... ... G.C TC.
#DR   ... ... ..A ... TGT ..T ... ..A ..A ..G ... ... ..G ..A ..T ..G ..A ... ... ..C ..C ..T ... ... G.G CCA
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#AH1   ATA GCT CAT AGG GGG GCA TCT GTA GAC TTA GCC ATC TTT TCC TTA CAT TTA GCG GGA ATG TCT TCG ATT TTG GGT GCT
#AH2   ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH4   ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH5   ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH7   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ..G ... ... ... ... ... ... ...
#AH8   ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH9   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH10  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH12  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH14  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ...
#AH17  ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ...
#AH20  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH21  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH25  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AD1   ... ... ..T ... ..G ..A ..T ..T ... ..T ..T ... ..T ... ..T ... ..T ... ..A ... ..A ..G ..A
#AD2   ... ... ..T ... ..G ..A ..T ..T ... ..T ..T ... ..T ... ..T ... ..T ... ..A ... ..A ..G ..A
#AD3   ... ... ..T ... ..G ..A ..G ..T ... ..T ..T ... ..T ... ... ... ..T ... ..A ... ..A ..G ..A
#AD4   ... ... ..T ... ..G ..A ..T ..T ... ..T ..T ... ..T ... ... ... A.T ... ..A ... ..A ..G ..A
#AD6   ... ... ..T ... ..G ..A ..T ..T ... ..T ..T ... ..T ... ... ... ..T ... ..A ... ..A ..G ..A
#AD7   ... ... ..T ... ..G ..A ..T ..T ... ..T ..T ... ..T ... ... ... ..T ... ..A ... ..A ..G ..A
#AD8   ... ... ..T ... ..G ..A ..T ..T ... ..T ..T ... ..T ... ... ... ..T ... ..A ... ..A ..G ..A
#AD9   ... ... ... ... ..G ..A ..T ..T ... ..T ... ... ..T ... ... ... ..T ... ..A ... ..A ..G ..A
#AD11  ... ... ..T ... ..G ..A ..T ..T ... ..T ..T ... ..T ... ... ... ..T ... ..A ... ..A ..G ..A
#AO1   C.. ... ..C ..A ..T ... ..T ..T ... ..T ... ... ..T ... ... ... ..A ... ..A ..T ... ..A ..G ...
#AO2   C.. ... ... ..A ..T ... ..T ..T ... ..T ... ... ..T ... ... ... ..A ... ..A ..A ..T ... ..A ..G ...
#AO4   C.. ... ... ..C ..A ..T ... ..T ..T ... ..T ... ... ..T ... ... ... ..A ... ..A ..A ..T ... ..A ..G ...
#A1    ... ... ..C ..A ..A ..T ..G ..G ..T ... ..T ... ... ..T C.T ... ... ..G ..A ... ..T ... ..A ... ...
#DR    T.. ..G. ... GCT ... ... ... ..G ..T ... ..T ..T ..C ..A ... ... ... ..A ..G ... ... ..T ... ..A ...
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```
#AH1  ATT AAT TTT ATT ACG ACT ATT TTT AAC ATA CGG CCA AAA GTA TTA ACT TTT GAA CGG TTA AGT TTA TTT GTC TGG TCT
#AH2  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH7  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH8  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH9  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ..T ... ...
#AH10 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH12 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH14 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH17 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
#AH20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ...
#AH21 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH25 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ...
#AD1  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AD2  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AD3  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AD4  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A A.G ..A A.. ..G G.. ... .CT ..A ...
#AD6  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AD7  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AD8  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AD9  ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AD11 ... ... ... ... ..T ..A ... ... ... ..G ..A ..T ... ..T ... ..C ..A ..G ..A A.. ..G ... ... ..T ..A ...
#AO1  ... ... ... ... ..A ..A ... ... ..T ... ..C ..T ... ... ... ... ... ... ..T A.G ..A ... ... ..G ... ..A
#AO2  ... ... ... ... ..A ..A ... ... ..T ... ..C ..T ... ... ... ... ... ... ..T A.G ..A ... ... ..G ... ..A
#AO4  ... ... ... ... ..A ..A ... ... ..T ... ..C ..T ... ... ... ... ... ... ..T A.G ..A ... ... ..G ... ..A
#AI   ... ... ... ... ..T ... ... ... ..T ..G ..A ..T ... ... C.T ..A ..A ..G ..T A.. ..G ... ... ..A ..A ..C
#DR   ... ... ..C ... ..T ..A ... ... ..T ... ... T.. CCT .GG A.G .GA A.A ... ..T ... ..A ... ... ..T ..A ..A
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#AH1  ATT TTA ATT ACC GTA TTT CTT TTA CTC CTC TCA TTG CCG GTC TTA GCT GGA GCT ATT ACG ATA TTG CTA ACC GAT CGA
#AH2  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH7  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH8  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH9  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH10 ... ... ... ... .G. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
#AH12 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH14 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH17 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AH21 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ...
#AH25 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ...
#AD1  ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD2  ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD3  ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD4  ... ..G ... ... .G. ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD6  ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD7  ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD8  ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD9  ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AD11 ... ..G ... ... ... ... T.A ... ..A ... ... ..A ... ..A C.T ... ... ... ... ..C ... ..A ..G ..A ... ..T
#AO1  ... ... ... ... ..G ... T.A ... ..A ..T ..T ... ... ..G ... ... ... ..G ..G ... ..T ... ..A ... ..A ... ...
#AO2  ... ... ... ... ..G ... T.A ... ..A ..T ..T ... ... ..G ... ... ... ..G ..G ... ..T ... ..A ... ..A ... ...
#AO4  ... ... ... ... ..G ... T.A ... ..A ..T ..T ... ... ..G ... ... ... ..G ..G ... ..T ... ..A ... ..A ... ...
#AI   ... ... ... ..T ..T ..C ..C C.. ... T.A ..T ..A ... ..A C.T ... ... ..C ... ..T ... C.T T.. ..A ... ..T
#DR   ... ... G.. ..T ..G ... T.A C.T T.A T.A ..T ..A ..T ..T ... ..G ..G ..A ... ..T ... C.T T.. ..T ... ...
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#AH1  AAT TTT AAT ACA AGT TTT TTT GAT CCT GCT
#AH2  ... ... ... ... ... ... ... ... ... ...
#AH4  ... ... ... ... ... ... ... ... ... ...
#AH5  ... ... ... ... ... ... ... ... ... ...
#AH7  ... ... ... ... ... ... ... ... ... ...
#AH8  ... ... ... ... ... ... ... ... ... ...
#AH9  ... ... ... ... ... ... ... ... ... ...
#AH10 ... ... ... ... ... ... ... ... ... ...
#AH12 ... ... ... ... ... ... ... ... ... ...
#AH14 ... ... ... ... ... ... ... ... ... ...
#AH17 ... ... ... ... ... ... ... ... ... ...
#AH18 ... ... ... ... ... ... ... ... ... ...
#AH20 ... ... ... ... ... ... ... ... ... ...
#AH21 ... ... ... ... ... ... ... ... ... ...
#AH25 ... ... ... ... ... ... ... ... ... ...
#AD1  ... ... ... ... ... ... ... ... ..C ..A
#AD2  ... ... ... ... ... ... ... ... ..C ..A
#AD3  ... ... ... ... ... ... ... ... ..C ..A
#AD4  ... ... ... ... ... ... ... ... ..C ..A
#AD6  ... ... ... ... ... ... ... ... ..C ..A
#AD7  ... ... ... ... ... ... ... ... ..C ..A
#AD8  ... ... ... ... ... ... ... ... ..C ..A
#AD9  ... ... ... ... ... ... ... ... ..C ..A
#AD11 ... ... ... ... ... ... ... ... ..C ..A
#AO1  ... ... ... ... ... ... ... ... ..A ..A
#AO2  ... ... ... ... ... ... ... ... ..A ..A
#AO4  ... ... ... ... ... ... ... ... ..A ..A
#AI   ... ... ... ..C ..A ... ... ... ..A ...
#DR   ... ... ... ... ..G ... ... ..C ... ..G
```

Appendix 2

Sequence divergence tables

Appendix 2a: Pairwise uncorrected distences (lower diagonal) and corrected distances (upper diagonal) for 12S rRNA sequences. Uncorrected distance estimator is uncorrected-p, and corrected distance estimator is K81uf +G.

	AH1	AH2	AH3	AH4	AH5	AH6	AH7	AH8	AH9	AH10	AH11	AH12
AH1	-	0.00291	0.00292	0.00587	0.00291	0.00000	0.00000	0.00291	0.00000	0.00000	0.00291	0.00000
AH2	0.00294	-	0.00297	0.00884	0.00000	0.00291	0.00291	0.00000	0.00291	0.00291	0.00584	0.00291
AH3	0.00294	0.00294	-	0.00886	0.00297	0.00292	0.00292	0.00297	0.00292	0.00292	0.00585	0.00292
AH4	0.00588	0.00882	0.00882	-	0.00884	0.00587	0.00587	0.00884	0.00587	0.00587	0.00884	0.00587
AH5	0.00294	0.00000	0.00294	0.00882	-	0.00291	0.00291	0.00000	0.00291	0.00291	0.00584	0.00291
AH6	0.00000	0.00294	0.00294	0.00588	0.00294	-	0.00000	0.00291	0.00000	0.00000	0.00291	0.00000
AH7	0.00000	0.00294	0.00294	0.00588	0.00294	0.00000	-	0.00291	0.00000	0.00000	0.00291	0.00000
AH8	0.00294	0.00000	0.00294	0.00882	0.00000	0.00294	0.00294	-	0.00291	0.00291	0.00584	0.00291
AH9	0.00000	0.00294	0.00294	0.00588	0.00294	0.00000	0.00000	0.00294	-	0.00000	0.00291	0.00000
AH10	0.00000	0.00294	0.00294	0.00588	0.00294	0.00000	0.00000	0.00294	0.00000	-	0.00291	0.00000
AH11	0.00294	0.00588	0.00588	0.00882	0.00588	0.00294	0.00294	0.00588	0.00294	0.00294	-	0.00291
AH12	0.00000	0.00294	0.00294	0.00588	0.00294	0.00000	0.00000	0.00294	0.00000	0.00000	0.00294	-
AH14	0.00000	0.00294	0.00294	0.00588	0.00294	0.00000	0.00000	0.00294	0.00000	0.00000	0.00294	0.00000
AH16	0.00588	0.00882	0.00882	0.00588	0.00882	0.00588	0.00588	0.00882	0.00588	0.00588	0.00882	0.00588
AH17	0.00294	0.00000	0.00294	0.00882	0.00000	0.00294	0.00294	0.00000	0.00294	0.00294	0.00588	0.00294
AH18	0.00000	0.00294	0.00294	0.00588	0.00294	0.00000	0.00000	0.00294	0.00000	0.00000	0.00294	0.00000
AH20	0.00294	0.00588	0.00588	0.00294	0.00588	0.00294	0.00294	0.00588	0.00294	0.00294	0.00588	0.00294
AH21	0.00000	0.00294	0.00294	0.00588	0.00294	0.00000	0.00000	0.00294	0.00000	0.00000	0.00294	0.00000
AH23	0.00294	0.00588	0.00588	0.00882	0.00588	0.00294	0.00294	0.00588	0.00294	0.00294	0.00588	0.00294
AH25	0.00294	0.00588	0.00588	0.00294	0.00588	0.00294	0.00294	0.00588	0.00294	0.00294	0.00588	0.00294
AH26	0.00294	0.00588	0.00588	0.00882	0.00588	0.00294	0.00294	0.00588	0.00294	0.00294	0.00588	0.00294
AH27	0.00294	0.00588	0.00588	0.00882	0.00588	0.00294	0.00294	0.00588	0.00294	0.00294	0.00588	0.00294
AD1	0.22875	0.22876	0.22569	0.23166	0.22876	0.22875	0.22875	0.22876	0.22875	0.22875	0.23175	0.22875
AD2	0.22631	0.22632	0.22322	0.22926	0.22632	0.22631	0.22631	0.22632	0.22631	0.22631	0.22931	0.22631
AD3	0.22320	0.22320	0.22011	0.22618	0.22320	0.22320	0.22320	0.22320	0.22320	0.22320	0.22620	0.22320
AD4	0.22875	0.22876	0.22569	0.23166	0.22876	0.22875	0.22875	0.22876	0.22875	0.22875	0.23175	0.22875
AD5	0.22882	0.22883	0.22574	0.23172	0.22883	0.22882	0.22882	0.22883	0.22882	0.22882	0.23182	0.22882
AD6	0.22667	0.22666	0.22354	0.22964	0.22666	0.22667	0.22667	0.22666	0.22667	0.22667	0.22968	0.22667
AD7	0.22631	0.22632	0.22322	0.22926	0.22632	0.22631	0.22631	0.22632	0.22631	0.22631	0.22931	0.22631
AD8	0.22631	0.22632	0.22322	0.22926	0.22632	0.22631	0.22631	0.22632	0.22631	0.22631	0.22931	0.22631
AD9	0.22875	0.22876	0.22569	0.23166	0.22876	0.22875	0.22875	0.22876	0.22875	0.22875	0.23175	0.22875
AD10	0.22677	0.22676	0.22364	0.22973	0.22676	0.22677	0.22677	0.22676	0.22677	0.22677	0.22978	0.22677
AD11	0.22882	0.22883	0.22574	0.23172	0.22883	0.22882	0.22882	0.22883	0.22882	0.22882	0.23182	0.22882
AO1	0.22224	0.22524	0.22526	0.22530	0.22524	0.22224	0.22224	0.22524	0.22224	0.22224	0.22217	0.22224
AO2	0.22532	0.22831	0.22833	0.22838	0.22831	0.22532	0.22532	0.22831	0.22532	0.22532	0.22525	0.22532
AO4	0.22532	0.22831	0.22833	0.22838	0.22831	0.22532	0.22532	0.22831	0.22532	0.22532	0.22525	0.22532

AH14	AH16	AH17	AH18	AH20	AH21	AH23	AH25	AH26	AH27	AD1	AD2	AD3	AD4
0.00000	0.00587	0.00291	0.00000	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00291	0.00884	0.00000	0.00291	0.00586	0.00291	0.00585	0.00586	0.00586	0.00585	0.43607	0.43168	0.41665	0.43607
0.00292	0.00886	0.00297	0.00292	0.00587	0.00292	0.00586	0.00587	0.00587	0.00586	0.42262	0.41830	0.40366	0.42262
0.00587	0.00587	0.00884	0.00587	0.00292	0.00587	0.00885	0.00292	0.00886	0.00885	0.44246	0.43803	0.42281	0.44246
0.00291	0.00884	0.00000	0.00291	0.00586	0.00291	0.00585	0.00586	0.00586	0.00585	0.43607	0.43168	0.41665	0.43607
0.00000	0.00587	0.00291	0.00000	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00000	0.00587	0.00291	0.00000	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00291	0.00884	0.00000	0.00291	0.00586	0.00291	0.00585	0.00586	0.00586	0.00585	0.43607	0.43168	0.41665	0.43607
0.00000	0.00587	0.00291	0.00000	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00000	0.00587	0.00291	0.00000	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00291	0.00884	0.00584	0.00291	0.00586	0.00291	0.00585	0.00586	0.00586	0.00585	0.44074	0.43632	0.42117	0.44074
0.00000	0.00587	0.00291	0.00000	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
-	0.00587	0.00291	0.00000	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00588	-	0.00884	0.00587	0.00292	0.00587	0.00885	0.00292	0.00886	0.00885	0.44246	0.43803	0.42281	0.44246
0.00294	0.00882	-	0.00291	0.00586	0.00291	0.00585	0.00586	0.00586	0.00585	0.43607	0.43168	0.41665	0.43607
0.00000	0.00588	0.00294	-	0.00292	0.00000	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00294	0.00294	0.00588	0.00294	-	0.00292	0.00586	0.00000	0.00587	0.00586	0.43261	0.42823	0.41329	0.43261
0.00000	0.00588	0.00294	0.00000	0.00294	-	0.00291	0.00292	0.00292	0.00291	0.43261	0.42823	0.41329	0.43261
0.00294	0.00882	0.00588	0.00294	0.00588	0.00294	-	0.00586	0.00586	0.00586	0.44214	0.43771	0.42249	0.44214
0.00294	0.00294	0.00588	0.00294	0.00000	0.00294	0.00588	-	0.00587	0.00586	0.43261	0.42823	0.41329	0.43261
0.00294	0.00882	0.00588	0.00294	0.00588	0.00294	0.00588	0.00588	-	0.00586	0.42292	0.41860	0.40394	0.42292
0.00294	0.00882	0.00588	0.00294	0.00588	0.00294	0.00588	0.00588	0.00588	-	0.42916	0.42480	0.40995	0.42916
0.22875	0.23166	0.22876	0.22875	0.22861	0.22875	0.23168	0.22861	0.22568	0.22874	-	0.00281	0.00575	0.00000
0.22631	0.22926	0.22632	0.22631	0.22619	0.22631	0.22923	0.22619	0.22323	0.22630	0.00281	-	0.00867	0.00281
0.22320	0.22618	0.22320	0.22320	0.22311	0.22320	0.22612	0.22311	0.22011	0.22319	0.00568	0.00848	-	0.00575
0.22875	0.23166	0.22876	0.22875	0.22861	0.22875	0.23168	0.22861	0.22568	0.22874	0.00000	0.00281	0.00568	-
0.22882	0.23172	0.22883	0.22882	0.22868	0.22882	0.23175	0.22868	0.22576	0.22881	0.00000	0.00283	0.00566	0.00000
0.22667	0.22964	0.22666	0.22667	0.22655	0.22667	0.22955	0.22655	0.22356	0.22668	0.00847	0.00565	0.01411	0.00847
0.22631	0.22926	0.22632	0.22631	0.22619	0.22631	0.22923	0.22619	0.22323	0.22630	0.00281	0.00000	0.00848	0.00281
0.22631	0.22926	0.22632	0.22631	0.22619	0.22631	0.22923	0.22619	0.22323	0.22630	0.00281	0.00000	0.00848	0.00281
0.22875	0.23166	0.22876	0.22875	0.22861	0.22875	0.23168	0.22861	0.22568	0.22874	0.00000	0.00281	0.00568	0.00000
0.22677	0.22973	0.22676	0.22677	0.22666	0.22677	0.22966	0.22666	0.22368	0.22678	0.00849	0.00567	0.01412	0.00849
0.22882	0.23172	0.22883	0.22882	0.22868	0.22882	0.23175	0.22868	0.22576	0.22881	0.00000	0.00283	0.00566	0.00000
0.22224	0.22530	0.22524	0.22224	0.22228	0.22224	0.22518	0.22228	0.21922	0.21924	0.23017	0.23285	0.23364	0.23017
0.22532	0.22838	0.22831	0.22532	0.22536	0.22532	0.22826	0.22536	0.22230	0.22232	0.23017	0.23285	0.23364	0.23017
0.22532	0.22838	0.22831	0.22532	0.22536	0.22532	0.22826	0.22536	0.22230	0.22232	0.23017	0.23285	0.23364	0.23017

AD5	AD6	AD7	AD8	AD9	AD10	AD11	AO1	AO2	AO4
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.43607	0.43352	0.43168	0.43168	0.43607	0.43352	0.43607	0.43048	0.43530	0.43530
0.42262	0.42007	0.41830	0.41830	0.42262	0.42007	0.42262	0.43214	0.43697	0.43697
0.44246	0.43991	0.43803	0.43803	0.44246	0.43991	0.44246	0.42710	0.43189	0.43189
0.43607	0.43352	0.43168	0.43168	0.43607	0.43352	0.43607	0.43048	0.43530	0.43530
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.43607	0.43352	0.43168	0.43168	0.43607	0.43352	0.43607	0.43048	0.43530	0.43530
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.44074	0.43819	0.43632	0.43632	0.44074	0.43819	0.44074	0.42595	0.43074	0.43074
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.44246	0.43991	0.43803	0.43803	0.44246	0.43991	0.44246	0.42710	0.43189	0.43189
0.43607	0.43352	0.43168	0.43168	0.43607	0.43352	0.43607	0.43048	0.43530	0.43530
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.41764	0.42236	0.42236
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.42258	0.42735	0.42735
0.44214	0.43959	0.43771	0.43771	0.44214	0.43959	0.44214	0.43182	0.43666	0.43666
0.43261	0.43005	0.42823	0.42823	0.43261	0.43005	0.43261	0.41764	0.42236	0.42236
0.42292	0.42037	0.41860	0.41860	0.42292	0.42037	0.42292	0.41319	0.41789	0.41789
0.42916	0.42660	0.42480	0.42480	0.42916	0.42660	0.42916	0.41476	0.41948	0.41948
0.00000	0.00855	0.00281	0.00281	0.00000	0.00857	0.00000	0.48378	0.48378	0.48378
0.00281	0.00567	0.00000	0.00000	0.00281	0.00568	0.00281	0.49105	0.49105	0.49105
0.00574	0.01463	0.00867	0.00867	0.00575	0.01467	0.00574	0.50074	0.50074	0.50074
0.00000	0.00855	0.00281	0.00281	0.00000	0.00857	0.00000	0.48378	0.48378	0.48378
-	0.00855	0.00281	0.00281	0.00000	0.00857	0.00000	0.48378	0.48378	0.48378
0.00847	-	0.00567	0.00567	0.00855	0.00000	0.00855	0.48632	0.48632	0.48632
0.00283	0.00565	-	0.00000	0.00281	0.00568	0.00281	0.49105	0.49105	0.49105
0.00283	0.00565	0.00000	-	0.00281	0.00568	0.00281	0.49105	0.49105	0.49105
0.00000	0.00847	0.00281	0.00281	-	0.00857	0.00000	0.48378	0.48378	0.48378
0.00849	0.00000	0.00567	0.00567	0.00849	-	0.00857	0.48632	0.48632	0.48632
0.00000	0.00847	0.00283	0.00283	0.00000	0.00849	-	0.48378	0.48378	0.48378
0.23016	0.23095	0.23285	0.23285	0.23017	0.23097	0.23016	-	0.00582	0.00582
0.23016	0.23095	0.23285	0.23285	0.23017	0.23097	0.23016	0.00583	-	0.00000
0.23016	0.23095	0.23285	0.23285	0.23017	0.23097	0.23016	0.00583	0.00000	-

Appendix 2b: Pairwise uncorrected distences (lower diagonal) and corrected distances (upper diagonal) for COI sequences. Uncorrected distance estimator is uncorrected-p, and corrected distance estimator is TVM+G.

	AH1	AH2	AH4	AH5	AH7	AH8	AH9	AH10	AH12	AH14	AH17	AH18	AH20
AH1	-	0.00344	0.00883	0.00519	0.00519	0.00519	0.00344	0.00528	0.00171	0.00344	0.00694	0.00344	0.00350
AH2	0.00347	-	0.00524	0.00171	0.00519	0.00171	0.00344	0.00529	0.00171	0.00696	0.00343	0.00344	0.00521
AH4	0.00868	0.00521	-	0.00703	0.01067	0.00703	0.00883	0.01087	0.00703	0.01251	0.00882	0.00884	0.01070
AH5	0.00521	0.00174	0.00694	-	0.00697	0.00344	0.00519	0.00709	0.00344	0.00875	0.00518	0.00519	0.00699
AH7	0.00521	0.00521	0.01042	0.00694	-	0.00697	0.00519	0.00709	0.00344	0.00875	0.00874	0.00171	0.00699
AH8	0.00521	0.00174	0.00694	0.00347	0.00694	-	0.00519	0.00710	0.00344	0.00876	0.00518	0.00519	0.00699
AH9	0.00347	0.00347	0.00868	0.00521	0.00521	0.00521	-	0.00529	0.00171	0.00696	0.00695	0.00344	0.00521
AH10	0.00521	0.00521	0.01042	0.00694	0.00694	0.00694	0.00521	-	0.00350	0.00891	0.00890	0.00529	0.00712
AH12	0.00174	0.00174	0.00694	0.00347	0.00347	0.00347	0.00174	0.00347	-	0.00519	0.00518	0.00171	0.00345
AH14	0.00347	0.00694	0.01215	0.00868	0.00868	0.00868	0.00694	0.00868	0.00521	-	0.01055	0.00696	0.00709
AH17	0.00694	0.00347	0.00868	0.00521	0.00868	0.00521	0.00694	0.00868	0.00521	0.01042	-	0.00695	0.00877
AH18	0.00347	0.00347	0.00868	0.00521	0.00174	0.00521	0.00347	0.00521	0.00174	0.00694		-	0.00521
AH20	0.00347	0.00521	0.01042	0.00694	0.00694	0.00694	0.00521	0.00694	0.00347	0.00694	0.00868	0.00521	-
AH21	0.00174	0.00521	0.01042	0.00694	0.00694	0.00694	0.00521	0.00694	0.00347	0.00521	0.00868	0.00521	0.00521
AH25	0.00521	0.00694	0.01215	0.00868	0.00868	0.00868	0.00694	0.00868	0.00521	0.00868	0.01042	0.00694	0.00174
AD1	0.20312	0.20139	0.20486	0.19965	0.20312	0.19965	0.20139	0.20139	0.20139	0.20312	0.19792	0.20139	0.20139
AD2	0.20312	0.20139	0.20486	0.19965	0.20312	0.19965	0.20139	0.20139	0.20139	0.20312	0.19792	0.20139	0.20139
AD3	0.20139	0.19965	0.20312	0.19792	0.20139	0.19792	0.19965	0.19965	0.19965	0.20139	0.19965	0.19965	0.19965
AD4	0.21181	0.21007	0.21354	0.20833	0.21181	0.20833	0.21007	0.20660	0.21007	0.21181	0.20660	0.21007	0.21007
AD6	0.20139	0.19965	0.20312	0.19792	0.20139	0.19792	0.19965	0.19965	0.19965	0.20139	0.19618	0.19965	0.19965
AD7	0.20312	0.20139	0.20486	0.19965	0.20312	0.19965	0.20139	0.20139	0.20139	0.20312	0.19792	0.20139	0.20139
AD8	0.20486	0.20312	0.20660	0.20139	0.20486	0.20139	0.20312	0.20312	0.20312	0.20486	0.19965	0.20312	0.20312
AD9	0.20486	0.20312	0.20486	0.20139	0.20486	0.20139	0.20312	0.20312	0.20312	0.20486	0.19965	0.20312	0.20312
AD11	0.20486	0.20312	0.20660	0.20139	0.20486	0.20139	0.20312	0.20312	0.20312	0.20486	0.19965	0.20312	0.20312
AO1	0.20139	0.20139	0.20486	0.19965	0.20139	0.19965	0.20312	0.20312	0.20139	0.19792	0.20312	0.19965	0.20139
AO2	0.19965	0.19965	0.20312	0.19792	0.19965	0.19792	0.20139	0.20139	0.19965	0.19618	0.20139	0.19792	0.19965
AO4	0.19792	0.19792	0.20139	0.19618	0.19792	0.19618	0.19965	0.19965	0.19792	0.19444	0.19965	0.19618	0.19792

AH21	AH25	AD1	AD2	AD3	AD4	AD6	AD7	AD8	AD9	AD11	AO1	AO2	AO4
0.00171	0.00527	0.60521	0.60521	0.59274	0.66116	0.59690	0.60521	0.61430	0.61835	0.61411	0.64825	0.63865	0.63032
0.00519	0.00698	0.59578	0.59578	0.58347	0.65106	0.58756	0.59578	0.60476	0.60875	0.60458	0.64652	0.63694	0.62864
0.01065	0.01255	0.61945	0.61945	0.60669	0.67662	0.61096	0.61945	0.62873	0.62583	0.62854	0.67361	0.66369	0.65511
0.00696	0.00878	0.58796	0.58796	0.57578	0.64268	0.57981	0.58796	0.59685	0.60079	0.59667	0.63811	0.62864	0.62041
0.00696	0.00878	0.60408	0.60408	0.59163	0.65997	0.59578	0.60408	0.61316	0.61721	0.61298	0.64670	0.63712	0.62881
0.00696	0.00878	0.58756	0.58756	0.57539	0.64224	0.57942	0.58756	0.59644	0.60038	0.59626	0.63768	0.62820	0.61998
0.00518	0.00698	0.59884	0.59884	0.58647	0.65431	0.59059	0.59884	0.60784	0.61185	0.60766	0.65564	0.64596	0.63756
0.00708	0.00894	0.59481	0.59481	0.58253	0.61958	0.58661	0.59481	0.60376	0.60775	0.60359	0.65519	0.64551	0.63712
0.00344	0.00520	0.59731	0.59731	0.58497	0.65268	0.58907	0.59731	0.60630	0.61030	0.60612	0.64670	0.63712	0.62881
0.00519	0.00890	0.60571	0.60571	0.59322	0.66171	0.59739	0.60571	0.61480	0.61886	0.61462	0.63061	0.62122	0.61305
0.00873	0.01058	0.57816	0.57816	0.58244	0.63208	0.57012	0.57816	0.58691	0.59078	0.58674	0.65801	0.64831	0.63990
0.00519	0.00698	0.59578	0.59578	0.58347	0.65106	0.58756	0.59578	0.60476	0.60875	0.60458	0.63786	0.62838	0.62015
0.00528	0.00171	0.60101	0.60101	0.58859	0.65674	0.59273	0.60101	0.61006	0.61409	0.60988	0.64168	0.63218	0.62393
-	0.00707	0.61361	0.61361	0.60100	0.67017	0.60521	0.61361	0.62280	0.62690	0.62261	0.63885	0.62937	0.62113
0.00694	-	0.60896	0.60896	0.59641	0.66526	0.60060	0.60896	0.61811	0.62219	0.61792	0.65011	0.64051	0.63218
0.20486	0.20312	-	0.00349	0.00352	0.00904	0.00174	0.00349	0.00527	0.00174	0.00174	0.33143	0.32576	0.32063
0.20486	0.20312	0.00347	-	0.00713	0.01281	0.00174	0.00000	0.00173	0.00529	0.00527	0.32046	0.31490	0.30987
0.20312	0.20139	0.00347	0.00694	-	0.01292	0.00532	0.00713	0.00897	0.00533	0.00532	0.33816	0.33240	0.32720
0.21354	0.21181	0.00868	0.01215	0.01215	-	0.01091	0.01281	0.01473	0.01096	0.01091	0.36639	0.36032	0.35486
0.20312	0.20139	0.00174	0.00174	0.00521	0.01042	-	0.00174	0.00349	0.00351	0.00349	0.32603	0.32041	0.31533
0.20486	0.20312	0.00347	0.00000	0.00694	0.01215	0.00174	-	0.00173	0.00529	0.00527	0.32046	0.31490	0.30987
0.20660	0.20486	0.00521	0.00174	0.00868	0.01389	0.00347	0.00174	-	0.00710	0.00707	0.31538	0.30987	0.30488
0.20660	0.20486	0.00174	0.00521	0.00521	0.01042	0.00347	0.00521	0.00694	-	0.00351	0.33930	0.33353	0.32832
0.20660	0.20486	0.00174	0.00521	0.00521	0.01042	0.00347	0.00521	0.00694	0.00347	-	0.33711	0.33138	0.32621
0.19965	0.20312	0.15278	0.14931	0.15451	0.16146	0.15104	0.14931	0.14757	0.15451	0.15451	-	0.00175	0.00352
0.19792	0.20139	0.15104	0.14757	0.15278	0.15972	0.14931	0.14757	0.14583	0.15278	0.15278	0.00174	-	0.00175
0.19618	0.19965	0.14931	0.14583	0.15104	0.15799	0.14757	0.14583	0.14410	0.15104	0.15104	0.00347	0.00174	-