British reptile conservation: phylogeography and translocation studies

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This thesis is dedicated to Jo Cable,
Mike Bruford, Mum and Alan
for their kindness, support and belief
over the many years of study.
I will be forever indebted to you all.

Abstract

This thesis reviews the availability and suitability of non-invasive samples for examining genetic diversity of reptiles. Such samples were used to examine the phylogeography of British snakes, namely *Natrix natrix*, *Vipera berus* and *Coronella austriaca*. In addition, *Anguis fragilis* was used as a model reptile to assess the impact of land development and consequent habitat loss on present day reptile populations.

For the first time, we demonstrate that snake faecal, egg and foetal tissues, as well as sloughed skin and carcasses, are valuable sources of non-invasively sampled (NIS) material permitting genetic studies with minimal disturbance to the individual and its population. Using mitochondrial cytochrome *b* primers, 500 and 758 bp length sequences were successfully amplified from a variety of NIS tissues. Furthermore, a new method was developed for obtaining snake faeces in the field.

Non-invasively collected samples supplied sufficient quality DNA to reconstruct cyt *b* mtDNA phylogenetic histories for *V. berus* (434 bp), *C. austriaca* (141 bp) and *N. natrix* (265 bp). Median spanning networks, Bayesian inference and Neighbour joining analyses grouped all three British snake species within Italian lineages. *V. berus* showed greater genetic variability (5 haplotypes) than the other two monophyletic British snake species. It is likely that *V. berus* survived in British Younger Dryas refugia whilst both *C. austriaca* and *N. natrix* retreated to more southerly European refugia.

Using the common slow worm, *Anguis fragilis,* as a model species, the impact of land development on present day reptile populations was calculated by accessing the success of established translocation protocols. During monitoring, we achieved a slow worm recapture rate of 24% with all animals maintaining or increasing body condition in the first year following translocation. We recommend a minimum of 5 year post-translocation monitoring of receptor site with preferably four receptor site visits per year. This number of visits is a compromise between ideal recapture rate and mitigation costs.

The effect of parasitic load on translocated slow worm populations was investigated though the first British record and field study of *Neoxysomatium brevicaudatum* (83% prevalence, n=100). Increased parasite load negatively affected slow worm body condition with parasitic loads varying amongst host populations. We discuss the value of post-translocation monitoring and disease surveillance as an important conservation tool in preserving threatened reptile species.

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CHAPTER 1: General introduction

1.1 Introduction

European reptiles are currently affected by a range of conservation issues, all of which have consequences to the future survival of these animals across their range (Araújo et al., 2006; Henle et al., 2004). Perhaps the greatest challenges are presented by a changing environment compounded by increased anthropogenic expansion. House developments have decreased connectivity between reptile metapopulations and the expanding network of highways has increased reptile roadkills (Ashley and Robinson, 1996). In addition, these land developments have diverted natural water runoff which increases reptile exposure to flooding through the withdrawal of natural drainage channels (Kelman, 2001; Penning-Rowsell, 2001). Compounded by climate change, the surrounding habitats can become tinder dry and vulnerable to fires (Araújo et al., 2006; Hailey, 2000). The increasing human population size has driven the construction industry to design smaller accommodation plots, which has proportionally affected the traditional garden size, reducing suitable reptile breeding and egg laying sites (Shine et al., 2002). Reptiles are further affected by the consequent reduction in garden ponds and loss of garden microhabitat, including with fringe end scrub, both within and connecting between gardens. The current trend for high maintenance amenity grassland and concrete driveways offers little to assist reptile habitation (Hitchings and Beebee, 2002). Also, the demand for exotic pets can leave native animals out competed in the remaining ideal habitat by escaped aliens (Cadi and Joly, 2003).

In addition to domestic concerns, intense agricultural practices put reptiles at increased risk of exposure to pesticide and manufactured chemical spillages. Finally, many important reptile habitats are lost or damaged each year due to the increasing trend for monoculture habitats such as bio-fuel crop, pine forests and rapeseed (Bloomfield and Pearson, 2004; Shine et al., 2002).

Within this thesis six key questions are addressed:

(I) Do non-invasive samples collected from the three British snake species, *Vipera berus*, *Natrix natrix* and *Coronella austriaca*, yield sufficient quantity and quality mtDNA to construct phylogenies?

(II) Which European mtDNA lineages of these three snake species do UK populations comprise?

(III) Assuming *V. berus* was the first snake species to populate the British Isles, does it possess greater genetic diversity compared to the grass and smooth snakes?

(IV) Are the isolated populations of *C. austriaca* in the UK (Surrey, Dorset and Hampshire), genetically distinct and if so, what are the implications for the management of this locally threatened species.

(V) Using *Anguis fragilis* as a model species, how efficient is reptile translocation mitigation and can population management be improved?

(VI) Do the parasite loads of translocated slow worms affect host growth rates?

Britain is populated by six native reptile species; three lizards (*Lacerta (Zootoca) vivipara*, *Lacerta agilis* and *Anguis fragilis*) and three snakes (*Coronella austriaca*, *Natrix natrix* and *Vipera berus*). To date, few studies have examined the genetic structure of these animals across their range (but see Santos et al., 2008; Ursenbacher et al., 2006; Guicking et al., 2006; Carlsson et al., 2004). The cryptic, shy nature of these reptiles in the wild makes sample collection challenging and non-invasively collected sample material is essential to support current sampling efforts. Currently, European reptile species are threatened from climate change and habitat loss; the latter almost exclusively the result of human expansion. Consequently, a high number of reptile population translocations occur across Europe every year. However, currently no consideration is given to the influence of parasites during such programmes with resistance to new host-parasite relationships disregarded in the equation of population survival.

1.1.1 *Squamata*

Snakes and lizards belong to the Order Squamata. Europe currently has 34 of the ~2900 extant species of snakes of which only three occur in the UK (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). Two of the species, the grass, or ringed snake (*Natrix natrix*) and the smooth snake (*Coronella austriaca*), are non-venomous, and belong to the Family Colubridae. The third, the adder or European viper (*Vipera berus*), is mildly venomous and belongs to the Family Viperidae. Over twice as many lizard species occur in Europe (n=79; Arnold and Ovenden, 2002; Beebee and Griffiths, 2000) but again with only three native UK species; the sand lizard (*Lacerta agilis*), the common lizard (*Lacerta* (*Zootoca*) *vivipara*) and the slow worm (*Anguis fragilis*).

This thesis focuses on the three indigenous British snake species and the most common lizard species (*Anguis fragilis*). The annual activity of these ectothermic species generally extends from spring to autumn with an over-winter brumation period. Consequently, sampling and studies of these reptiles in the field is only possible for six to eight months of the year, dependent on species and gender (see Figure 1.1).

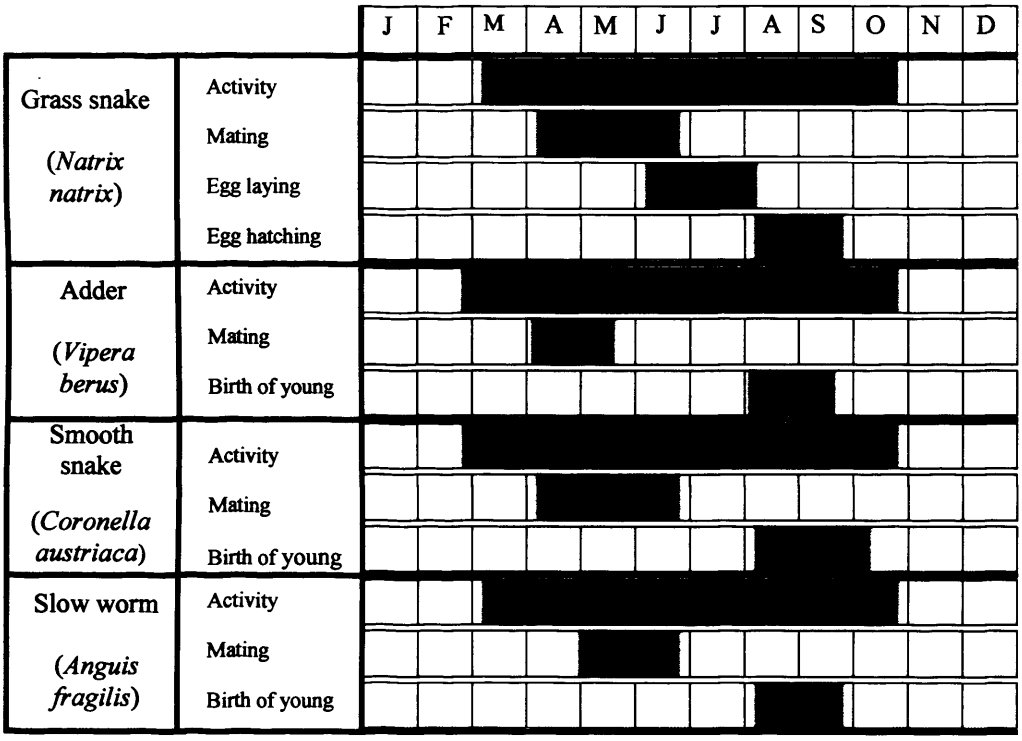

Figure 1.1 Annual activity of British snakes (grass snake, adder and smooth snake) and the slow worm lizard studied within this project (Data derived from Beebee and Griffiths, 2000; personal observations).

1.1.2 *Adder, Common or Northern viper* (Vipera berus)

Vipers (subfamily Viperinae) are distributed throughout Europe, Asia and Africa and are related to the Asian and American Pit vipers. Pit vipers are the most evolutionary advanced snakes, differing from true vipers in having a depression in front of each eye (Fry et al., 2003; Phelps, 1981). This heat sensitive, pit organ enables the snake to locate and track warm-blooded prey sensing a difference of as little as 0.003°C (Arnold and Ovenden, 2002; Phelps, 1981). The subfamily Viperinae is taxonomically and ecologically diverse containing 13 genera and over 70 species (Roelke and Childress, 2007).

The adder displays size and colour sexual dimorphism (Madsen, 2006, Madsen et al., 1988; Forsman, 1991; Shine, 1978; Figure 1.2). The males are generally lighter in colour than females, ranging from dirty yellow to grey or even white, while females are reddish brown. Both sexes have a characteristic dark zigzag stripe running along the back and dark bars along the sides (Niskanen and Mappes, 2005; Wüster et al., 2004; Forsman, 1995, 1993). The ventral surface is grey, grey-brown or black, occasionally with white spots. The tail tip can be yellow, orange or even red from beneath and an X- or V-shaped mark is present on top of the head (Sheldon and Bradley, 1989; Burgess and Shuttleworth, 1977). This mark is quite distinct and presents a non-invasive means of identification for each animal (Phelps 1981).

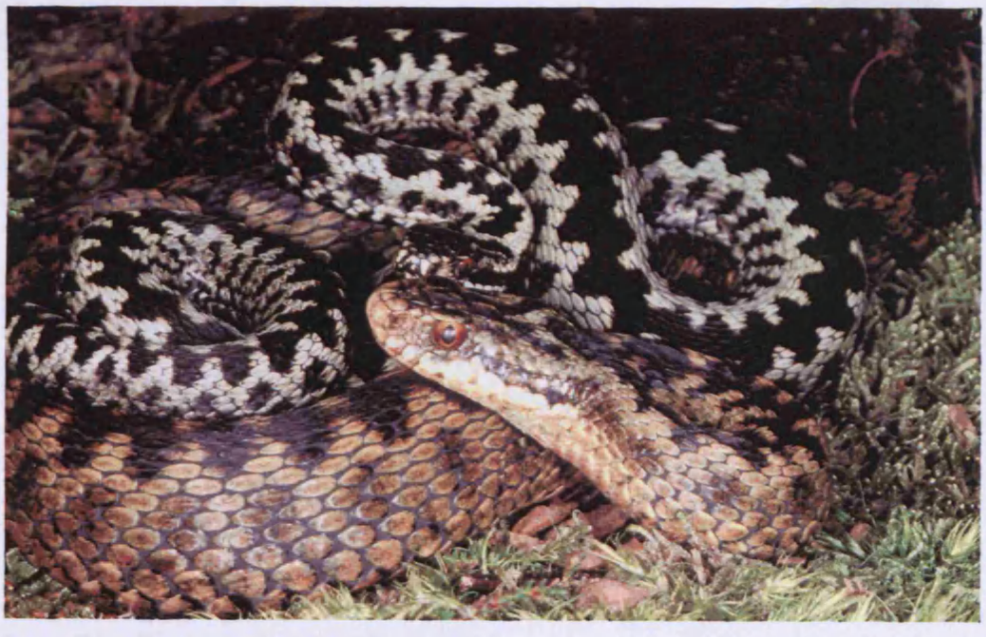

Figure 1.2 Sexual dimorphism between the male (top) and female adder (*Vipera berus*) (Reproduced with permission of T.E. Phelps).

Adders are small, heavy-bodied snakes with flat snouts that usually attain a length of around 65 cm (Olsson et al., 1997; Schoener and Schoener, 1984). The females are proportionally longer than males and can grow up to 90 cm (Andrén and Nilson, 1981; Boulenger, 1913). This viper can be identified by 21 dorsal scale rows along the mid-body, several large scales (including frontal and parientals) on top of the head and a single row of small scales (suboculars) beneath the eye (Beebee and Griffiths, 2000; Phelps 1981). The adder is largely diurnal, especially in the north of Europe (Bollmann and Reyer, 2001). Prey includes small mammals, such as voles and mice, but adders will take common lizards and even frogs on occasion (Beebee and Griffiths, 2000; Luiselli et al., 1995; Luiselli and Anibaldi, 1991).

1.1.2.1 *Range*

The adder occurs over much of Europe (Figure 1.3) from the Balkans in the south extending north to beyond the Arctic Circle; the only snake species to inhabit this niche and the most northerly distributed reptile in the world (Andersson, 2003; Meliadou and Troumbis, 1997). It occupies a broader range than any other terrestrial snake species extending from Scotland across Europe, through Russia to the Pacific coast (Ursenbacher et al., 2006; Carlsson et al., 2004; Beebee and Griffiths, 2000).

Figure 1.3 Distribution of adder (*Vipera berus*) populations throughout Britain and Europe. (Reproduced from Beebee and Griffiths (2000) with permission from Harper Collins Publishers).

Adders have a wide distribution throughout mainland Britain, Anglesey and the Isle of Wight (Figure 1.3), but are absent from the Isle of Mann and Ireland (Arnold and Ovenden, 2002; Phelps, 1981). They occur in small populations of 10-40 adults in open habitats with heathland, moorland dunes, woods and field edges (Spellerberg, 2008; Reading et al., 2007). The adder prefers lighter chalk or sandy soils to heavy clay residing in undisturbed areas. Its distribution is interrupted by densely populated human development throughout Europe (Herczeg et al., 2007; Bonnet et al., 1999; Gasc et al, 1997; Madsen et al., 1996; Nilson, 1980).

Comparing the current distribution maps of the adder to the maximum extent of ice and permafrost at the end of the last ice age (*ca.* 20,000 years ago) indicates that the adder has migrated north from glacial refugia which it has currently abandoned. With climate change and retreating permafrost the original refugia may have become too warm to sustain the adder populations resulting in a range shift (Ursenbacher et al., 2006; Carlsson et al., 2004; Beebee and Griffiths, 2000).

1.1.3 *Grass or ringed snake* (Natrix natrix)

The grass snake is the largest terrestrial reptile in the British Isles (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). The snake is typically olive green, grey or brown in colour with black bars running along the side and sometimes two rows of black marks along the back (Madsen, 1987b; Nilson and Andren, 1981).

Figure 1.4 *Natrix natrix*, the Grass or Ringed snake (Reproduced with permission of T.E. Phelps).

It is a slender snake displaying sexual size dimorphism (Borczyk, 2007; Gregory, 2004; Gregory and Isaac, 2004; Rivas and Burghardt, 2001; Shine, 2000; Thorpe, 1989) reaching lengths of over 80 cm (males 65 cm) but on exception females can reach 1.8 m in length (Arnold and Ovenden, 2002; Madsen and Shine, 1993c; Madsen, 1983). Both sexes have similar markings (Arnold and Ovenden, 2002). The most reliable way to sex this snake is from the hemi penal swelling and proportionally longer, thinner tail in males. Thus, the sub caudal scale count is higher in males (61-73) than in females (49-63) and any snake larger than 85 cm is generally a female (Beebee and Griffiths, 2000; Luiselli et al., 1997; Thorpe, 1989).

The underside of the grass snake is an off cream or white colour with dark triangular markings. These remain consistent throughout the snake's life and are sufficiently variable to present a means of non-invasive identification for this species (Mertens, 1995; Carlström and Edelstam, 1946). This is particularly apparent in the first

twenty ventral scales (Beebee and Griffiths, 2000). The iris is copper coloured with dark circular pupils (Figure 1.4). Its alternative common name of the Ringed snake comes from its distinctive yellow and black 'collar' marking around the neck which can be absent in older females (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000).

The grass snake is Britain's only oviparous snake species (Wisler et al., 2008; Isaac and Gregory, 2004; Reading and Davies, 1996). Females lay their eggs in June to July (see Figure 1.1), normally in rotting vegetation (Isaac and Gregory, 2004; Boulenger, 1913). The eggs hatch into miniature versions of the adults in September (Arnold and Ovenden, 2002; Madsen, 1987a). Males compete for mates forming a mating ball around the female snake (Shine and Mason, 2005; Shine et al., 2000b; Luiselli, 1996). The largest male is normally successful in tail mêlées and consequently in copulation (Borczyk, 2004; Shine et al., 2000b; Shetty and Shine, 2002; Luiselli, 1996; Madsen & Shine, 1993c). Large females tend to be more attractive to males than smaller ones. This could be due to a greater release of pheromone from the larger female or for her ability to produce a greater egg clutch (or both). Females mate only once and then become unattractive to males (Shine and Mason, 2001; Beebee and Griffiths, 2000).

In Britain, large females will lay an average of 20 eggs each and up to 250 communally (Beebee and Griffiths, 2000). On mainland Europe over 2000 eggs were recorded to have been laid in a single superclutch with females choosing to lay together once ideal conditions have been identified (Gregory, 2004; Arnold and Ovenden, 2002; Golder, 1984). Clutch size is directly related to the size and age of the female with older, larger, fitter, females attracting larger, fitter males and consequently having larger clutches. Eggs measure 15×25 mm when laid but soon swell as they absorb water and hatch at around 35 days (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000).

The snake brumates throughout the winter months from October in burrows and under rock piles, re-emerging in late March (see Figure 1.1). Although the snakes do not feed during brumation, in North America snakes do move in colder spells preferring to either huddle together with other snakes or to retreat deeper into burrows to maintain body temperatures (Beebee and Griffiths, 2000). Their body temperatures can fall as low as 0.4°C during hibernation (Mertens, 1994). On their re-emergence in March, the grass snake is lethargic and spends most of its time openly basking. Although a diurnal species, the snake will hunt at night if conditions are warm enough (Isaac and Gregory, 2004). Body temperature is maintained at between 26 and 30°C with no snakes being

seen when the air temperature drops below 10°C (Wisler et al., 2008; Beebee and Griffiths, 2000; Mertens, 1994).

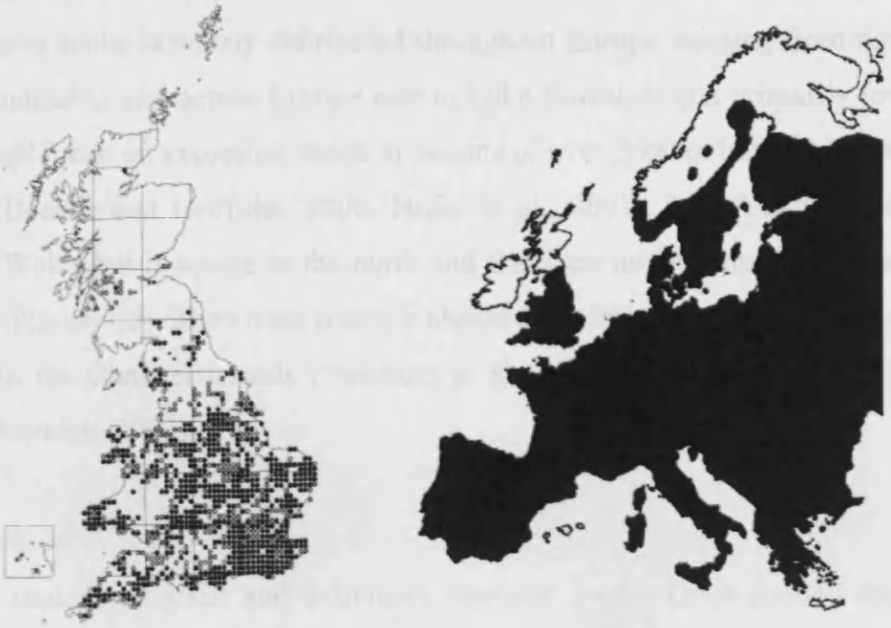

Figure 1.5 Current day distribution of the grass snake *Natrix natrix* throughout the UK and Europe (Reproduced from Beebee and Griffiths (2000) with permission from Harper Collins Publishers).

Grass snakes use hedgerows and ditches as corridors of movement (Wisler et al., 2008). Although they move an average of 120 m per season some snakes have been recorded moving as far as 4 km. Grass snakes have been recorded using the same habitat in consecutive years (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000; Madsen, 1984). A key part of the conservation strategy for the grass snake is education and tolerance of this species within urban environments. Grass snakes are non-venomous (at least to humans) and despite a seemly aggressive defensive display they rarely bite, commonly striking with their mouths closed (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). Their primary defence is their speed, alertness and responsiveness. If cornered they can feign death and commonly defecate a foul smelling excretion (Gregory, 2008; Mori and Burghardt, 2008; Gregory et al., 2007; Ushakov, 2007). Their diet comprises of mostly newts, frogs and toads but on occasions will eat fish, tadpoles, slugs, snails, and sometimes even birds and other snakes (Luiselli et al., 2005; Gregory and Isaac, 2004; Sorace et al., 2000; Luiselli and Rugiero, 1991). Grass

snakes generally live for up to 15 years but have been recorded living as long as 28 years in the wild (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000).

1.1.3.1 *Range*

The grass snake is widely distributed throughout Europe, ranging from northern Africa to Scandinavia and across Europe east to Lake Baikal. It is a primarily lowland animal although it can on exception reside at heights of over 2000 m in the Alps (Wisler et al., 2008; Beebee and Griffiths, 2000; Hofer et al., 2001). It is found throughout England and Wales but is scarce in the north and there are no confirmed records from Scotland (see Figure 1.5). The Grass snake is absent from Ireland and is the only snake to be found in the Channel Islands (Guicking et al., 2006; Gregory and Isaac, 2004; Arnold and Ovenden, 2002).

1.1.3.2 *Behaviour*

Grass snakes are swift and extremely spatially aware. Once spotted they are challenging to approach with field study requiring skill and patience (Isaac and Gregory, 2007; Van Roon et al., 2006). Rather than adopting an 'ambush' policy, the grass snake is an active hunter constantly on the move (Beebee and Griffiths, 2000). After egg laying in June the snake engages in hunting and hiding only basking in long grasses and other such protected habitats (Arnold and Ovenden, 2002). On maturation, ecdysis occurs twice a year for males (after brumation and at the close of the year) and once for females (directly following egg laying; see Figure 1.1) (Beebee and Griffiths, 2000; Landmann, 1979; Maderson, 1965).

1.1.4 *Smooth snake* (Coronella austriaca)

The smooth snake is a small colubrid reaching an average length of 60 cm but can reach a length of 80 cm (Arnold and Ovenden, 2002; Luiselli et al., 1996; Spellerberg and Phelps, 1977; Boulenger, 1913). *C. austriaca* has a cylindrical body, poorly defined neck, small head and eyes with round pupils and a golden yellow iris (Beebee and Griffiths, 2000). Colouration varies between grey, brown pink and red, often colouration is more intense on the mid-line giving the effect of two streaks running along the body. A dark stripe runs from the nostril, through the eye to the neck often forming a bridle across the snout (see Figure 1.6). A series of small poorly defined dark spots run along the length of the body (Arnold and Ovenden, 2002; Boulenger, 1913).

Figure 1.6 Smooth snake (*Coronella austriaca*) captured by the author during field research in Dorset (Photograph T.E. Phelps).

The ventral surface ranges from dark red, orange, grey or even black with mottling or fine spotting (Najbar, 2006; Beebee and Griffiths 2000; Spellerberg and Phelps, 1977; Boulenger, 1913). As the common name suggests, the smooth snake has smooth unkeeled scales along its entire body permitting immediate field identification of sloughed skin for this species when comparing to Britain's other two snake species (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000; Luiselli et al., 1996; Boulenger, 1913).

Both sexes of the smooth snake are of similar size but males have a proportionally longer broader tail than females, especially in the breeding season (Spellerberg and Phelps, 1977). Males also have more subcaudal scales (50-64 compared to 43-57 in females) and less ventral scales (153-165 compared to 166-180 in females) (Najbar, 2006; Beebee and Griffiths, 2000; Boulenger, 1913). In contrast to females, grey colouration of males is rarely encountered (Van Gelder et al., 1988; Gent, 1988)

1.1.4.1 *Range*

The smooth snake has a wide range across Europe from Portugal in the east to Greece in the south as far north as Sweden and extending east to Russia (Figure. 1.7). In Britain, its range is restricted to southern England, occurring in just three counties (Dorset, Hampshire and Surrey) (Bond et al., 2005; Beebee and Griffiths, 2000; Luiselli et al., 1996; Arnold and Ovenden, 2002; Boulenger, 1913).

Figure 1.7 Current day distribution of the smooth snake *Coronella austriaca* throughout the UK and Europe (Reproduced from Beebee and Griffiths (2000) with permission from Harper Collins publishers).

The smooth snake occurs from sea level to over 1800 m in mountain habitat to the south of its range (Luiselli et al., 1996; Boulenger, 1913). It has an estimated home range of between 0.5 and 3 hectares, is found in a variety of different habitats including woodland, embankments and hedgerows but in Britain is restricted to sunny, sandy heathland (Van Gelder et al., 1988). In England, the smooth snake is essentially a lowland heathland species and does not occupy the conifer plantations, woodland and

urban habitats that surround and limit its further expansion (Reading, 2004; Beebee and Griffiths, 2000; Braithwaite et al., 1989). Smooth snakes generally favour a small-scale mosaic of low-level vegetation, bare ground and limited scrub (Natural England, 2007; Marrs and Watt, 2006; Spellerberg and Phelps, 1977). Only small isolated patches of heathland would have existed between the retreating ice of the Younger Dryas (a brief period that saw a rapid return to glacial conditions in the higher latitudes of the Northern Hemisphere, 12,900-11,500 years ago) and the quickly establishing forests of the British Isles following a period of rapid warming 10000 BP (Jones et al., 2004; Alley, 2000; Hewitt, 1999; 1996; Winifred, 1977). Afforestation quickly spread and by 9000 BP pollen records suggest that almost all of Britain was under canopy (Vincent, 1990; Yalden, 1982). As the smooth snake requires an open lowland heathland habitat, at the north of its range it must have reached the extent of its present day distribution around this time (Natural England, 2007; Beebee and Griffiths, 2000). Up until the 17th century man had a continuous impact on heathland creation and maintenance by the felling large stretches of forest (Green, 1990; Heil and Diemont, 1983). Inadequate habitat management in conjuction with improvements in agricultural and transport technology has lead to a steady decline in heathland habitat allowing non heathland crops species to grow and nutrients to be put back into the previously nutrient poor soil (Gleed-Owen, 1997; Snow and Marrs, 1997; Barr et al., 1993; Green, 1990; Eversham et al., 1996; Heil and Diemont, 1983). This has restricted the smooth snake's current day distribution and restrains it from future population expansion (Beebee and Griffiths, 2000).

1.1.4.2 *Behaviour*

The smooth snake exits brumation later than the other two British snake species (Phelps, 1978) with full yearly activity displayed in Table 1.2. It is diurnal and highly secretive participating in a substantial subterranean existence to regulate ideal body temperatures of between 28°C to 33°C. During summer nights, smooth snakes bury themselves under a few cm of topsoil allowing their temperature to drop to as low as 11°C (Spellerberg and Phelps, 1975). The snake is commonly found in areas of sparse vegetation residing in crevices and rock piles. They bask in sunshine but do so intertwined around heather, gorse or other vegetation. This so-called mosaic basking (cryptic heliothermy) allows thermoregulation whilst remaining practically invisible to predators. Arnold and Ovenden (2002) state that the smooth snake sheds its skin between 4 to 6 times a year. However, Spellerberg and Phelps (1977) previously

reported the smooth snake as shedding its skin twice and on occasion three times a year. These latter observations are consistent with the author's findings from a three year field research project with T.E. Phelps.

The smooth snake is slow moving and easily captured but bites readily when handled and can exude a foul smelling secretion form the anal gland. Smooth snakes are opportunistic hunters but the diet consists of mainly (70%) *Lacerta* lizards (Goddard, 1984). However, slow worms and small snakes (including vipers) are also consumed constricting larger prey in its coils and the smooth snake will on occasion eat small mammals or conspecifics (Luiselli et al., 1996; Reading, 2004). The smooth snake commonly reaches maturity at 4 years and produces between 2 and 15 fully formed young (Luiselli et al., 1996; Beebee and Griffiths, 2000; Arnold and Ovenden, 2002).

1.1.5 *Slow worm* (Anguis fragilis)

The slow worm is a legless lizard growing up to 40 cm, with females usually larger than males. Twenty six rows of scales form a smooth covering over overlapping bony plates, called osteoderms, which distinguish the firm, rigid body from that of a snake. Once classified as a snake, and still frequently mistaken for one, the slow worm's common name in Welsh, 'Neidr dafad' or sheep snake, refers to the lizard's snake like appearance and its fondness of urban environments; here referring to sheep and cattle sheds. However, the slow worm is easily distinguishable from a serpent in that it has movable eyelids and a broader notched (rather than forked) tongue that can only be flicked with an open mouth (Beebee and Griffiths, 2000).

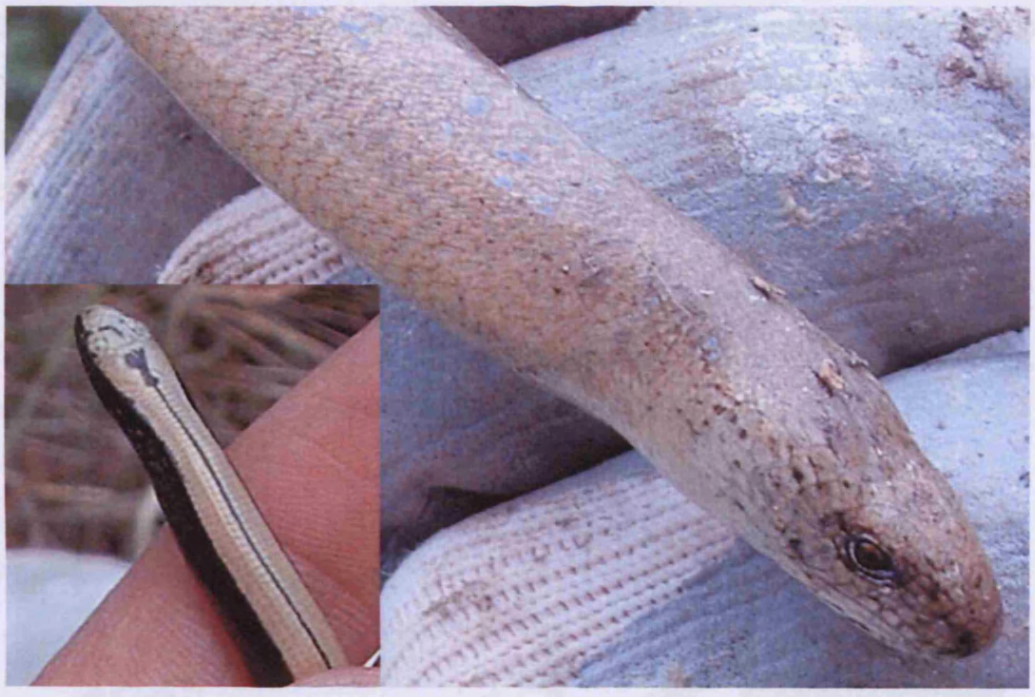

Figure 1.8 A large male slow worm (*Anguis fragilis*) displaying turquoise spots during mating season captured by the author in Pontypool, South Wales. Male spots faded darker over time disappearing completely within two weeks of mating. Inset: Neonate slow worm (Photographs by J. Jones).

Male and female adult slow worms differ in colour, pattern and often in size. Females are generally coppery red, often with a thin dark line running down the dorsal length of the lizard beginning behind the head. In addition, paler stripes, often broken into dashes or spots, run along each side of the female lizards. Males are more uniform in their markings lacking the stripes of the female and are generally a paler grey to dark brown (Figure 1.8). The ventral surface can be slate grey, black mottled or bluish and

tends to be paler in males. Males generally have a larger, broader head with stronger looking jaw and their intact tails are proportionally longer. The pineal and throat markings are unique to each individual and in conjunction with weight and body length offer an excellent and robust means of non-invasive identification (Webster, 2007; Beebee and Griffiths, 2000; Platenberg and Griffiths, 1999). The most robust means of sex determination is through identifying the presence or absence of the hemipen.

All the anguids are carnivores, predominantly consuming arthropod prey (Zug, 1993). However, studies of slow worms in the wild have shown that they take a variety of prey including slugs, snails and earthworms (Webster, 2007; Luiselli, 1992b). Slow worms can be observed 'death rolling' after capturing prey before consumption although this behaviour is displayed more frequently by some individuals than others. The slow worm can commonly be observed directly after rainfall, presumably a profitable time to hunt its mollusc prey (Luiselli, 1992b; personal observations). Slow worms are morphologically cryptic and functionally adapted to a semi subterranean life (Cabido et al., 2004).

1.1.5.1 *Range*

The anguid lizards are found throughout America with a few forms in Asia, north west Africa with just two species found in Europe (see Figure 1.9).

Figure 1.9 Current day distribution of the slow worm *Anguis fragilis* throughout the UK and Europe (Reproduced from Beebee and Griffiths (2000) with permission from Harper Collins Publishers).

The slow worm has a wide European distribution sharing the mainland with the largest of the anguids, the glass lizard (Arnold and Ovenden, 2002; Platenberg and Griffiths, 1999; Luiselli, 1992b). Although absent from Southern Spain and Portugal, southern Greece, Ireland and most of the Mediterranean Islands, north west Iran and Adriatic Turkey, it generally resides at high population densities throughout the remainder of mainland Europe (Arnold and Ovenden, 2002; Beebee and Griffiths 2000). It is the most common British reptile preferring to occupy herbaceous microhabitats with high vegetation cover but can be found in a range of habitats. Slow worm population densities have been predicted at 1700, 600-2000 and 1000/ ha (Ferreiro et al., 2004; Arnold and Ovenden, 2002; Herpetofauna groups of Britain and Ireland (HGBI), 1998 respectively). The slow worm can be found at high altitudes to the south of its range attaining heights of 2-2400 m in the Alps (Luiselli, 1992b; Beebee and Griffiths 2000) but have been estimated to occur at lower population densities of >80/ ha (Capula et al., 1998).

1.1.5.2 *Behaviour*

As a thigmotherm, this semi-fossorial lizard prefers to thermoregulate under refugia and is rarely observed in open ground but can, on occasion, be seen mosaic basking amongst long vegetation (Platenberg and Griffiths, 1999; Beebee and Griffths, 2000; Meek, 2005). They endeavour to maintain a body temperature between 14.5 and 28°C (mean 25.3 to 26.4°C) throughout their active period of March-October (Brown and Roberts, 2008; Patterson, 1990).

Although highly weather dependant, males will leave brumation sites earlier than females, commonly mid-February in Wales (Ferreiro et al., 2004; Platenberg and Griffiths, 1999; personal observations). Slow worms are difficult to see during both very cold and extremely warm days when they reportedly maintain a mostly subterranean existence (Beebee and Griffiths, 2000). Slow worms are not strictly social animals but will communally brumate, aggregated within the same hibernacula and will readily share refugia with conspecifics (Beebee and Griffiths, 2000; Leighton, 1903).

Unlike other British lizards, the slow worm nearly always 'freezes' rather than flees when disturbed. Mechanically it would find it difficult to 'outrun' its predators and instead deprives them of key behavioural stimuli (i.e. movement) by 'freezing' in the position it was discovered. If picked up the lizard thrashes about wildly and defecates. Its Latin name, *Anguis fragilis* or 'fragile snake', refers to the lizard's ability to shed its tail in response to such an attack. This is made possible by means of a fracture plane

that exists across the caudal vertebra. This behaviour is, however, observed more commonly in adult slow worms (Beebee and Griffiths, 2000; Holman, 1998). To a predator, the slow worm head may not be completely distinguishable from its tail, and therefore a bird will frequently attack the 'wrong end' of the slow worm flying away with the lizard's tail whilst the slow worm survives. When a tail is shed, the slow worm 'freezes' whilst the tail, packed with mitochondria, thrashes around for up to 15 minutes; long enough to secure the animals safety in the majority of cases. Slow worms regularly exhibit scarring and up to 70% found in the field have shed tails (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). Although the tail grows back it never attains its original length and in the majority of cases retains a small stump. This does not affect reproduction as the slow worm, unlike other British lizards, does not store extensive fat reserves in its tail (Bryant and Bellairs, 1967). Thus, Ferreiro et al. (2004) was not able to determine any relationship with female tail length and reproductive success or broad size.

Like most cold tolerant reptiles in the north of their range, slow worms are viviparous and breed biannually (Capula et al., 1992; Patterson, 1983). In the breeding season some males can take on spectacular turquoise spots along the dorsal surface (Beebee and Griffiths, 2000; Capula et al., 1997). Mating has been observed in early June (2008) at two separate South Wales sites by the author (personal observations; see Figure 1.8). Males are highly aggressive during mating pinning females into position by clamping their jaws behind her neck prior to copulation, which can last up to 10 hours. Post copulation females are often visibly scarred but heal quickly over a few months (Böhme, 2006; Beebee and Griffiths, 2000; Capula et al., 1998; personal observations). After mating in late May, females give birth to live young in the following September (see Figure. 1.1). Slow worms typically give birth to between 6-12 young but can produce a brood of up to 26 offspring. The young are cryptically coloured polished gold with a black dorsal stripe to camouflage them amongst dry autumn grasses (Arnold and Ovenden, 2002; see inset Figure. 1.8).

1.2 *Historical distribution and biogeography*

Expansion into northern Europe presented reptiles with a multitude of challenges and obstacles to overcome. In order to survive reptiles had to exploit their current survival strategies to better adapt to the harsher northern environment.

1.2.1 *Ice ages, refugia and colonisation of the UK*

Severe climatic change, such as ice ages, produces dramatic changes in species distributions. Many species can become extinct over large areas of their range, some dispersing to new locations whilst others survive in refugia. Species then expand from their refugia south of ice and permafrost, dispersing to new locations as the climate warmed and the polar ice sheets receded (Hewitt, 2000, 1996).

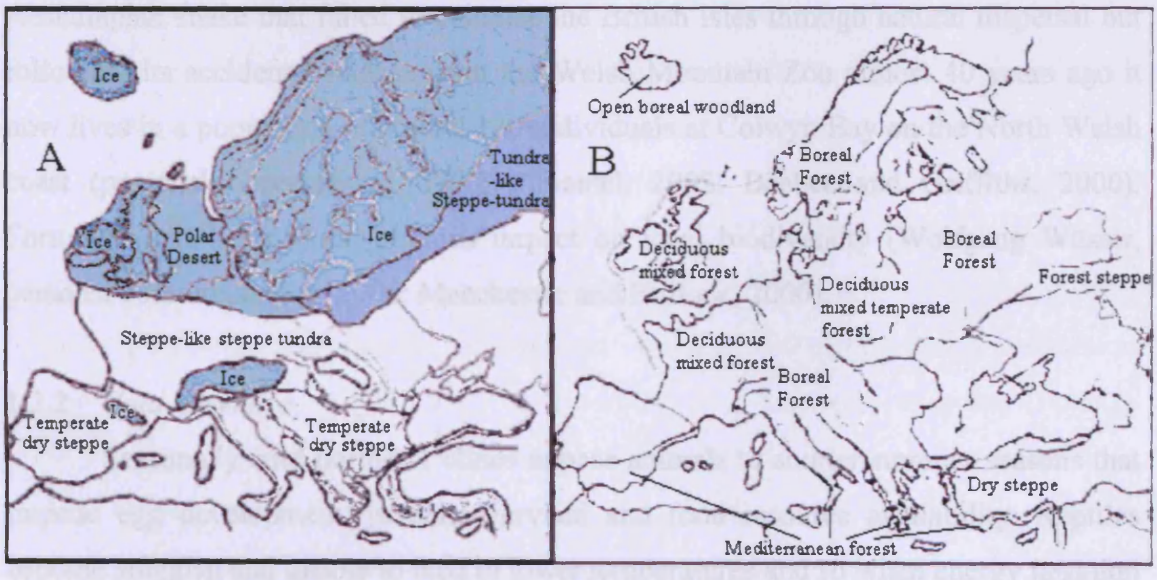

Figure 1.10 Map of Europe at the time of (A) the last ice age 18,000 years ago showing the extent of ice and permafrost at the last glacial maximum and (B) showing the returning forest after the last glacial maximum some 8,000 years ago (Velichko and Isavea, 1992).

Britain lay mostly under a thick sheet of ice during the last ice age some 18,000 years ago with permafrost expanding as far as southern France (Figure 1.10). Land bridges between mainland Europe and Britain were established by low sea levels, a consequence of water being bound up in the polar ice caps. Land bridges continued to exist as the ice and permafrost receded allowing new species to colonise Britain and other islands (Figure 1.10) (Graham et al., 2003; Hewitt, 2000). As glaciers melted and the permafrost receded, temperatures rose sufficiently for the expansion of many reptile

species into Europe. These animals would have expanded as rapidly as prey, temperature, geographical boundaries and their intrinsic dispersal ability would have allowed (Jansen et al., 2007). With the rising sea levels, certain reptile populations would become isolated on islands as land bridges gave way to ocean expanses (Graham et al., 2003; Hewitt, 1996). Only three snake species naturally populated the British Isles from their more southerly Younger Dryas refugia. All three species exhibited adaptation and behaviour that facilitated a more rapid northerly dispersal than their competitors, establishing populations in Britain prior to the severing of the land bridge connection to mainland Europe (Petit et al., 2003; Haggett, 2001; Gibb and Foster, 2000; Hewitt, 2000, 1999, 1998; Taberlet et al., 1998; Frazer, 1989). Many non-native snake species are released in the UK each year but few survive to sustain viable populations (Pimentel, 2002; Beebee and Griffiths, 2000). One exception is the Aesculapian snake that failed to colonise the British Isles through natural dispersal but following its accidental release from the Welsh Mountain Zoo almost 40 years ago it now lives in a population of around 120 individuals at Colwyn Bay on the North Welsh coast (personal observations, 2006; Pimentel, 2002; Beebee and Griffiths, 2000). Fortunately, it has had no obvious impact on local biodiversity (Wolfgang Wüster, personal communication, 2006; Manchester and Bullock, 2000).

1.2.2 *Cold tolerance*

Seasonally cold northerly clines expose animals to shorter summer seasons that impede egg development, juvenile survival and food resource availability. Reptiles become sluggish and unable to feed in lower temperatures and so when energy negation continues to exceed energy absorption, reptiles retire to thermally-buffered over-wintering refugia (Voituron et al., 2004; Beebee and Griffiths, 2000; Ultsch, 1989). Brumation only terminates when sufficient solar energy resources are available that permit energy absorption. If exposure to temperatures below 0°C is unavoidable then reptiles can employ one of two survival strategies; freeze avoidance (i.e. supercooling) or freeze tolerance (Storey, 2006; Beebee and Griffiths, 2000; Storey and Storey, 1992). The latter refers to reptiles that can tolerate freezing of extracellular body fluids during brumation. Such reptiles can revive many weeks later from a frozen state where up to 50% of their total body water had been locked in ice (Storey, 2006; Costanzo et al., 1995a; Storey and Storey, 1992). However, under laboratory conditions, Andersson and Johansson (1991) found that although newborn adders recovered completely from limited freezing at -3.1°C for up to 3 hours, they did not survive exposures of 22-30

hours (Burke et al., 2002). British reptiles appear to favour supercooling strategies and the selection of adequate overwintering facilities (Voituron et al., 2004; Andersson and Johansson, 2001). The exception being the common lizard, *Lacerta* (*Zootoca*) *vivipara*, that exhibits the unusual capacity to tolerate sub-zero temperatures by both supercooling and freeze tolerance. This lizard can remain in a supercooled state for 21 days at -3.1°C (a record duration for any vertebrate) and can survive the conversion of 50% of its total body water into ice for 1 day at-3.1°C (Voituron et al., 2004, 2002; Costanzo et al., 1995b).

1.2.3 *Advantage of ectothermy in cooler climates*

Although cold and darkness increasingly hinders the northern expansion of reptiles there are some advantages to being ectothermic under such dispersal trends. Sustaining the high body temperatures required by endothermic animals is energetically demanding and heavy on food resources (Arnold and Ovenden, 2002; Burghardt et al., 1996; Bennett, 1988). In contrast, in times of resource depletion ectotherms can cut back substantially on their food intake without losing body condition and prolonging their chances of survival (Beebee and Griffiths, 2000). Due to their low metabolism, reptiles can survive on as little as 10% of the food resources of their endothermic counterparts (Burness et al., 2001; Beebee and Griffiths, 2000). This enables reptiles to occupy niches at higher population densities than endotherms, in some instances occupying regions unable to sustain endothermic animals (Arnold and Ovenden, 2002; Bonnet et al., 1998). Food availability, however, does dictate reproductive effort in reptiles (Beebee and Griffiths, 2000). When food is abundant, it is common for clutches to be produced that are equal in weight to the parent, but when resources are limited clutch size is reduced to accommodate growth (Wapstra and O'Reilly, 2001; Beebee and Griffiths, 2000; Madsen, 1987a; Shine, 1980).

1.2.4 *Body temperature*

Reptiles need to regulate body temperatures above ambient conditions to extend their range into more northerly territories (Shine, 2004). This can be achieved internally (limited endothermy) through morphological features and behaviour, i.e., through selection of appropriate thermal microhabitats (Lutterschmidt et al., 2003; Brodie and Russell, 1999; Goff and Stenson, 1988; Crowley and Pietruszka, 1983). Reptiles are commonly categorised as purely ectothermic, only able to regulate their body temperature from their surrounding environment (Davies and Johnston, 2000; Vittand et

al., 1999; Bonnet et al., 1998). However, Davenport (1990) suggests that reptiles exhibit a spectrum of thermoregulation strategies ranging from endo- to ectothermic (Gledhill, 2007; Seymour et al., 2004; Davenport, 1990).

In Britain the adder will actively expand its ribcage in order to increase the surface area to body volume ratio (Beebee and Griffiths, 2000; Phelps, 1981). This enables the snake to absorb maximum solar energy more efficiently at cooler, higher altitudes than other British snakes. Once the adder has achieved the desired body temperature it will revert back into its more familiar cylindrical body shape to retain the achieved temperature (Shine, 2004; Arnold and Ovenden, 2002; Phelps, 1981; personal observations). Large reptiles are more thermally stable than small ones and larger body size in colder environments is adaptive (Meiri, 2008; Chiaraviglio, 2006; Tanaka, 2005; Angilletta et al., 2004; Pearson et al., 2003).

In addition to body size, body colour plays a key role in reptile thermoregulation (Beebee and Griffiths, 2000; Forsman, 1995; Gibson and Falls, 1979). Melanism is one of the most common colour variants with black phenotypes far more efficient at absorbing solar radiation, permitting them to exploit more northerly and montane niches unfavourable to other snakes, even conspecific specimens (Clusella-Trullas et al., 2008; Luiselli, 1993, 1992b; Forsman and Ås, 1987; Gibson and Falls, 1979). Melanistic adders are rare but can, on occasion, occur sporadically at high frequency of up to 50% of a population. Melanism is uncommon in smooth snakes and grass snakes, the exception being a number of small Mediterranean islands where a higher than normal frequently of melanistic grass snake females have been observed (Arnold and Ovenden, 2002; Taborowskii and Mynarski, 1962). In adders, there is a higher frequency of melanism in females than males. As with all British snake species, melanistic females tend to be bigger and give birth to large, stronger broods due to their ability to thermoregulate more efficiently (Forsman, 1995; Monney et al., 1995; Andrén and Nilson, 1981). In addition, melanistic males tend to be larger and commonly win food disputes and mating combats (Luiselli, 1993; 1992a; Andrén and Nilson, 1981). However, size variation amongst polymorphic populations is disputed by Forsman (1995). In contrast to the obvious advantages in expressing a melanistic phenotype, specimens experience diminished crypsis within their environment which in turn leads to higher rates of predation (Forsman, 1995; King, 1992).

The frequency of albinism is, not surprisingly, low for all British snake species with such conspicuous specimens under a higher risk of predation. However, an albino grass snake was held in captivity at London Zoo in 1951 (Smith, 1951) and an albino

adder was captured on a Cornish hedge bank along the A40 by the author in 2002 (personal observation, 2002).

1.2.5 *Predation*

Remaining cryptic within the environment is essential to reptile survival. In northerly clines, reptiles are under a constant threat from an array of predators including ravens (*Corvus corax*), crows (*Corvus corone cornix*), magpies (*Pica pica*), hedgehogs (*Erinaceus europaeus*), weasels (*Mustela nivalis*), foxes (*Vulpes vulpes*), buzzards (*Buteo buteo*), pheasants (*Phasianus colchicus*), kestrels (*Falco tinnunculus*), and in accordance with the vicinity urban habitat, domestic dogs and cats (Herczeg et al., 2007; Arnold and Ovenden, 2002; Beebee and Griffiths, 2000; Andrén and Nilson, 1981; Phelps, 1981). Also in northern regions, snakes tend to be more sluggish having to bask for longer time periods than their southern conspecifics.

Snake fight or flight antipredatory response behaviour varies considerably between northerly and southerly regions. If a snake's locomotor ability is compromised by cold conditions whilst migrating north, then it is more likely to display and strike rather than flee (Shine et al., 2000a; personal observations). Under such conditions, measurable distinctions have been observed between faster male and slower female locomotion in the garter snake (*Thamnophis sirtalis*) (see Shine et al., 2000a). However, rather than size or sexual divergence in antipredator behaviour, this reaction was attributed to a range of other factors including fatigue, poor condition, digesting a meal, being gravid or if an effective escape route is hindered by conspecifics or propinquity (Herzog and Bailey, 1989; Goode and Duvall, 1988; Duvall et al., 1985; Arnold and Bennet, 1984; Andrén, 1982).

In general, venomous or faster moving reptile species tend to practice heliothermy to more efficiently thermoregulate in extreme northern (or southern) clines (Herczeg et al., 2003; Klingenböck et al., 2000; Spellerberg, 1972). Standard colour morph adders are able to bask cryptically within their environment (Andrén and Nilson, 1981). As a heliotherm, the grass snake basks in open long grasses but can, on occasion, be encountered climbing bushes to take full advantage of solar radiation (Spellerberg and Phelps, 1977). In contrast, the slower moving reptiles, such as the smooth snake and slow worm are best described as semi-fossorial thigmotherms (Luiselli, 2006; Cabido et al., 2004; Platenberg and Griffiths, 1999; Agrimi and Luiselli, 1994). They primarily absorb their heat energy through conduction and are most often encountered sheltering under refugia and rarely bask in the open (although mosaic basking has been observed

by the author in both species). For slower moving reptiles, thigmothermy offers a means of thermoregulating whilst remaining cryptic to predators (Arnold and Ovenden, 2002; Shine, 2002; Beebee and Griffths, 2000; Spellerberg and Phelps, 1977).

1.2.6 *Parasitism*

Understanding the epizoology of wild reptile populations is important for conservation and evolutionary biology (Jacobson et al., 2005; Cheatwood et al., 2003; Uller et al., 2003; Calle et al., 2001; Karstad, 1961). However, declines in snake populations are largely unreported as qualitative or anecdotal evidence frequently substitutes quantitatively sampling for these reptiles (Winne et al., 2007). This is not true for all reptiles with sampling of certain lizard populations (e.g. *Anguis fragilis*) possibly due to ease of capture, limited vagility and their comparatively higher population density. Pathogens such as fungi (e.g. chytridiomycosis [*Batrachochytrium dendrobatidis*], coccidioidomycosis [*Coccidioides immitis* or *C. posadasii*], cryptococcosis [*Cryptococcus neoformans* species complex]; Harris et al., 2006; Knapp et al., 2006; Rachowicz et al., 2006; Briggs et al., 2005; Cheatwood et al., 2003), bacteria (e.g. *Aeromonas*, *Citrobacter*, *Enterobacter*, *Pseudomonas* and *Salmonella*; Lips et al., 2006; West, 2004; Cambre et al., 1980; Shotts et al., 1972; Caldwell et al., 1940) or viruses (e.g. Ranavirus, West Nile virus and arboviruses; western equine encephalitis, eastern equine encephalitis, Venezuelan equine encephalitis; Johnson et al., 2008; Klenk and Komar, 2003; Steinman et al., 2003; Hyatt et al., 2001; Bowen, 1977; Thomas et al., 1958) can reach epidemic proportions changing species richness and the diversity of an ecological community through local extinctions across a wide range of species. However, transmission rates can be lowered in reptiles at the fringe edge of northern dispersal due to lower population densities and limited contact between reptiles (Lips et al., 2006; Daszak et al., 2003; Johnson et al., 2003).

Perhaps a greater threat to northerly migrating reptiles is the introduction of new pathogens, to which the reptile may have limited or no resistance. Parasites can adversely affect natural populations posing significant threat to species that are residing at low population density or at an endangered status (Pedersen et al., 2007; Castro and Bolker, 2005; Lafferty and Gerber, 2002; Gibbon et al., 2000; Woodroffe, 1999; Cunningham and Daszak, 1998; McCallum and Dobson, 1995; Scott, 1988). Examples of parasites effecting reptiles across their range include protozoa (e.g. *Cryptosporidium*; *Trypanosoma brucei*, *T. bufophlebotomi n.* sp. *T. scelopori*, *Plasmodium floridense*, *P. azurophilum* and *P. azurophilum*; Ayala, 2007; Perkins et al., 2007; Green et al., 2003;

Schall and Staats, 2002; Schall et al., 2000; Njagu et al., 1999; Upton et al., 1989), helminths (e.g. *Mesocestoides* sp. Tetrathyridia [cestode], *Mesocoelium* sp, *Allogyptus crenshawi* [trematode], *Thelandros cubensis*, *Skrjabinodon* sp, *Spinicauda amarili* [nematode], and *Centrorhynehus* sp. [acanthocephalan]; Sharpilo et al., 2001; Hanley et al., 1995; Dobson et al., 1992; Aho et al., 1990) and ticks (e.g. *Amblyomma marmoreum*, *A. cajennense* and *A. hebraeum*; Fielden et al., 2008; Allan et al., 2000).

1.2.7 *Reproduction*

In temperate regions, the activity season (*ca.* mid-April to mid-October) for reptiles, is generally thought to be too short to allow females, particularly those of viviparous species, to bask, accumulate sufficient food resources and store adequate fat reserves to breed in consecutive years (Reading, 2004; Biebach, 1996; Naulleau & Bonnet, 1995; Madsen & Shine, 1993a; Whittier & Crews, 1990). Thus, the female of the three native British snake species breed on a biennial or even triennial schedule to the extreme north of their range (Beebee and Griffiths, 2000; Luiselli and Zimmermann, 1997; Luiselli et al., 1996; Madsen et al., 1996; Blem, 1982) with males breeding annually (Madsen and Shine, 1992; Andrén and Nilson, 1981). Snake species at such northern clines are at the limits of ectotherm reproduction with British snakes commonly found in a state of anorexia after giving birth (Gregory et al., 2006; Filippi,

Viviparity evolved from egg laying in many lineages of squamates in response to extreme climates (Chiaraviglio, 2006). Both *Vipera berus* and *Coronella austriaca* are viviparous as this reproductive mode ensures offspring are large enough to hunt independently from birth and be of sufficient size to maintain body heat to survive at higher altitudes and more northerly vicinities. It eliminates the need to guard or incubate young permitting parental movement, hunting and basking (Arnold and Ovenden, 2002; Madsen et al., 1996; Luiselli et al., 1995).

Compared to sympatric viviparous snakes, the oviparous grass snakes can achieve a much higher reproductive rate owing to larger clutch size and more frequent reproduction (occasionally annual, rather than biennial or triennial) (Beebee and Griffiths, 2000; Luiselli et al., 1997). The grass snake commonly lays communally exploiting a range of heat sources from residential buildings to decomposing plant matter to incubate its eggs. This strategy has assisted the grass snake in attaining the most northerly cline of any egg laying snake (Arnold and Ovenden, 2002). Alternative strategies, although not observed for UK grass snakes, include shivering thermogenesis during egg incubation which can result in large and prolonged metabolic increments

(Wang et al., 2003; Vinegar et al., 1970; Hutchison et al., 1966). Oviparous snakes bask significantly more often before oviposition than after, whereas males bask at a comparatively low rate throughout the same period (Madsen, 1987a). Further studies of oviparous temperate-zone snakes using radiotelemetry tracking of the Rat snake (*Elaphe obsolete*) revealed gravid females maintained higher body temperatures and thermoregulated more precisely than nongravid females or males (Blouin-Demers and Weatherhead, 2001). In this instance, gravid oviparous snakes, such as *Natrix natrix,* may be exhibiting the same set of behaviours that characterize pregnancy in viviparous species (Shine, 2004; Gregory et al., 1999).

Animals will generally exploit two extreme strategies to accumulate sufficient resources to fuel reproduction becoming either capital or income breeders (Drent and Daans, 1980). Income breeders tend to be mostly endothermic and are not particularly suited to long-term storage with investment in reproduction directly related to foraging success. Ectothermic snakes are considered typical capital breeders (Bonnet et al., 1998; Jönsson, 1997; Else and Hulbert, 1981) as a consequence of their low metabolism accumulating body reserves to fuel their reproductive effort (Bonnet et al., 1998; Pough, 1980). However, recent studies have highlighted the plasticity of snakes in their breeding strategy (Santos et al., 2007). Female *Vipera aspis* combine resources from both capital and income strategies to maximize litter size (Lourdais et al., 2003; Bonnet et al., 2001), while breeding success in *C. austriaca* is influenced by both a combination of capital and income energy, directly correlated to prey density in the breeding year (Reading, 2004). Tropical natricine snakes are able to produce two annual clutches by using endogenous resources for the first and exogenous resources for the second clutch (Santos et al., 2007; Brown and Shine et al., 2002).

For snakes, reproduction is the most intensive energy drain over a short time period (Santos et al., 2007; Drent and Daans, 1980). Body reserves can become rapidly exhausted through physiological (vitellogenesis, spermatogenesis) and behavioural (female searching, female attraction, male–male combats) reproductive processes (Bonnet et al., 1998; Jönsson, 1997; Else and Hulbert, 1981). In viviparous snakes, anorexia during pregnancy can generally be assigned to the conflicting demands of feeding and thermoregulation (Gregory et al., 2006), both essential to successful reproduction (Gregory and Isaac, 2004). Snakes accumulate energy, in the form of lipids, when resources are plentiful. Lipids are a highly concentrated source of metabolic energy (Thompson and Speake, 2003) that in snakes are stored in fat bodies

located along the abdominal cavity from the liver to the cloaca (Santos and Llorente, 2004; Dougthy and Shine, 1997; Derickson, 1976).

Top predators, such as snakes, exist at low densities within an ecosystem. Expanding into more northerly clines can further lower population density. Consequently, mate encounter rates are lowered. Under such circumstances prolonged sperm storage is favoured in females (Gist & Congdon 1998; Olsson & Madsen 1998). Sperm storage has been observed in *Vipera berus* (365 days; Stille et al., 1986.), *Natrix natrix* (180 days; Petter-Ronseaux, 1953; Rollinat, 1946) and *Coronella austriaca* (150 days; Rollinat, 1946) although snakes do not exhibit any specific organ adaptation for prolonged sperm storage compared to, for example, insects (Sever & Hamlett, 2002; Pitnick et al. 1999). Sperm storage for snakes at extreme northerly clines could be mandatory supporting mating in the autumn and fertilization in the following spring (Aldridge & Duvall, 2002; Oring et al., 1992; Schuett, 1992). It might also support multiple paternity, increasing genetic diversity of small populations through fertilization from mixed sperm stored from previous matings (Uller and Olsson, 2008; Birkhead, 1993). Sperm stored over a long period is known to be capable of fertilizing snake eggs (Olsson et al., 2007; Pearse et al., 2002; Cuellar, 1966). Furthermore, multiple paternities resulting from the mixing of stored sperm has been documented in captive *Agkistrodon contortrix* (see Birkhead, 1993; Schuett & Gillingham, 1986).

1.2.8 *Diet*

Predators, such as snakes, remain nested within the geographical range of their prey. Snake diet can be restricted by gape and body size and, as a consequence, this can restrict their range (Meiri, 2008; Okuyama, 2007; Case et al., 2005; Laurich et al., 2003). The smooth snake's primarily reptile diet would limit its northerly expansion to the expansion rate and density of its ectothermic prey (Reading, 2004; Luiselli et al., 1996). However, large smooth snakes become far more opportunistic in their choice of prey with the incorporation of mammals being common place (Reading, 2004; Luiselli et al., 1996; Cohen et al., 1993; Spellerberg and Phelps, 1977). Inclusion of endothermic mammalian prey would present opportunities to expand into areas devoid of small reptilian prey and niches too cold to support them.

Diet not only limits a snake's northerly expansion but can also influence the migration route taken. The basic amphibian diet of *N. natrix* would restrict this snake to low wetlands or within 1-2 km of river or marsh; the accepted range of frogs and toads from a watercourse, respectively (Beebee and Griffiths, 2000). It is highly unlikely that

arid desert regions would offer a suitable terrain for northerly migrations of small grass snakes (Gregory and Isaac, 2004; Luiselli et al., 1997). However, larger specimens could overcome limited stretches of arid habitat by incorporating a more diverse diet. In contrast, *V. berus* would generally avoid wetter habitats, preferring to hunt its mammalian prey amongst heath, wood and grasslands.

Prey availability may restrict the grass snake to low altitudes as it primarily hunts amphibians and fish. This diet is a limiting factor to the grass snakes northerly migration as prey abundance enables energy to be recouped more rapidly, which in turn permits annual breeding.

1.2.9 *Altitude*

Although *N. natrix* is found in mountainous habitat to the south of its range (Kryštufek, 2008; Thorpe, 1975) it remains a primarily lowland species to the north with substantial mountain regions proving a limiting factor to migration (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). Similarly, the smooth snake is a montane specialist to the south of its range but it remains a primarily lowland animal to the north (Beebee and Griffiths, 2000; Luiselli et al., 1996). The behaviour of the adder differs from the aforementioned snakes in that it is a cold tolerant specialist (Bhagwat and Willis, 2008; Meliadou and Troumbis 1997) and is out-competed to the south of its range by locally adapted *V. latasti* in the Iberian Peninsular, *V. aspis* in Italy and by *V. ammodytes* in the Balkans (see Ursenbacher et al., 2006; Carlsson et al., 2004; Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). In the Italian Alps, the adder is found at altitudes of up to 2600 m (Rauter et al., 2002; Bollmann and Reyer 2001; Luiselli and Anibaldi, 1991). At the northern edge of its range mountainous expanses present little obstacle to the adder, but would potentially present barriers to dispersal for both the smooth and grass snake.

1.2.10 *Aquatic environments*

In addition to mountain ranges, many river systems and wetlands dissect the migration route of reptiles to north Europe. Although all snakes can swim, not all are routinely found in the water. Tercafs (1961) found that *V. berus* cannot co-ordinate its movements or keep its head above water when attempting to swim for prolonged periods. Although adders are reasonable swimmers over short distances and may enter water briefly to evade predators (personal observation) negotiation of substantial faster flowing river systems and waterways would be challenging and may present a barrier to

dispersal. However, Forsman (1995) confirmed two individual adder migrations between islands in the Baltic Sea off the east coast of Sweden; one >2 km and another of ~20 km. He also reported gull activity across all his study islands. It is highly unlikely that an adder negotiated a single 20 km open sea swim (Beebee and Griffiths, 2000; Phelps, 1981) and amongst the more plausible explanations for such a migration would be island hopping to final destination or accidental distribution by tidal current, with or without detritus. However, another possible dispersal method would be capture and consecutive escape from an airborne gull. In 2006, the author witnessed a slow worm seemly falling from the sky some 3 m away. On closer observation, deep cuts indicative of a bird's beak were seen running along the back of the slow worm.

Smooth snakes are often observed near bogs but are not thought to regularly swim (Beebee and Griffiths, 2000). However, isolated reports do exist of smooth snakes swimming for prolonged periods (Smith, 1951) so small slow flowing river systems and lakes may not provide a sufficient barrier to smooth snake dispersal. In contrast, the grass snake is semi-aquatic and is often associated with ponds, ditches, marshes and water (Aubret and Shine, 2008; Gregory and Isaac, 2007). They are excellent swimmers keeping their heads above water in a periscope type pose whilst the lateral undulations propel the snake forward (Beebee and Griffiths, 2000). Grass snakes enter ponds regularly to hunt amphibians and will even dive possessing the ability to remain under water for up to 30 minutes at a time in order to ambush prey (Arnold and Ovenden, 2002; Griffiths et al., 1998). During hunting in ponds precise thermoregulation is abandoned with short basking periods countering the cold dips to boost body temperatures (Madsen, 1984). Lakes and river systems do not present a barrier to migrating grass snakes. On rare occasions *N. natrix* has even been sited far out to sea (Guicking et al., 2006; Foufopoulos and Ives, 1999; Boulenger, 1913), and although presumably these animals have drifted with currents, snakes carried amongst detritus could survive for periods long enough to founder nearby island populations (Schoener and Schoener, 1984; Heatwole and Levins, 1973).

1.2.11 *Climate change*

The current large scale declines in herpetofauna across the globe cannot be directly attributed to human expansion and the consequent loss of reptile and amphibian habitat (Araújo et al., 2006; Gibbon et al., 2000; Houlahan et al., 2000; Alford & Richards, 1999; Daszak et al., 1999; Blaustein and Wake, 1998; Halliday, 1998). This is perhaps best illustrated by the decline of reptile populations in the tropical rainforests of

Australia and South America where man's environmental impact (agriculture, deforestation, or pollution) is negligible (Araújo et al., 2006; Gibbon et al., 2000; Daszak et al., 1999; Pounds et al., 1997; Mahony, 1996). Whitfield et al. (2007) found a 75% decline in total densities of leaf litter amphibians and reptiles in primary forest since 1970 from their 35 year study at La Selva Biological Station, lower Central America. However, although global reptile declines have not been directly attributed to Man, anthropogenic impact on the global environment through increased ultraviolet (UV-B) irradiation (e.g. embryo development; Anzalone et al., 1998; disputed by Licht, 2003), acid precipitation, temperature (e.g. temperature dependent sex determination; Godfrey et al., 1999; Janzen, 1994), environmental pollution and consequently adverse weather patterns adversely affect populations (Araújo et al., 2006; Corn, 2005; Carey and Alexander, 2003; Collins and Storfer, 2003; Kiesecker et al., 2001; Gibbon et al., 2000; Alford & Richards, 1999).

1.2.12 *Urban environment*

Snake habitat has been influenced by human activity since forest felling following the Younger Dryas (Green, 1990; Heil and Diemont, 1983). Modern day dispersal of snakes is challenged by the continual expansion of urban and suburban environment (Beebee and Griffiths, 2000). Black tar road surfaces offer excellent basking habitat, which may account for the large number of roadkills due to snakes' limited ability to evade traffic (Shine et al., 2004; Ashley et al., 1996). Urban habitat, along with woodland fringe, has restricted the smooth snake to its southern English niche, south of the industrial and urban zones (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). One exception is a small population of smooth snakes inhabiting a busy motorway verge in Surrey (Beebee and Griffiths, 2000; Spellerberg and Phelps, 1977). The grass snake has adapted better to urban environments than the other two British snakes. It will commonly hunt from residential fish ponds and lay eggs in compost heaps (Isaac and Gregory, 2004; Boulenger, 1913). Along with adders, it will use major road systems and railway embankments as corridors to expand its range (Wisler et al., 2008; Arnold and Ovenden, 2002; Mertens, 1995). Apart from such routes, adders do not readily enter inner urban habitat. All snake species are further affected by continued human expansion and intervention through direct killing, introduction of new predators or competitors (by accidental release or biological control), trade, habitat loss, or deliberate and accidental fire (Winne et al., 2007; Araújo, 2003; Spellerberg, 1977).

1.2.13 *Human expansion and translocation*

Consideration must be given to selection pressure from human development and reptile population translocation. Slow worms, along with adders, grass snakes and common lizards are only partly protected under the Wildlife and Countryside Act (1981, Section 9, as amended) requiring developers to avoid death or injury to the reptile within the timescale of the translocation exercise and making trade of the animal illegal (JNCC, 1998). Unlike the smooth snake and the sand lizard, the slow worm does not attract full European protection. Slow worm translocations do not currently require the conditions of monitoring of a licensed translocation. The slow worm is one of the three native lizard species of the British Isles; the others being the common lizard (*Lacerta* (*Zootoca*) *vivipara*), and the sand lizard (*Lacerta agilis*) (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000). Of these, *A. fragilis* is the only species that could be practically used to assess the feasibility of translocations due to its ease of capture, non-invasive identification, limited vagility and, most importantly, comparatively high population density. (Webster, 2007; Ferreiro et al., 2004; Platenberg and Griffiths, 1999; Capula et al., 1998; Capula and Luiselli, 1993; Smith, 1990; Stumpel, 1985). Although it is the most common British reptile, little is known of their ecology (Webster, 2007; Ferrerio et al., 2004; Capula et al., 1997; Capula and Luiselli, 1993).

1.2.14 *Genetics and evolution*

Finally, in the face of the aforementioned selection pressures it is essential for reptiles to maintain genetic variation for the long term survival of the population (Amos and Balmford, 2001). Demographic stochasticity, genetic drift or inbreeding can reduce genetic variation inturn reducing population fitness and compromising the populations' ability to respond to environmental change. The necessity to maintain genetic variation within a population is best illustrated by Madsen et al. (1999) who introduced new genotypes into a severely inbred and isolated population of *Vipera berus* to the extreme north of its range. This not only halted the populations' steep decline towards extinction but resulted in extensive population expansion.

1.3 Phylogeography

A phylogeographic approach can be a useful analytical tool in uncovering cryptic speciation, although its main focus is generally on intraspecific genealogies and their relation with geography, history and demography (Avise, 1994). Phylogeography is a branch of biogeography studying the spatial patterns of genetic diversity within and between species. Phylogeographic reconstructions have been used to infer the geographic origin of populations, genetic bottlenecks and range expansions in many species including reptiles (Ursenbacher et al., 2006; Taberlet et al., 1998; Avise et al., 1987). Biogeographic barriers, such as mountain ranges (Hewitt, 2000) and rivers (Goossens et al., 2005; Eriksson et al., 2004; Telfer et al., 2003), may disrupt gene flow thus moulding genetic structure. Phylogeography departs from classical population genetics by explicitly focusing on the species' history, in particular the biogeographic past, when studying patterns of genetic variation through inferred gene trees, but still owes its statistical and mathematical foundations to it (Knowles and Maddison, 2002).

1.3.1 *Migration routes*

The current distributions of reptiles in the British Isles imply that these species survived ice age events in warmer southern European refugia (Beebee and Griffths, 2000). As the Mediterranean Sea forms a deep trench it did not dry out during the lower sea levels of ice age episodes and hence would have formed a barrier to the southern dispersal of European terrestrial reptile species (but see Hsü et al., 1977, regarding the Messinian salinity crisis). With climate change events some 15-18,000 years ago came the rapid range expansion of reptile species into Northern Europe (Hewitt, 2000). Temperatures rose to as high as, or even higher than, those of modern day for several millennia until the rapid cooling of the Younger Dryas. Reptile species sought shelter in relatively northerly refugia before climate change and colonisation of Northern Europe and Britain some 10,000 years ago. Major river systems and mountain ranges, such as the Alps and Pyrenees, would have presented challenging barriers to dispersal for many reptile species. These mountain ranges supported considerable glaciations with bound ice reducing sea levels by up to 120 m (compared to current day levels).

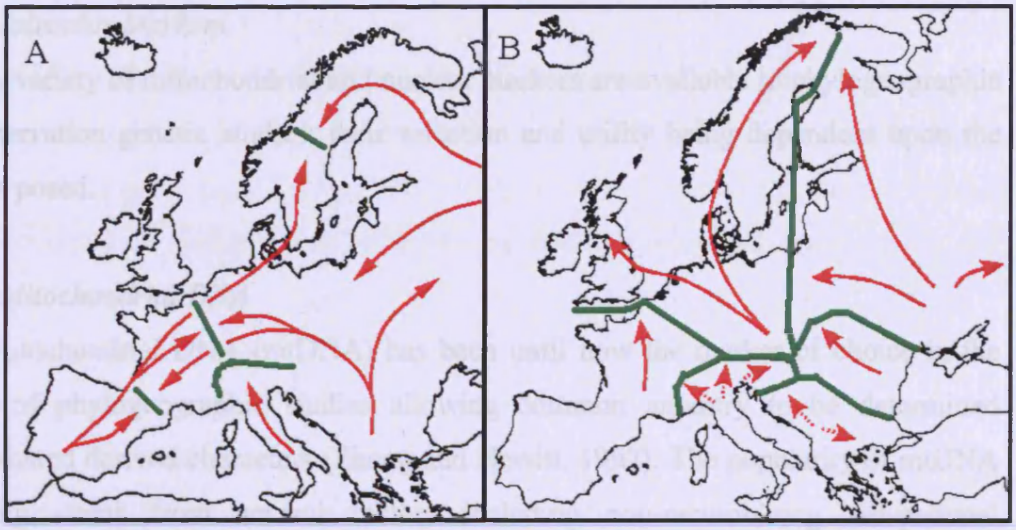

Figure 1.11 (A) The main post-glacial colonisation routes and subsequent suture zones in Europe (Taberlet et al., 1998) and (B) European recolonisation routes used by *Vipera berus* (see Ursenbacher et al., 2006). Green lines symbolize probable contact zones between clades/ subclades and red arrows represent altitudinal migrations.

As a consequence, land bridges were established including between France, Belgium, the Netherlands and Britain (Hewitt, 2000). Figure 1.11 shows the probable post-glacial colonisation routes and contact zones for European fauna (Taberlet et al., 1998; also see Hewitt, 2000) and for *Vipera berus* (see Ursenbacher et al., 2006). Although colonisation routes have been proposed for *V. berus* little consideration was given to the Britain and other northern clades although limited study was undertaken in Scandinavia by Carlsson et al. (2004). Currently, no complete phylogeographical study of *Natrix natrix* nor *Coronella austriaca* has been undertaken across their range (but see Santos et al., 2008 and Guicking et al., 2006).

1.3.2 *Molecular Markers*

A variety of mitochondrial and nuclear markers are available to phylogeographic and conservation genetic studies; their selection and utility being dependent upon the questions posed.

1.3.2.1 *Mitochondrial DNA*

Mitochondrial DNA (mtDNA) has been until now the marker of choice in the majority of phylogeographic studies allowing common ancestry to be determined through shared derived characters (Zhang and Hewitt, 1997). The popularity of mtDNA sequencing stems from several factors including non-recombining uni-parental (maternal; with the exception of paternal leakage/ hetroplasmy) inheritance, few duplication/ insertion/ deletion events, a conserved gene order and number (Kaneda et al., 1995; Avise et al., 1987; Fischer-Lindahl 1985; Giles et al., 1980) and its rapid rate and well understood mode of evolution (Kocher et al., 1989; Moritz et al., 1987). The effective population size of haploid mtDNA is approximately 25% when compared to diploid nuclear autosomal genes with biparental transmission (Moore, 1995). MtDNA is therefore likely to be congruent with a species phylogeny due to high probability of coalescence even when divergence events have occurred over short time periods. Mitochondrial DNA exists in high copy number in each cell presenting excellent opportunity to amplify from non-invasively collected sample material such as ancient, faecal substrates and weather degraded field samples. The rate of evolution is 5-10 times higher in mtDNA than the nuclear DNA of most animals (DeSalle et al., 1986; Ferris et al., 1983; Brown and Stimpson, 1982). However, the rate of evolution is not constant throughout the mitochondrial genome or for the same genes between species (Ballard and Kreitman, 1995; Cann et al., 1984). Factors such as substitution rate heterogeneity must therefore be considered and corrected for, especially when a molecular clock is used to date the coalescence of mitochondrial lineages.

Inherited as a single linkage group (Hoech et al., 1991), animal mtDNA is a circular molecule of 15-20 kb and in vertebrates contains genes for 22 transfer-RNAs, 2 ribosomal-RNAs and 13 messenger–RNAs coding for proteins involved in the electron transport and oxidasive phosphorylation. The only non-coding region of the mtDNA is the ~1.5 kb long control region which is involved in the regulation and initiation of mtDNA replication and transcription (Ballard and Rand 2005; Moritz et al., 1987). MtDNA is prone to oxidative damage since the mitochondria are the sites of cellular respiration and consequently rich in free oxygen radicals (Martin and Palumbi, 1993).

This oxidative damage and higher replication errors when compared to that of the nuclear DNA genome (Brown, 1979) coupled with more relaxed constraints on the DNA repair mechanisms (Cann et al., 1984) result in rapid accumulation of selectively neutral mutations (principally base substitutions). These nucleotide substitutions make it possible to establish phylogenetic relationships, even between the lower taxonomic ranks of species and subspecies (Cann et al., 1984).

1.3.2.2 *Cytochrome* b

Cytochrome *b* is an integral membrane protein that contains two hemes in a protein of molecular mass of about 40 kDa. Cytochrome *b* is one of the best known of the 9-10 proteins that make up complex III of the mitochondrial oxidative phosphorylation system (Hatefi, 1985) and is the only one encoded by the mitochondrial genome. It contains two redox centres involved in electron transfer. The knowledge of structure-function relationships in cytochrome *b* enhances the utility of its gene for evolutionary investigations (Irwin *et al* 1991). Cytochrome *b* has been used to establish the evolutionary relationships between true vipers (Lenk et al., 2001), *Vipera berus* genetic structure and phylogeography (Ursenbacher et al., 2006; Carlsson et al., 2004), the evolution and phylogeny of the genus *Natrix* (see Guicking et al., 2006) and the molecular systematics of racers (Nagy et al., 2004) amongst others. Partial cytochrome *b* sequence was chosen as a 'phylogenetic probe' since as a functional protein-coding gene it would be easier to align than non-coding genes (i.e. the D-loop) and in reptilia, cytochrome *b* exhibits a similar rate of evolution as the D-loop (Ursenbacher et al., 2006).

1.3.3 *Molecular clocks*

The relative high levels of sequence variation in the animal mtDNA control region (and cytochrome *b* in reptiles) allow calculation of a 'molecular clock' to be established. This has previously been estimated in the region of 0.47-1.32% per million year (my $^{-1}$) for mtDNA divergence rates for small to medium sized ectotherms (Zamudio and Greene, 1997). There have been few specific estimates for the rate of sequence evolution of the cytochrome *b* gene in squamate reptiles. In bushmasters (*Lachesis* sp.) the divergence rate for cytochrome *b* has been estimated at 0.6-0.76% my^{-1} (Pook et al., 2000).

1.4 Aims and layout of the thesis

This study investigates the genetic diversity in the increasingly threatened *Natrix natrix, Coronella austriaca* and *Vipera berus* populations across Europe and aims to define how these species came to populate the British Isles from their ice-age refugia using phylogeographic analysis of cytochrome *b* mitochondrial DNA sequences. In addition, current day reptile population management issues are examined using *Anguis fragilis* as a British model reptile to assess translocation protocols. For the first time, parasite loads of translocated slow worms are assessed and correlated with host growth rates.

Three data chapters allow the following hypotheses to be tested:

Hypothesis I: Non-invasive materials from British snake species are present in sufficient quantity and quality to construct mtDNA phylogenies.

Hypothesis II: *V. berus* was the first snake species to populate the British Isles and maintained a presence in the UK during the Younger Dryas.

Hypothesis III: The *C. austriaca* populations of Surrey and isolated populations in Dorset and Hampshire, UK are monophyletic.

Hypothesis IV: Current reptile translocation mitigation is inefficient and can be updated to reduce both effort and cost.

Hypothesis V: The parasite loads of translocated slow worms affect host growth rates and present a previously undetected challenge to reptile mitigation.

The thesis is presented as five self contained chapters. Methodologies are outlined in each specific data chapters. Chapter 2 on non-invasive sampling has been recently published in the Herpetological Journal. Chapter 3 concerns comparative British snake phylogeography and in Chapter 4 *A. fragilis* is used as a model organism to assess the efficiency of reptile translocation. Finally, Chapter 5 gives an overview of the entire study and highlights future research avenues.

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CHAPTER 2: An evaluation of non-invasive sampling for genetic analysis in northern European reptiles

Abstract

Genetic studies of native herpetofauna populations are important for the conservation of European biodiversity. However, previous studies have been largely dependent on invasive sample collection. Here we explore the efficiency of non-invasive sampling (NIS) for molecular studies and review the potential different sources of such samples. Snakes produce a multitude of by-products, such as sloughed skin, faeces and eggs or embryos, which along with road kills, predated specimens and museum samples, could potentially be used in molecular studies. We describe a new method for obtaining snake faeces in the field and, using mitochondrial cytochrome *b* primers, we successfully amplified 500 and 758 bp sequences from a variety of tissues collected by NIS. The availability and degradation of such material differed greatly, and both DNA extraction and PCR success appeared dependent upon sample origin and storage. Nevertheless, for the first time we demonstrate that faecal, egg and foetal tissues, as well as sloughed skin and carcasses, represent valuable NIS source material permitting genetic studies with minimal disturbance to the individual and its population.

2.1 Introduction

The study of indigenous herpetofauna in the field is important for conservation and our understanding of reptile ecology in a changing landscape. Increasingly, prime reptile habitat in northern Europe is being modified, destroyed or fragmented (Beebee and Griffiths, 2000), with reptiles having to adapt to changes instigated by anthropogenic expansion. In general, translocation success rates for amphibians and reptiles are lower than those for mammals and birds (Platenberg and Griffiths, 1999; Reinert and Rupert, 1999; Dodd and Seigel, 1991; Griffith et al., 1989), and yet translocation exercises rarely employ genetic data. This is perhaps surprising, given the now seminal study of Madsen et al. (1999), who demonstrated unequivocally that the introduction of new genotypes into a severely inbred and isolated population of *Vipera berus* not only halted its precipitous decline towards extinction but resulted in dramatic population expansion. However, molecular ecological approaches are now more commonly used in the conservation of herpetofauna (e.g. Leaché and Reeder, 2002; Morrison and Scott, 2002; Ciofi and Bruford, 1999; Madsen et al., 1999) and cryptic snake taxa are being re-assigned following genetic analysis (Wüster et al., 2002;

Burbrink, 2002; Puorto et al., 2001). Molecular characterization of individuals may also resolve novel insights into how genetic variation is partitioned within and among populations (e.g. Carlsson et al., 2004). Invasively obtained material has tended to be the source for herpetological genetic studies to date (Feldman and Spicer, 2002; Voris et al., 2002; Keogh, 1998) but this is now less acceptable with the wide availability of commercial non-invasive sampling (NIS) DNA extraction kits. These offer affordable, alternative methods for molecular studies with minimal disturbance to the animal and its population (Morin and Woodruff, 1996). Below we summarize the various sources of NIS material that could potentially be utilized for molecular studies, concentrating on our target taxa, the three snake species indigenous to northern Europe: the adder (*Vipera berus*), the grass snake (*Natrix natrix*) and the smooth snake (*Coronella austriaca*).

2.1.1 *Sloughed skin*

Sloughs can be found fragmented or whole around hibernation sites, often entwined within gorse, bracken and other coarsely textured plants, and beneath tins or other such cover. With warm weather conditions, sloughs dry quickly in the field and can be folded into an envelope or sample bag on collection and stored dry.

2.1.2 *Aborted embryo, egg, road kill and museum samples*

Muscle is available for DNA extraction from semi-predated and stillborn carcasses, road kills and museum samples (Dallas et al., 2003). Stillborn offspring from both the adder and the smooth snake can be collected in the vicinity of hibernation sites. Grass snake eggs, laid from late June to July, are found in compost and manure heaps, decaying tree stumps, woodchip piles and even rotting seaweed. Around 30% of eggs fail to hatch in the autumn or are infertile (Beebee and Griffiths, 2000), therefore nonviable unfertilized eggs should only be collected after this period, being identifiable by their discoloured state. Although museums are a potential source of animal tissue, traditional specimen fixatives (commonly formalin) are optimized for morphological study, which degrades DNA (Chang and Loew, 1994; Pääbo, 1989; Chaw et al., 1980). Extracting DNA from formalin-fixed material is possible but laborious and PCR amplification success rates are low (Serth et al., 2000). The analysis of tissue stored in formalin pH <7 for longer than 12 months should be restricted to analysis of short (<100–200 bp) DNA fragments (Bucklin and Allen, 2003).

2.1.3 *Faeces*

Faecal samples typically contain low quantities of degraded target DNA (Taberlet et al., 1999; Gerloff et al., 1995), but have proved to be a valuable source of DNA from avian and mammalian samples (e.g. Regnaut et al., 2006; Robertson et al., 1999; Taberlet et al., 1999) and should also be useful in herpetological studies. Snake faeces are challenging to obtain in the field non-invasively but can potentially be found throughout the snakes' active period from late April to October. As faecal material contains a range of micro-organisms and is particularly prone to deterioration by endogenous nucleases, the highest quality DNA is found in freshly collected faeces (Wehausen et al., 2004; Taberlet et al., 1999). Faecal matter gathered in the field should be immediately sealed and cooled in a collection bag. This can either be frozen at –20°C or stored in ethanol (>95% molecular grade), RNALater (Ambion) or silica gel (Nsubuga et al., 2004) at 4°C.

2.1.4 *Cloacal and buccal swabs*

Cloacal and/or buccal swabbing is a rapid, inexpensive and potentially easy to implement field method for obtaining reptile DNA samples (Miller, 2006). However, in addition to the delicate bone structure of British reptiles, there are obvious hazards associated with buccal swabbing from both venomous and non-venomous reptiles. Therefore, for both these reasons this procedure is not recommended for British snakes.

2.1.5 *Teeth and bone*

Teeth and bone samples, from semi-predated and stillborn carcasses, shed teeth, road kill and museum samples, might yield DNA of sufficient quality for certain studies, but because of the time involved in sample preparation and the number of replicates required (see Rohland et al., 2004; Wandeler et al., 2003), they should only be considered in the absence of other more suitable tissues. This study aimed to demonstrate the utility of a range of non-invasively collected samples for mitochondrial DNA PCR in the three native UK snake species.

2.2 Material and methods

2.2.1 *Origin of samples*

Table 2.1 shows the preservation method of the samples collected during the current study. An additional 20 sloughs and 20 carcasses from *Vipera berus* were collected 1–30 days prior to DNA extraction. Field collected carcasses, road kills and foetal samples were directly frozen at –20°C or preserved in 95% ethanol. Ancient ethanol preserved tissue (A.E.P.T.; collected pre-1907 to 1969) consisted of museum samples donated by the National Museum of Wales, Cardiff. Non-viable grass snake eggs, located in compost heaps, were preserved at –20°C. Sloughed skins, mostly collected at the entrance of identified adder domains, were preserved dry at room temperature for up to two years. An additional fresh slough was obtained from an adder observed in the process of ecdysis. Snake faeces (associated with sloughs) collected from a range of UK sites were either immediately frozen at –20°C or preserved in 95% ethanol. A 10 µl blood sample, obtained by caudal extraction from an adder and stored in 90 µl of Seutin's buffer (Seutin et al., 1991) at room temperature, was collected as a positive PCR control.

2.2.2 *DNA extraction*

Sloughed skin required a rehydration step to remove impurities prior to DNA extraction. A fragment (1–2 cm^2) of slough was placed in a 1.5 ml eppendorf tube containing 1 ml of double distilled (dd) water at 55°C in a rocking incubator. After 4–6 h the water was removed and a further 1 ml of dd water added to each sample prior to incubation at 55°C for a further 8–12 h. DNA extraction was performed on these rehydrated samples, egg yolks (approximately 0.2 cm^3), NIS muscle (1 cm^3) and blood (5 ml in Seutin's buffer) following the manufacturer's protocols for Qiagen DNeasy® (Cat. # 69504) tissue extraction kit. Faecal material (1 cm^3) was extracted following the manufacturer's protocol for the QIAamp® DNA stool mini kit (Cat. # 51504). DNA extraction was not attempted from teeth or bone in the current study but relevant protocols and commercial kits are available (see QIAamp® DNA minikit protocol). A maximum of three extraction attempts were performed for each sample, with second and third extractions only prepared on failure to successfully amplify a product after three PCR attempts from the previous extraction. Partial cytochrome *b* gene amplification and sequencing two different snake PCR primer sets were used that generated approximately 500 and 758 bp amplicons. The first primer pair consisted of a

forward primer (UKsnakecyto_F, 5' CAACATCAACTTAGCCTTCTC 3') adapted from cytochrome *b* primer, 703bot (Pook et al., 2000) and a reverse primer (UKsnakecyto_R, 5' GTGGAATGGGATTTTATCG 3') designed from an alignment of partial cytochrome *b* gene from *Vipera berus* (GenBank accession number AJ275728) and *Natrix natrix* (AF471059). The second primer set, 5' TCAAACATCTCAACCTGATGAAA 3' and 5' GGCAAATAGGAAGTATCATTCTG 3', were previously used by Pook et al. (2000) to generate a 758 bp cyt *b* fragment. Primer set 1 was tested on all tissue samples, whereas set 2 was only tested on the additional 20 sloughs and 20 carcasses from *Vipera berus*. Each PCR was performed in 25 µl comprising 1 µl of DNA, 1×Invitrogen buffer (200mM Tris-HCL, pH 8.4, 500 mM KCl), 3 mM $MgCl_2$, 250 mM of each dNTP, 0.5 mM of each primer and 1 U of Invitrogen *Taq* polymerase. DNA amplification was performed at 96°C for 4 min and then 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min with a final extension cycle of 72°C for 3 min in an Applied Biosystems GeneAmp® PCR system 9700 thermocycler. Both negative (dd water) and positive (adder blood) controls were included with each PCR. PCR success was determined by running products on an agarose gel. Fragment length of PCR product was determined by interpolation using a 100 bp ladder. PCR products were purified using the GeneClean® Turbo for PCR kit. For each DNA sequencing reaction, 3 µl of PCR product, 2.5 µl Better Buffer (Webscientific Ltd, Cat. # 3BB-10), 0.5 µl ABI Big Dye Vs. 3 and 2.4 pmol of primer was made up to a final volume of 11 µl volume with deionised dd water. Sequencing of the isopropanol purified products was performed in both forward and reverse directions. Sequencing PCR entailed a step of 94°C for 90 s followed by 25 cycles of 96°C for 15 s, 50°C for 10 s and 60°C for 4 min. Samples were run on an ABI 3100 DNA semi-automated DNA analyser (Perkin Elmer) and sequences were aligned using Sequencer™ and corrected by eye.

2.3 Results

2.3.1 *Faecal sample collection*

Although potentially accessible, snake faeces are challenging to identify and rarely located in the field. During the course of this study, we developed a simple semi-invasive method of faeces collection following the observation that snakes often defecate in response to direct disturbance or handling. In a series of trials, individual snakes were carefully scooped from the ground in an upright motion whilst the tail was quickly transferred into a small plastic collection bag. As adders are venomous and have particularly delicate cervical vertebrae, extreme care is necessary when securing a hold on these animals and the mid-body should also be supported while the snake is held aloft and the tail placed into the collecting bag. However, unless suitably qualified we strongly advise the use of restraint tubing to secure the adder allowing safer handling. Repeated sampling (n=100) revealed that the majority of British snakes handled in this fashion would defecate in the bag, usually within 20 s of capture. This procedure was most reliable with grass snakes (about 95% defecation) compared to smooth snakes (about 75%) and adders (about 70%). These animals could then be released after photography, measurement and other details had been recorded. The sample bag was quickly cooled *in situ* before storage at –20°C.

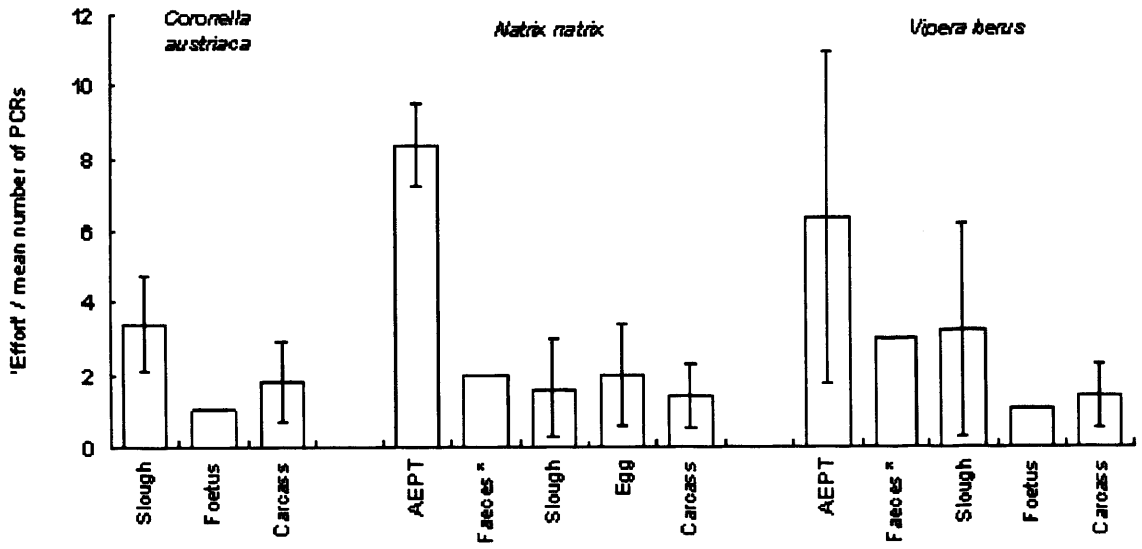

Figure 2.1 Mean number (±SE) of PCR reactions required to generate a positive 500 bp partial cytochrome *b* gene amplification product from non-invasive snake sample material. Road kill and other carcasses are grouped together.

*Error bars are not shown for the faecal samples as for both species only one of the two PCRs successfully amplified.

2.3.2 *DNA extraction and amplification*

Table 2.1 displays the success rates of extraction and amplification of the 500 bp mtDNA products from non-invasively collected sample material. These tissue types were sampled for all three British snake species with the exception of faeces and ancient ethanol preserved tissue (A.E.P.T.) for *Coronella austriaca*, which were not available in the current study. All different types of tissue samples eventually amplified and sequenced, but with varying rates of success both between tissue types and species (Figure 2.1). As predicted, the highest success rates for DNA extractions and amplification were obtained from recently preserved muscle samples, namely fresh foetus and other carcasses including road kill.

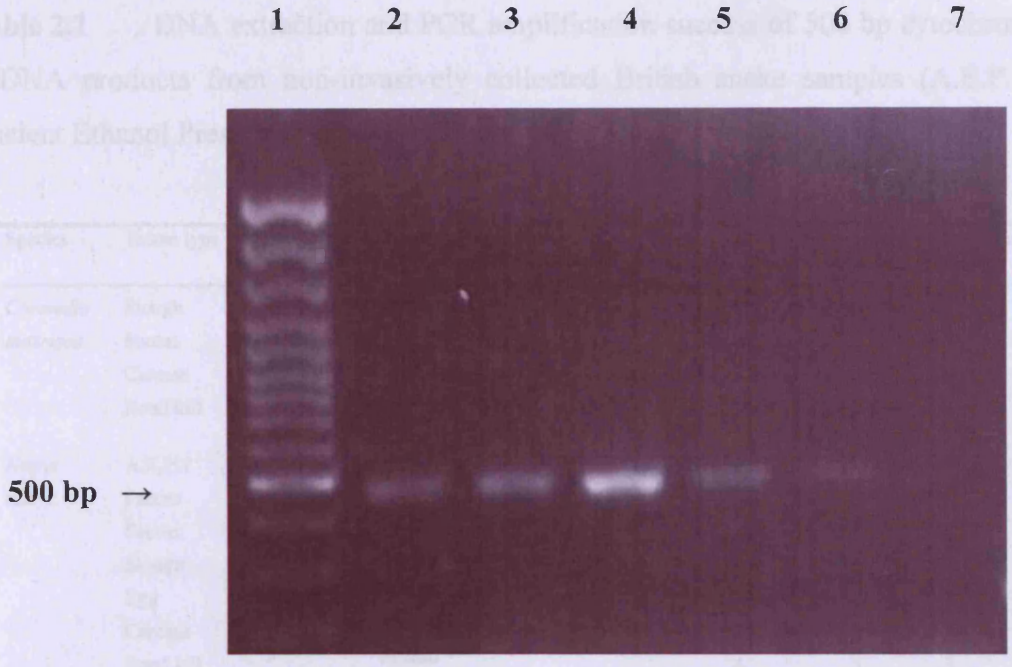

Figure 2.2 Gel electrophoresis of partial cytochrome *b* amplification products from *Vipera berus* and *Natrix natrix* non-invasively collected tissues and blood. Lane (1) 100 bp DNA size ladder; (2) blood; (3) sloughed skin; (4) carcass (road kill); (5) Ancient Ethanol Preserved Tissue (museum sample); (6) egg; and (7) faeces.

Theses samples consistently produced the strongest intensity 500 bp fragment (based on comparison to 100 bp ladder luminosity) with an amplification success of 84% (*V. berus* 90%; *N. natrix* 86%; *C. austriacas* 81%). There was no obvious difference between ethanol and frozen samples, but sample sizes were low. High amplification success (70%) was also obtained using a second primer set that consistently generated 758 bp of cyt *b* sequence from 20 *V. berus* carcasses. Three DNA extractions from eggs generated faint 500 bp bands in two out of four PCRs (Table 2.1). Dried sloughs, including a sample stored for two years, amplified strong 500 bp products with an overall 61% amplification success rate (*V. berus* 64%; *N. natrix* 85%; *C. austriacas* 40%), although the brightness of this product was more intense from the fresh slough (Figure 2.2). Surprisingly, 758 bp fragments were also obtained from 10 of 20 *V. berus* dried sloughs.

Table 2.1 DNA extraction and PCR amplification success of 500 bp cytochrome *b* mtDNA products from non-invasively collected British snake samples (A.E.P.T. = Ancient Ethanol Preserved Tissue).

Species	Tissue type	No. of samples	Preservation method	No. of extracts	No. of PCRs	Positive PCR
Coronella austriaca	Slough	5	Dried	9	17	5
	Foetus	2	Ethanol	2	2	2
	Carcass	2	Ethanol	2	2	2
	Road kill	3	Ethanol	2	7	3
Natrix natrix	A.E.P.T	3	Ethanol (one stored for 50-100 y)	9	26	3
	Faeces	1	Ethanol	1	2	0
	Faeces	1	Frozen	1	2	1
	Slough	5	Dried (one fresh)	6	8	5
	Egg	2	Frozen	3	4	2
	Carcass	2	Ethanol	2	2	2
	Road kill	3	Frozen	4	5	3
Vipera berus	Faeces	1	Ethanol	1	2	0
	Faeces	1	Frozen	2	3	1
	Slough	5	Dried (one 2 y old)	8	15	5
	Foetus	2	Ethanol	2	2	2
	Carcass	2	Ethanol	2	2	2
	Road kill	2	Frozen	3	4	2
	Road kill	1	Ethanol	1	1	1

From faeces, 500 bp fragments were amplified from one of five samples for both *V. berus* and *N. natrix,* but products were weak in intensity and the resulting sequences were of extremely poor quality. A.E.P.T. snake samples required up to three extractions before positive amplifications were achieved in five of the six samples tested (Table 2.1), with only 41% and 11% amplification success rate for *V. berus* and *N. natrix,* respectively. Sequences of 500 bp with very few or no ambiguous bases were obtained from all tissue types, apart from faeces. The 758 bp cyt *b* fragment was successfully sequenced from *V. berus* carcasses and sloughs; sequencing of this larger amplicon was not attempted from other tissues types. Interspecific sequence homology allowed alignment of partial cyt *b* sequences from all three species and yet sufficient base substitution existed to reliably determine and identify sequences at species level. BLAST searches revealed that all sequences generated during the current study matched with GenBank sequences of adder, grass snake or smooth snake.

2.4 Discussion

This is the first study to demonstrate the feasibility of DNA amplification from snake faeces, egg and foetal material, as well as from other non-invasive samples including slough skin and non-invasively sampled (NIS) muscle. Although snake faeces have previously been used to identify the morphological remains of specific prey items (e.g. in the black rat snake; Weatherhead et al., 2003), their utility as an NIS material for DNA extraction from snakes had not previously been investigated. The most reliable NIS sources in this study were muscle tissue and slough skin, with ancient tissue samples being the least reliable. Some snake by-products can be easily located in the field, but consideration must be given to exclude temporal duplication of samples from the same individual, for instance multiple skin sections from the same slough that have broken up and dispersed. If only a small number of samples are analysed, such non-random sampling can skew data and give a false representation of population genetic diversity. However, repeated sampling of the same individual over time can provide useful ecological data regarding movement and lifespan. Recording the exact location of collected samples is critical and advances in geographic information system (GIS) technology (Salem, 2003) allow patterns of genetic structure to be analysed in a geographical context (e.g. Kidd and Ritchie, 2006). It is also important to record the age of the sample and method of preservation. PCR from template DNA extracted from degraded tissue is problematic, due not only to general DNA degradation but also to the presence of inhibiting factors (Wehausen et al., 2004; Kohn and Wayne, 1997). In addition, using universal (highly conserved) mitochondrial PCR primers, there is an increased likelihood of inadvertently amplifying non-target organism DNA. Furthermore, even if DNA does amplify, decayed nuclear DNA is more commonly associated with genotyping errors, such as allelic dropout and false alleles (Taberlet et al., 1999). In the current study, we also developed a new method for faeces collection from wild-caught snakes. In the field, British snakes can be secured and scooped vertically from the ground, initiating a defecation defence response. The faeces can be simultaneously collected and appropriately preserved for subsequent DNA extraction. There appeared to be a relationship between stool consistency and successful sample collection: grass snakes (that have loose stools) are more likely to defecate when handled than either smooth snakes (with intermediate stools) or adders (firmer stools). Such variation in faecal consistency is related to diet, with grass snakes preferring amphibians and fish, smooth snakes eating mostly reptiles and rarely small mammals, and adders consuming mostly small mammals. Typically, snakes with loose stools

defecate more regularly than those with firm stools and so our observations are not surprising, but we demonstrate how this natural response can be exploited for semi-NIS. Faecal samples have previously been an overlooked source of reptile DNA. However, faeces are routinely used to genotype protected species (e.g. Chih-Ming et al., 2004; Garnier et al., 2001; Bayes et al., 2000; Goossens et al., 2000). Although DNA from faecal samples is degraded, microsatellite analyses and sequencing of short amplicons is usually possible, but DNA fragments of greater than 500 bp are difficult to sequence, as observed in the current study. More in-depth studies are required to assess the maximum size of amplicons than can be sequenced from snake faeces. In addition, storage methods for snake faeces should be optimized, as has been done for large mammals. For example, Roeder et al. (2004) described a two-step method of preservation whereby gorilla faecal samples stored in ethanol for 24–36 h were subsequently transferred into silica for optimal DNA preservation. The most reliable source of non-invasive sample material was muscle tissue from carcasses (road killed and semi-predated specimens), recent museum samples and foetal tissue. Roads provide excellent basking opportunities for snakes (under low-traffic conditions) as they heat up quickly, maintaining temperature throughout the day and into the cooler evening (Shine et al., 2004; Ashley and Robinson, 1996). Unfortunately, snakes are often not quick enough to evade vehicles, exhibiting momentary immobilization in response to traffic (Andrews and Gibbons, 2005), but no agency records snake road kill statistics for the UK, despite the fact that animal carcasses do provide high quality genetic data for a multitude of studies including phylogeography and phylogenetics (Piertney et al., 2005 Doyon et al., 2003; Keogh, 1998). DNA from grass snake eggs was successfully extracted and amplified, but success rates could not be assessed during the current study due to the small sample size. Unhatched eggs should only be collected late in summer/autumn after all viable eggs have hatched, but such samples are likely to be contaminated by microbial PCR inhibitors (Fernando et al., 2003). Avian eggshell membrane is established as a non-invasive DNA source (Fernando et al., 2003; Strausberger and Ashley, 2001), allowing genotyping of an identified egg-laying female without disturbance; however, this is the first study to show that yolk tissue can also be used to identify British reptiles such as the grass snake or sand lizard. We have not yet tested whether DNA can be successfully extracted from the membranous egg shells of grass snakes. Of the non-invasive materials tested in this study, sloughed skin was the easiest to collect and store directly from the field. DNA extraction from slough does require an initial rehydration step, but this is still a simple and quick method. The lower yield of DNA (compared to muscle)

and its potentially fragmented condition can make amplification above 500 bp intermittent, especially in older samples. However, with relatively fresh sloughs it is possible to amplify DNA fragments in excess of 750 bp, although previous studies indicate the ability to amplify such products would diminish with time (Fetzner, 1999). Sloughed skins are often subjected to moisture, UV and microbial damage before collection, which reduces DNA quality. Generally, it is recommended that studies based on dry, room-temperature-stored sloughs collected over a year (in various states of decay) should not aim to target sequences over 700 bp (e.g. Ursenbacher et al., 2006). The inclusion of slough as a source of DNA is becoming more frequent for snakes (Clark et al., 2003; Burbrink, 2002; Vidal et al., 2000) and other animals (e.g. Sigler et al., 2002; Fetzner, 1999; Valsecchi et al., 1998). Ancient tissue sources were amongst the least viable non-invasively collected source tissues for reptiles. This could be due to degradation of DNA (Krause et al., 2006; Thomas et al., 2005) or contamination of the sample storage medium, e.g. in the event of evaporation, alcohol preserved sample bottles being "topped up" from neighbouring bottles containing related sample material. Although a single band may apparently be amplified, it is common to produce a recombinant or multiple sequence comprising two or more individuals. Depending on storage media and sample age, extractions from preserved museum samples often prove labour-intensive (Serth et al., 2000; Pääbo, 1989). Non-invasively collected British herpetofaunal samples reliably yielded DNA of sufficient quality and quantity for sequencing; in fact the current study is a conservative estimate of the value of snake NIS tissues for genetic studies as we intentionally targeted relatively long DNA fragments (500 and 758 bp). Populations and species of an endangered, threatened or highly protected nature, such as the British sand lizard or smooth snake, can now be studied non-invasively, producing viable data and valuable insight into the ecology of these reclusive animals.

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CHAPTER 3: Comparative phylogeography of British snakes (*Vipera berus*, *Natrix natrix* and *Coronella austriaca*) inferred from non-invasive samples

Abstract

European temperate snake species used both southern glacial and more northerly Younger Dryas refugia during times of severe palaeoclimatic oscillations. In the current study, non-invasively collected samples were used to amplify cytochrome *b* mtDNA sequences to reconstruct phylogenetic histories for *Vipera berus* (434 bp), *Coronella austriaca* (141 bp) and *Natrix natrix* (265 bp). Median Spanning networks, Bayesian inference and Neighbour joining analyses grouped all these three British snake species within Italian lineages. *V. berus* showed greater genetic variability (5 haplotypes) in the UK than the other two monophyletic snake species. It is likely that *V. berus* survived in British Younger Dryas refugia whilst both *C. austriaca* and *N. natrix* retreated to more southerly European refugia. The re-colonisation of Britain by snakes was made possible through the existence of a land bridge (~18,000 years ago) prior to the warm preceding interstadial deglaciation and isolation of the British Isles (~9000 years ago).

3.1 Introduction

European temperate fauna and flora have experienced multiple range shifts driven by severe palaeoclimatic oscillations. Many species survived glacial episodes in southerly refugia situated around the Caucasus Mountains and the Iberian, Italian, and Balkan peninsulae. Re-colonisation of northern Europe emanated from these refugia, south of the ice and permafrost, during interglacial and interstadial climatic periods. Refugia and post glacial colonisation routes have now been corroborated by genetic data for a range of organisms (Hewitt, 2004, 2000, 1999, 1996; Taberlet et al., 1998). Land bridges between mainland Europe and Britain were established by low sea levels, a consequence of water being bound up in the polar ice caps. Britain lay under a thick sheet of ice, which extended from South Wales to the Thames basin during the last glacial maximum (circa 18,000 years ago). Land bridges continued to exist until 9,000 BP as the ice and permafrost receded allowing new species to colonise Britain and other islands (Graham et al., 2003; Hewitt, 2000).

The term phylogeography was first used by Avise et al. (1987) for the composite process of inferring the phylogenetic relationships among individual sequences (haplotypes) and superimposing the resulting haplotype phylogeny over the geographic locations of the samples to reveal spatio-temporal patterns of population structure.

Phylogeography can provide reasonably straightforward inferences where congruent patterns are evident, but such patterns are not always evident and depend on demographic history, life-history and dispersal patterns, which are population and species-specific. Comparative phylogeography derives its power from the analysis from many co-distributed species and can accommodate the incongruent patterns of interspecific disparate time scales. The comparison of phylogeographic patterns among broadly sympatric species shows whether those species have responded in parallel to recent isolating events or barriers to their dispersal (see Chapter 1). If so, geographical congruence in genetic structure and diversity may be recovered (Templeton, 1998). However, extinctions, dispersal, and overlapping events in earth history can obscure phylogeographic history with recovery of species information becoming more challenging to resolve over time (Zinc, 2002; Cracraft, 1988).

Britain is populated by three native snake species; the adder (*Vipera berus*), grass snake (*Natrix natrix*) and smooth snake (*Coronella austriaca*). These snakes can tolerate cooler temperate regions than other European species (Isaac and Gregory, 2004; Beebee and Griffiths, 2000; Meliadou and Troumbis, 1997; see Chapter 1). The adder is patchily distributed throughout mainland Britain from Cornwall to the Outer Hebrides. It is the most widely distributed terrestrial snake species (Arnold and Ovenden, 2002; Beebee and Griffiths, 2000; Phelps, 1981) ranging from the UK in the west through to Pacific Russia in the east, Greece to the south and Sweden to the north. It is the most cold-tolerant snake species in the world, being found at latitude of 69°N and altitudes of up to 2600 m at the south of its range (Gasc et al., 1997). The grass snake is the most commonly occurring British snake, although is absent from Scotland (Beebee and Griffiths, 2000). It is the only snake species found in the Channel Islands (Jersey). In Europe, its range extends from Spain in the west through to Lake Baikal in the east, the Balkans to the south and as far north as southern Scandinavia. Although primarily a lowland animal in the north of its range, it can be found at heights of 2000 m in the Alps. In Europe it ranges from northern Spain in the west through to the Caucasus to the east, Norway to the north and as far south as the Balkans. It is believed that the smooth snake arrived in southern England around 7500-8000 yrs ago (Strijbosch, 1997) establishing an historic range from Dorset to Surrey and as far north as Wiltshire and Somerset that was only restricted by the distribution of southern heaths (Herpetological Conservation Trust Rare Species Database, 2009). Continuing human development, including the expansion of conifer plantation, has seen a decrease in population size and

range with the current smooth snake population restricted to three southern counties; Dorset, Hampshire and Surrey (Beebee and Griffiths, 2000).

In addition to various morphometric analyses (e.g. Thorpe et al., 1984), evolutionary processes have been investigated at the genetic level using Amplified Fragment Length Polymorphism (AFLP) and both mitochondrial and nuclear DNA sequencing with several studies investigating the genetic variation of the adder and grass snake across their geographical range (Guicking et al., 2006; Ursenbacher et al., 2006; Bond et al., 2005; Carlsson et al., 2004; Carlsson and Tegelström, 2002; Stille et al., 1986) but with more restricted coverage of the smooth snake (Santos et al., 2008). Furthermore, there have been no comprehensive investigations of the genetic diversity of these snakes throughout mainland Britain. To date, most phylogeographic analysis has been made possible through the sequencing polymorphic regions of mitochondrial DNA (mtDNA). The cytochrome *b* gene has become a commonly used mtDNA coding region in snakes, often proven to resolve greater genetic variability than the control region (Ursenbacher et al., 2006) and is therefore used in this study.

In the current study we examine the utility of non-invasive sample material in the characterisation of the genetic structure of the adder, grass and smooth snakes focussing on their potential colonisation routes and glacial refugia. In particular, we asked the following questions:

(I) Can we identify mtDNA lineages for European populations of these three species and if so, which of these lineages do UK populations comprise.

(II) If *V. berus* was the first snake species to populate the British Isles does it possess greater genetic diversity compared to the grass and smooth snakes.

(III) Can we detect genetic differentiation between the *C. austriaca* population of Surrey and isolated populations in Dorset and Hampshire, UK and if so, what implications does this have for the management of this locally threatened species.

3.2 Materials and methods

3.2.1 *Origin of samples*

Where feasible, sample material was collected in a semi or fully non-invasive fashion. A total of 111 (55, *V. berus*; 10, *C. austriaca*; 46, *N. natrix*) samples from the UK were collected from, England, Wales, Scotland and the Channel Islands from localities listed in Appendix I. An additional 48 (16, *V. berus*; 5, *C. austriaca*; 27, *N. natrix*) field samples were collected and donated from professional bodies. Finally, 117 (41, *V. berus*; 35, *C. austriaca*; 41, *N. natrix*) sequences were obtained from Genbank (Appendix I).

3.2.2 *Preservation of sample material*

Field collected carcasses, road kills and foetal samples were directly frozen at -20°C or preserved in 95% ethanol. Museum ethanol preserved tissue (collected pre-1907 to 1969) consisted of museum samples donated by the National Museum of Wales, Cardiff. Non-viable grass snake eggs, located in compost heaps, were preserved at -20°C. Sloughed skins, mostly collected at the entrance of identified adder domains, were preserved dry at room temperature for up to two years. Additional fresh slough was obtained from snakes observed in the process of ecdysis. Snake faeces (associated with sloughs) collected from a range of UK sites were either immediately frozen at -20°C or preserved in 95% ethanol. The majority of samples (92%) were sloughed skins (see Jones et al., 2008).

3.2.3 *DNA extraction*

Sloughed skin required a rehydration step to remove impurities prior to DNA extraction. A fragment (1-2 cm^2) of slough was placed in a 1.5 ml eppendorf tube containing 1 ml of double distilled (dd) water at 55°C in a rocking incubator. After 4-6 h the water was removed and a further 1 ml of dd water added to each sample prior to incubation at 55°C for a further 8-12 h. DNA extraction was performed on these rehydrated samples, egg yolks (approximately 0.2 cm^3), NIS muscle (1 cm^3) and blood (5 ml in Seutin's buffer) following the manufacturer's protocols for Qiagen DNeasy® (Cat. # 69504) tissue extraction kit. Faecal material (1 cm^3) was extracted following the manufacturer's protocol for the QIAamp® DNA stool mini kit (Cat. # 51504).

3.2.4 *Primer design and cytochrome* b *amplification*

Two different snake PCR primer sets were used to generate approximately 500 and 758 bp fragments of cytochrome *b* (cyt *b*; see Chapter 2). Primer set 1 (Jones et al., 2008) was tested on all tissue samples, whereas set 2 (Pook et al., 2000) was only tested on the additional 20 sloughs and 20 carcasses from *Vipera berus*. Both primer sets were amplified under the same conditions. Each PCR was performed in 25 µl comprising 1 µl of DNA, 1×Invitrogen buffer (200 mM Tris-HCL, pH 8.4, 500 mM KCl), 3 mM MgCl2, 250 mM of each dNTP, 0.5 mM of each primer and 1 U of Invitrogen *Taq* polymerase. DNA amplification was performed at 96°C for 4 min and then 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min with a final extension cycle of 72°C for 3 min in an Applied Biosystems GeneAmp® PCR system 9700 thermocycler. Both negative (dd water) and positive (snake blood) controls were included with each PCR. PCR success was determined by running products on an agarose gel. Fragment length of PCR product was determined by interpolation using a 100 bp ladder.

3.2.5 *DNA sequencing and alignment*

PCR products were purified using the GeneClean® Turbo for PCR kit. For each DNA sequencing reaction, 3 µl of PCR product, 2.5 ml Better Buffer (Webscientific Ltd, Cat. # 3BB-10), 0.5 ml ABI Big Dye Vs. 3 and 2.4 pmol of primer was made up to a final volume of 11 ml volume with deionised dd water. Sequencing of the isopropanol purified products was performed in both forward and reverse directions. Sequencing PCR entailed a step of 94°C for 90 s followed by 25 cycles of 96°C for 15 s, 50°C for 10 s and 60°C for 4 min. Samples were run on an ABI 3100 DNA semi-automated DNA analyser. Sequences were aligned using Sequencer™ (vs. 3.1.2; Genecode Corporation). and corrected by eye. Resulting consensus sequences were aligned into a single file for each species and this data was then exported in 'Nexus' format.

3.2.6 *Genetic diversity*

Nucleotide (π) and haplotype (h) diversity were calculated in Arlequin vs. 3 (Excoffier et al., 2005; Nei 1987).

Table 3.1 Interpretation of differences between haplotype and nucleotide diversities (after Grant and Bowen, 1998).

		Haplotype diversity (*h*)	
		Low	High
Nucleotide Diversity (π)	Low	Recent population bottleneck. Founder effect with single or few lineages.	Bottleneck followed by rapid population growth and mutation accumulation.
	High	Divergence between geographically subdivided populations.	Large stable population with long evolutionary history. Secondary contact between differentiated lineages.

Due to low sample sizes, nucleotide diversity (π) was calculated by grouping populations into Northern, Italian, Balkan and (with the exception of *V. berus*) Iberian clades, and compared among groups, among populations within groups and within populations using AMOVA in Arlequin 3.11 (Excoffier et al., 2005).

3.2.7 *Phylogenetic analyses*

The 112 *Vipera berus* (434 bp), 50 *Coronella austriaca* (141 bp) and 114 *Natrix natrix* (265 bp) cyt *b* sequences were used for phylogenetic and phenetic analyses using Bayesian and neighbour joining (NJ) approaches. For Bayesian and NJ analyses, a total of 88 substitution models were evaluated using jModeltest version 0.1.1 (Posada, 2008; Guindon, 2003). The best observed data-fitting models were selected by the Akaike Information Criterion (AIC) for each species is shown in Table 3.2.

Table 3.2 Best fitting model selected by Akaike Information Criterion (AIC) for each study species as inferred by jModeltest version 0.1.1 (Podosa, 2008; Guindon, 2003).

Species	Best model (AIC)	Freq. A	Freq. C	Freq. G	Freq. T	Distribution Shape parameter
Vipera berus	TrN+G	0.2628	0.3217	0.1310	0.2846	Gamma 0.0100
Coronella austriaca	TrN+G	0.2892	0.2679	0.1340	0.3089	Gamma 0.2210
Natrix natrix	HKY+I	0.3097	0.2805	0.1396	0.2702	Kappa 10.6404 ti / tv = 5.1188 p-inv = 0.7790

Bayesian estimation of phylogeny was calculated using MrBayes vs. 3.1.2. (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bayesian inference of phylogeny employs Markov Chain Monte Carlo (MCMC) simulation to approximate the posterior probabilities of trees. In addition, each data set was analysed with Neighbour joining (NJ) methods conducted using MEGA vs. 4 (Tamura et al., 2007). The robustness of the branching of the trees was tested for both Bayesian and NJ methods by 100,000 bootstrap replicates. The resulting Bayesian trees were displayed using Microsoft® Paint vs 5.1. with supported NJ clades shown in parentheses.

3.2.8 *Networks*

Gene genealogies were investigated from the construction of networks in addition to evolutionary trees, as the latter assume no reticulate evolution: a common feature of intra-specific genealogies. Intraspecific genealogies can be affected by persistence of ancestral haplotypes, reticulated and large sample sizes and low divergence among individuals (Campbell et al., 2007; Posada and Crandall, 2001). Median spanning networks were implemented in Network vs. 4.1.1.1. (Bandelt et al. 1999). Median spanning networks are constructed by combining all parsimonious minimum spanning trees within a single network following an algorithm analogous to Excoffier and Smouse (1994). Median vectors (representing missing intermediates) are added to the network using parsimony criterion (Posada and Crandall, 2001; Bandelt et al., 1999).

3.2.9 *Haplotype and relative lineage incidence*

Haplotypes occurring at country level were grouped together in a proportional pie diagram to illustrate haplotype incidence across Europe. In addition, closely related and geographically connected haplotypes were grouped together into proportional pie diagrams to demonstrate relative lineage incidence. Haplotype incidence diagrams for each study species were independently presented on maps of Europe along with a combined study species map of relative lineage incidence. Due to generally low levels sampling at each country except the UK, we were unable to determine haplotype and lineage frequency but provide complementary information to the networks and trees and illustrate haplotype occurrence.

3.2.10 *Mismatch distributions*

Population demography was analysed using mismatch distributions: the distribution of the observed number of differences between pairs of haplotypes. Samples drawn from a population at demographic equilibrium that exhibit highly stochastic gene tree topologies usually have a multimodal distribution. In contrast, populations having passed through recent demographic bottleneck and expansion (see Rogers and Harpending, 1992; Slatkin and Hudson, 1991) or through a range of expansions with high levels of migration between neighbouring demes (see Excoffer, 2004; Ray et al., 2003) tend to be unimodal. Mismatch distributions can be calculated using three different models: sudden expansion, pure demographic expansion and spatial expansion. As the first two models are highly analogous only the pure demographic and spatial expansion models were calculated for the current study using Arlequin 3.11 (Excoffier et al., 2005).

A demic expansion will generally occur if the range of a population is initially restricted to a small area prior to the range of the population expands over time and space. This expansion model illustrates the generally expected ectotherm population expansion from thermally constrained ice age refugia. In the demic expansion model, the shape of the gene genealogies and the overall pattern of diversity within demes are influenced by the age of the expansion and the level of gene-flow, Nm (where N= size of deme; m= proportion of migrants) between neighbouring demes (Ray et al., 2003). Star-shaped and multimodal mismatch distributions represent gene genealogies generated by low gene-flow (<1 migrant per generation) producing a substantial proportion of coalescent events early in the genealogy. For large Nm values, most

coalescent events occurring around the time of the onset of spatial expansion will produce a mixture of both short and long branch-length gene genealogies resulting in multimodal mismatch distributions. Only where demes have high Nm values will tests of selective neutrality (i.e. Tajima's D or Fu's F_S; Fu, 1997; Tajima, 1989) show significant negative values after a spatial expansion.

3.2.11 *Selective neutrality and population history*

Statistics based on mismatch distribution are not always robust at detecting expansion, particularly where sample size is small. Consequently, a range of tests were employed to detect signatures of past population growth or stability based on DNA sequences (Ramos-Onsins and Rozas, 2002). Fu's F_S (Fu, 1997), Fu and Li, D* and F* (Fu and Li, 1993) and Tajima's D (Tajima, 1989) tests of neutrality Tests of neutrality (and demographic history) were based on the infinite-site model (ISM) without recombination (appropriate for mtDNA). The effects of background population growth or range expansion can be distinguished by Fu and Li's (1993) D* and F* statistics collectively with Fu's (1997) F_S. If F_S is significant but F* and D* are not then this would indicate population growth or range expansion, while the reverse indicates selection (Fu, 1997). All selective neutrality tests were carried out in Arlequin 3 (Excoffier et al., 2005) with the exception of Fu and Li's (1993) D* and F* which were calculated in DnaSP 4.10.3 (Rozas et al., 2003). In the current study, sample sizes for each snake species are relatively low (i.e. <115). In addition, haplotypes were grouped by country with sampling for each of these populations also small. Such small population sizes can lead to bias through the substantial misrepresentation of haplotype frequency (Beavis, 1994). Hence, although the current study accurately records haplotype occurrence throughout Europe it is unable to accurately establish haplotype frequency.

3.3 Results

Cytochrome *b* sequences were obtained from all three British snake species (all sequences and haplotypes used to infer phylogenetic reconstruction are listed in Appendices I and II). Despite the varying length of the sequences that could be obtained (434 bp for *Vipera berus*, 141 bp for *Coronella austriaca* and 265 bp for *Natrix natrix*), similar numbers of parsimony informative sites were observed in each species (Table 3.3; Figure 3.1).

Table 3.3 Haplotype / nucleotide diversity for each study species.

Species	Number of Samples	Number of Haplotypes	Number of Variable sites	Parsimony informative sites	Nucleotide diversity (π)	Haplotype diversity (h)
Vipera berus	112	26	37	27	0.00827	0.639
Coronella austriaca	50	20	32	26	0.05576	0.898
Natrix natrix	114	19	41	34	0.03124	0.686

The *V. berus* Median spanning network (MSN; Fig. 3.2) and the Bayesian and NJ phylograms (Fig. 3.3) revealed three well supported groups (Italian, Balkan and Northern). The most geographically widespread (Northern) group is composed of two subgroups; a basal Carpathian group and a group containing the rest of the northern European expanse. *V. berus* showed lower nucleotide diversity, but similar haplotype diversity to the other study species, indicative of a recent bottleneck followed by rapid population growth (see Table 3.3). Nucleotide diversity was significantly different among groups, among populations within groups and within populations (AMOVA, for all $P<0.000001$). Mismatch results for the Northern clade (Fig. 3.4) displayed uni-modal distribution and were significant ($r=0.0498$; $P<0.05$) indicative of population growth or range expansion. These results were supported by significant values for both Tajima's D (-1.97833; $P<0.05$) and Fu's F_s (-19.016; $P<0.001$). In addition, a trend was observed for both Fu and Li's D (-2.08164) and F (-2.37264) statistics, although results were not significant. Both the Italian and Balkan clades returned non significant results for both

mismatch and neutrality tests suggestive of stable populations. Haplotype occurrence across Europe for *V. berus* is shown Figure 3.5.

Although the data set for *C. austriaca* resulted in similar rates of parsimonious informative sites to the other two study species, the current genetic data and sample size offered limited phylogeographic structure. However, the *C. austriaca* MSN (Fig. 3.6) was in agreement with both the Bayesian and NJ trees (Fig. 3.7) revealing a complex structure of seven supported putative groups (Iberian western, Iberian central, Iberian eastern, Northern, Italian, Balkan and (albeit of low bootstrap support) Greek. *C. austriaca* displayed similar rates of haplotype and nucleotide diversity indicative of a large stable population. Conversely, mismatch results for the whole Iberian Peninsular (Fig. 3.8) were bi-modal in distribution and revealed a range expansion (r=0.0404) which was supported by Fu's F_s (-6.201; $P < 0.05$). However, there were no significant mismatch or neutrality results for the Iberian Peninsular when analysed as three separate clades or for any of the other identified clades. Haplotype occurrence across Europe for *C. austriaca* is presented in Figure 3.9.

The MSN (Fig. 3.10) and both Bayesian and NJ trees (Fig. 3.11) revealed five supported groups (Southern, Central, Northern, Eastern and Iberian clades) for *Natrix natrix*. The Southern group is composed of two supported subgroups, albeit of low bootstrap support; the Balkan and Greek subgroups. In addition, the Central group is composed of two well supported subgroups; in Italy and north western Europe. Similar rates of haplotype and nucleotide diversity were observed for *N. natrix* indicative of a large stable population. However, although not significant (Fu's F_s=0.068), the Southern group mismatch results were uni-modal in distribution and showed a trend towards expansion (r=0.0889). As with *C. austriaca*, AMOVA results for *N. natrix* were not significant as greater nucleotide diversity existed within than between groups. Haplotype occurrence across Europe for *N. natrix* is presented in Fig. 3.13.

Relative lineage incidence across Europe for all three study species is shown in Figure. 3.14.

A)

	CTT	AGC	CTT	CTC	ATC	CAT	TGT	TCA	CAT	CAC	CCG	AGA	TGT	CCC	ATA	CGG	TTG	AAT	TAT	ACA	AAA	CTC	ACA	CGC	CAT	CGG
HAP1																										
HAP2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP5	...	...	...	...	...	...	...	C..	...	...	...	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP6	.C.	...	...	...	...	...	...	C..	...	...	...	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP7	...	...	T..	...	...	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP8	...	...	T..	...	...	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP10	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP12	...	...	...	...	...	...	...	...	...	A..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP13	...	...	...	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP14	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP15	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP16	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP17	.C.	...	...	...	...	...	...	C..	...	...	...	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP18	...	...	...	...	...	...	...	C..	...	...	...	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP19	.C.	...	...	...	...	...	...	C..	...	...	...	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP21	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP22	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP23	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP24	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.C	...	...	...	...	...	...	...	...
HAP25	...	...	T..	...	...	...	...	C..	...	.T.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP26	...	...	T..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

B)

	TCC	ATA	TTC	TTC	ATC	TGC	ATT	TAT	ATC	CAT	ATC	GCA	CGC	GGC	TTA	TAC	TAC	GGG	TCG	TAC	TTA	AAT	AAA	AAC	GTC	TGA
HAP2	...	...	...	...	...	...	...	..C	...	...	...	...	..T	...	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP3	...	...	...	...	...	...	...	..C	...	...	...	...	...	..G	...	...	..T	...	..A	...	C..	...	...	...	..A	..G
HAP4	...	..G	...	...	...	...	...	..C	G..	...	...	...	..T	...	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP5	...	...	...	...	...	...	...	..C	...	...	...	...	..T	...	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP6	...	...	..T	...	...	...	...	..C	...	..C	...	...	..T	...	...	...	..T	...	..A	...	C..	...	...	...	...	...
HAP7	...	...	..T	...	...	...	...	..C	...	..C	...	...	..T	...	...	...	..T	...	..A	...	C..	...	...	...	...	...
HAP8	...	..G	...	..T	...	...	...	..C	...	..C	...	...	..T	..A	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAP10	...	...	...	...	...	...	...	..C	...	..C	...	...	..T	..A	...	...	...	...	...	...	C..	...	...	...	...	..T
HAP11	...	...	...	...	...	...	...	..C	...	...	...	G..	...	..T	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP12	...	...	..T	...	...	...	...	...	...	...	..C	...	...	..T	...	...	...	...	...	..A	C..	...	...	...	...	...
HAP13	...	...	...	...	...	...	..C	...	...	...	...	G..	...	..T	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP14	..A	...	...	..T	...	...	...	..C	...	...	...	...	...	..T	..T	C..	...	...	...	..A	C..	...	...	...	...	...
HAP15	..A	...	...	..T	...	...	...	..C	...	...	...	...	...	..T	...	C..	...	...	...	..A	C..	...	...	...	...	...
HAP16	..A	...	...	..T	...	...	...	..C	.C.	...	...	...	...	..T	...	C..	...	...	...	...	C..	...	...	...	...	...
HAP17	...	...	...	...	...	..T	...	..C	...	...	...	...	...	...	..G	...	...	..T	..A	..C	C..	...	...	...	...	...
HAP18	...	..G	...	...	...	...	...	..C	...	...	...	...	..T	...	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP19	...	..G	...	...	...	...	...	..C	...	...	...	...	..T	...	...	...	..T	...	...	...	C..	...	...	...	...	...
HAP20	..A	...	...	..T	...	...	...	..C	...	...	...	...	..T	..T	C..	...	...	...	...	...	C..	...	...	...	...	...

C)

	ATT	TTT	TAT	CTG	TAT	CTA	TAC	CCA	CAT	TGC	ACG	TGG	ACT	TTA	CTA	TGG	CTC	CTA	CCT	AAA	CAA	AGA	AGT	GTG	ACT	ATC
Hap1																										
Hap2	...	C..	C..	...	C..	T..	C..	...	...	...	...	...	...	...	T..	...	...	...	...	...	...	...	...	A..	...	...
Hap3	...	...	C..	...	C..	...	C..	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...
Hap4	...	...	...	...	C..	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hap5	...	...	C..	...	C..	...	C..	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...
Hap6	...	...	...	...	C..	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...
Hap7	...	...	...	...	C..	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hap8	...	...	...	...	C..	...	C..	...	T..	...	...	...	...	...	...	...	...	...	...	T..	...	...	...	A..	...	...
Hap9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hap10	...	...	...	...	C..	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hap11	...	...	...	...	C..	...	C..	...	T..	...	...	...	...	...	...	...	...	...	...	T..	...	...	...	A..	...	...
Hap12	...	...	...	...	C..	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hap13	...	...	C..	...	C..	...	C..	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	T..	...	...
Hap14	...	...	...	...	C..	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
HAp15	...	...	C..	...	C..	...	C..	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...
Hap16	...	...	...	...	C..	...	C..	T..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hap17	...	...	...	...	...	...	C..	...	T..	...	...	...	G..	...	...	...	...	...	...	...	...	...	...	...	...	...
Hap18	...	C..	C..	...	C..	T..	C..	...	...	...	...	...	...	...	T..	...	...	...	...	...	...	...	...	A..	...	...
Hap19	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...

Figure 3.1 Viable nucleotide positions in the alignment of mtDNA cytochrome *b* sequences for A) *Vipera berus*, B) *Coronella austriaca* and C) *Natrix natrix*. Dots indicate where bases are identical to haplotype1.

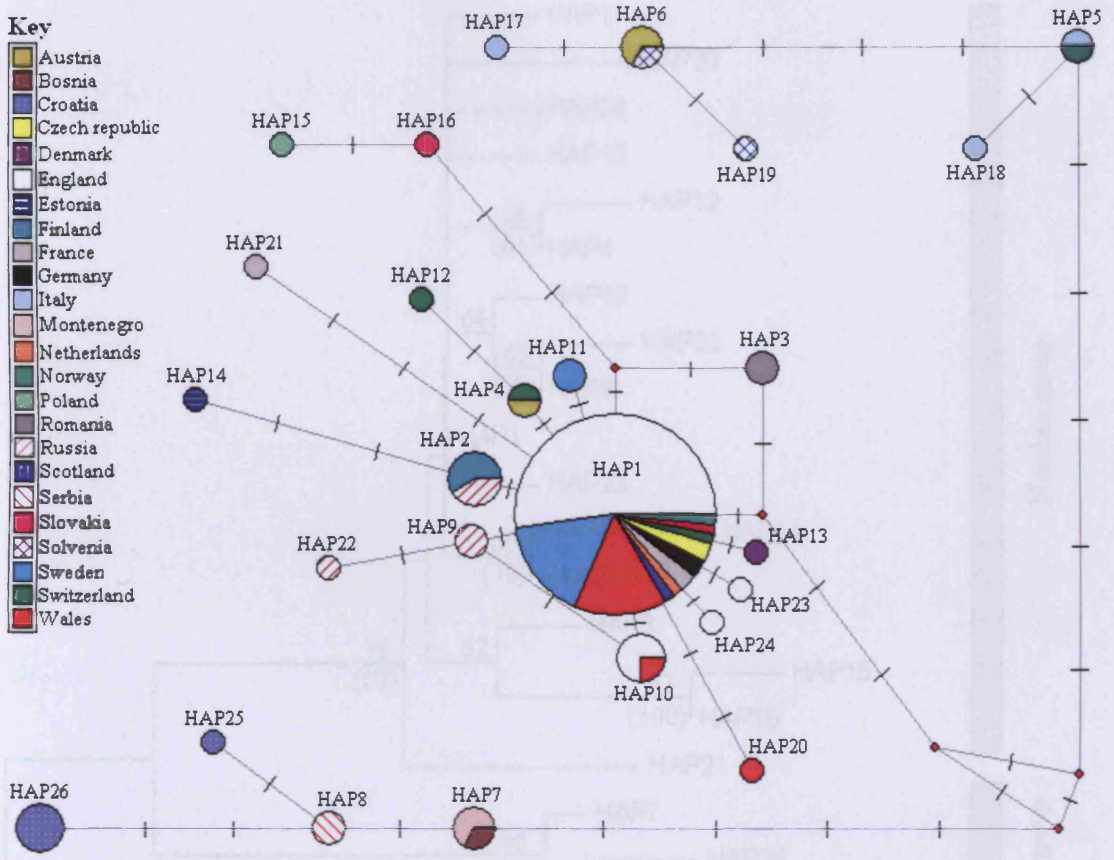

Figure 3.2 Median spanning network of *Vipera berus* mtDNA cytochrome *b*. Each circle represents a single haplotype with diameter indicating haplotype frequency. The smallest circle represents a singleton. Red dots represent median vectors presumed unsampled or missing intermediates and mutational steps are represented by black bars on lines connecting haplotypes.

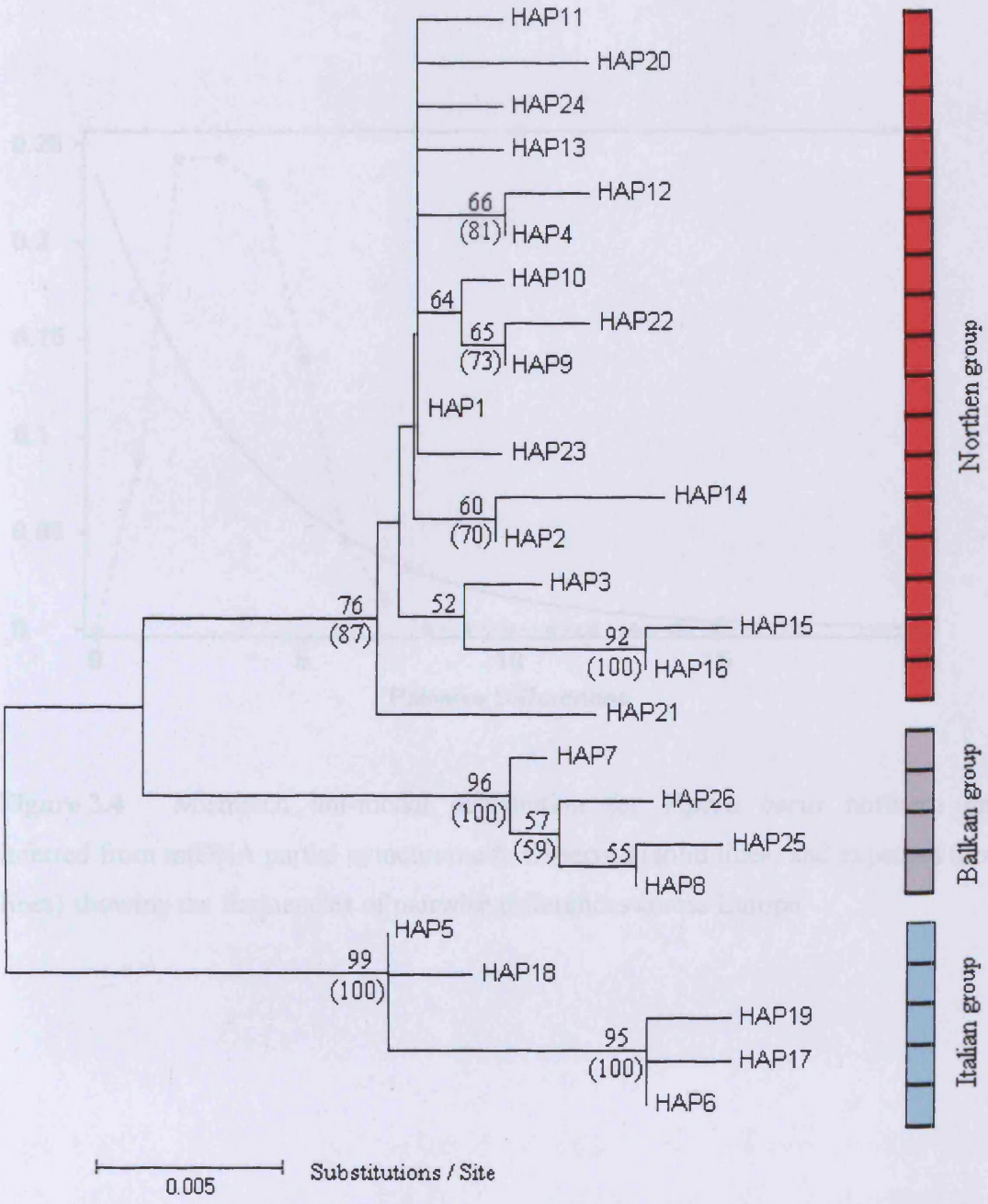

Figure 3.3 NJ tree from mtDNA cytochrome *b* sequences (112 sequences; 434 bp) for *Vipera berus*. Values for bootstraps support are shown for nodes found in more than 50% of 100,000 trees for NJ (kimura 2-parameter; top) and Bayesian analysis (bottom in parenthesis). Haplotypes are listed in Appendix II.

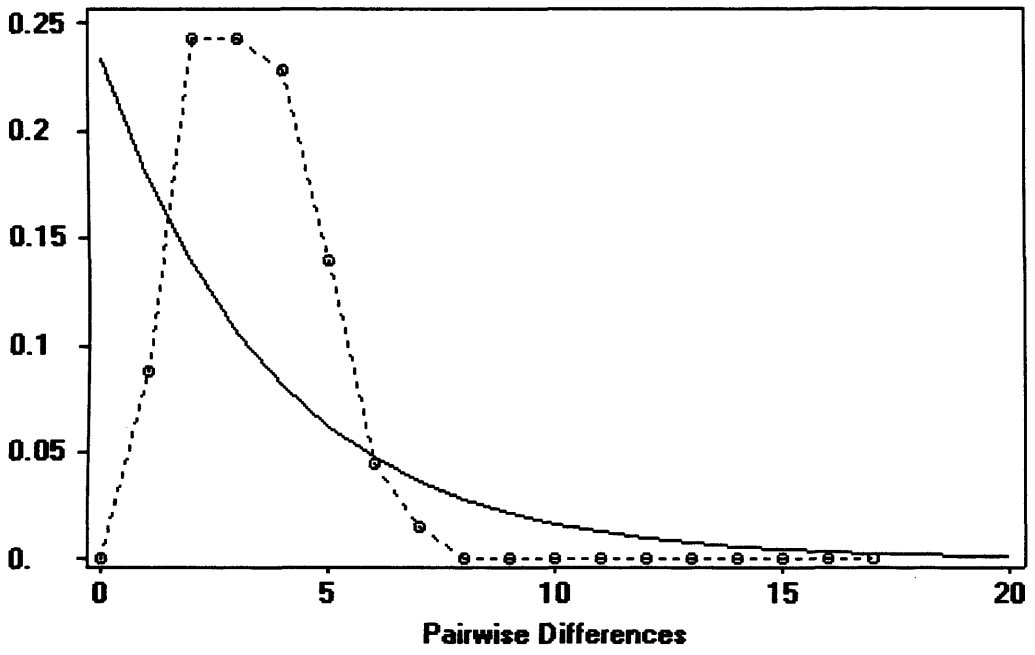

Figure 3.4 Mismatch uni-modal distribution for *Vipera berus* northern group inferred from mtDNA partial cytochrome *b*. Observed (solid lines) and expected (dotted lines) showing the frequencies of pairwise differences across Europe.

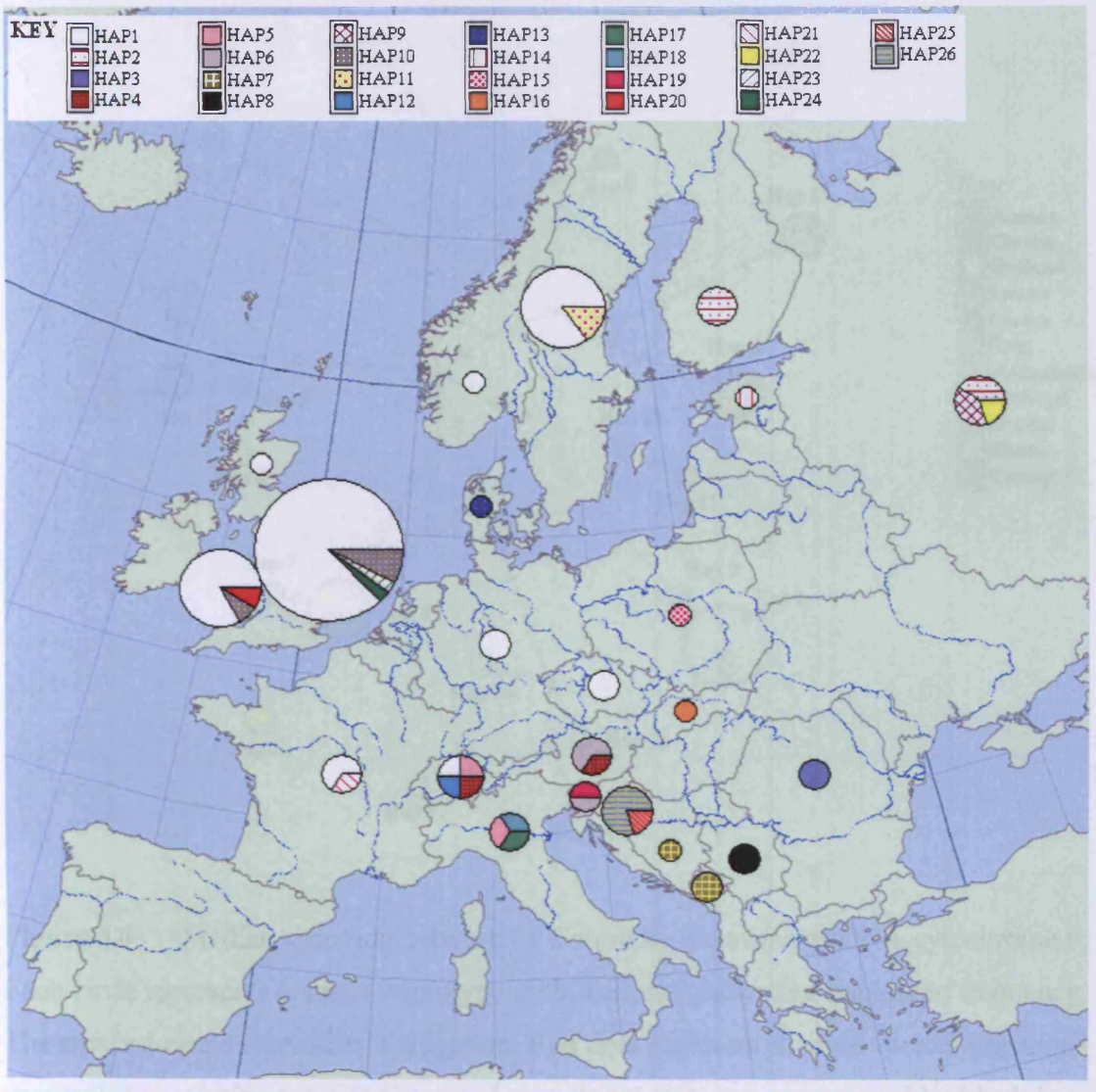

Figure 3.5 Diagram displaying haplotype incidence across Europe for *Vipera berus* from mtDNA cytochrome *b* sequences (112 sequences; 434 bp). Haplotypes are represented by a single pie diagram per country with diameter indicating haplotype incidence.

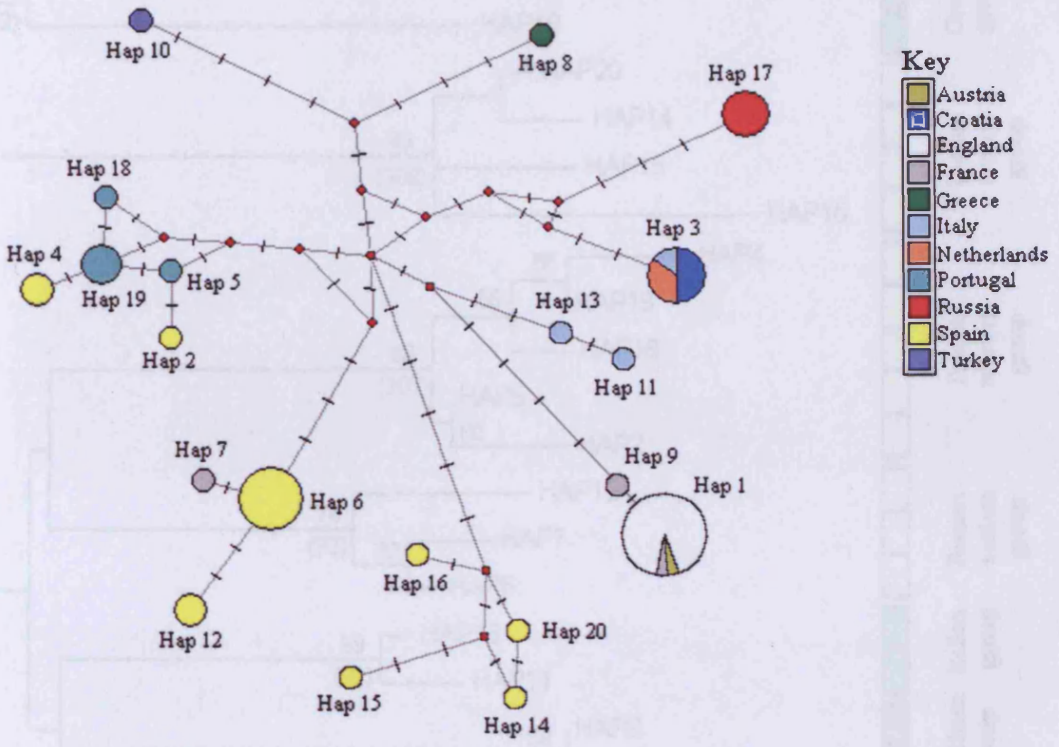

Figure 3.6 Median spanning network of *Coronella austriaca* mtDNA cytochrome *b*. Each circle represents a single haplotype with diameter indicating haplotype frequency. The smallest circle represents a singleton. Red dots represent median vectors presumed unsampled or missing intermediates and mutational steps are represented by black bars on lines connecting haplotypes.

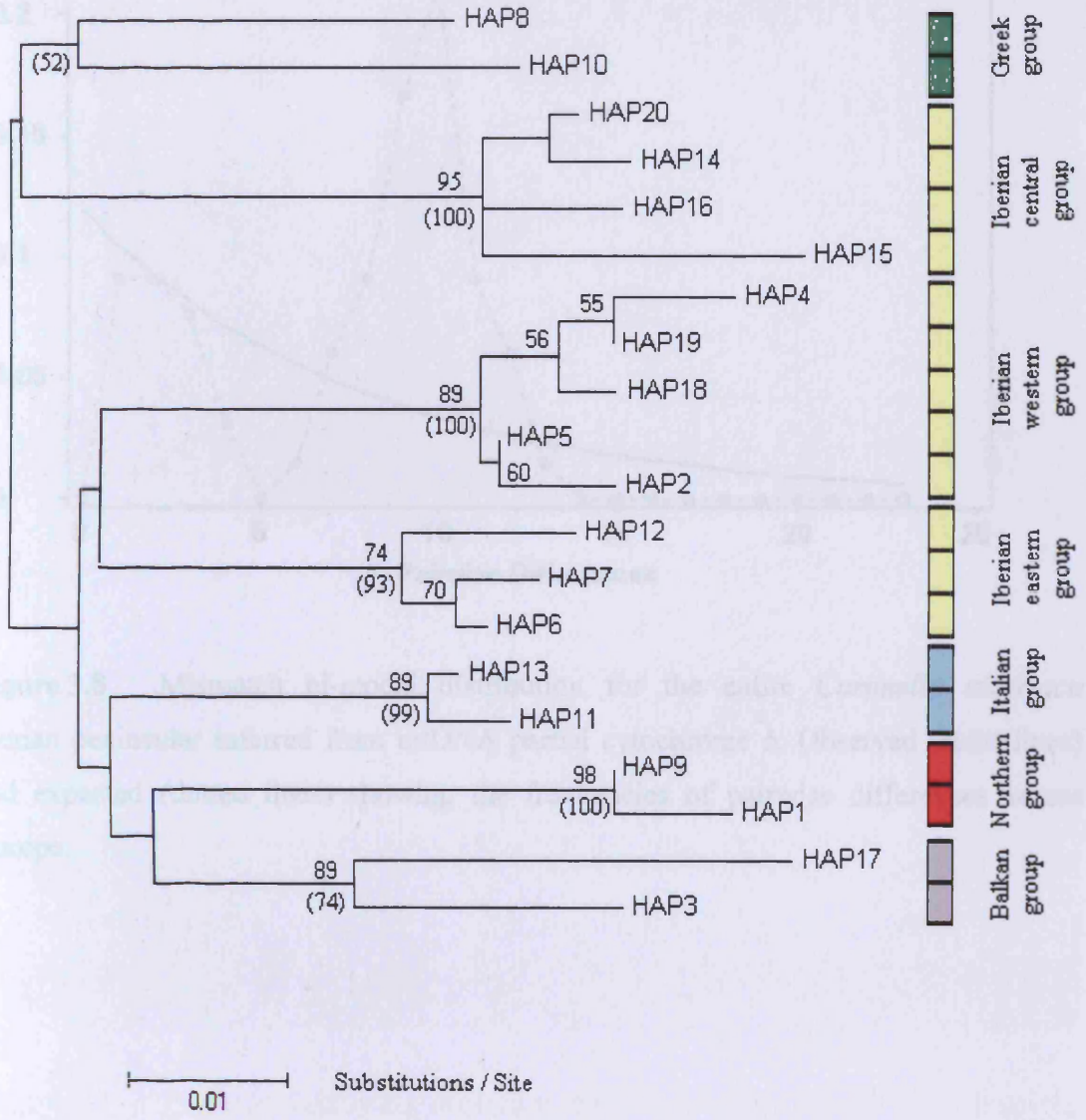

Figure 3.7 NJ tree from mtDNA cytochrome *b* sequences (50 sequences; 141 bp) for *Coronella austriaca*. Values for bootstraps support are shown for nodes found in more than 50% of 100,000 trees for NJ (kimura 2-parameter; top) and Bayesian analysis (bottom in parenthesis). Haplotypes are listed in Appendix II.

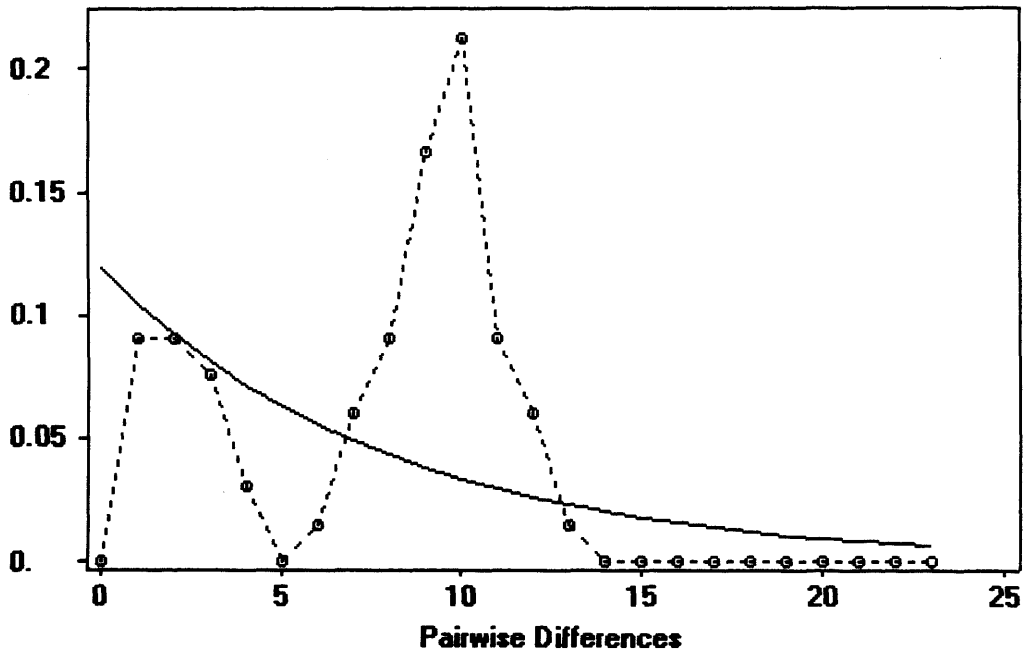

Figure 3.8 Mismatch bi-modal distribution for the entire *Coronella austriaca* Iberian peninsular inferred from mtDNA partial cytochrome *b*. Observed (solid lines) and expected (dotted lines) showing the frequencies of pairwise differences across Europe.

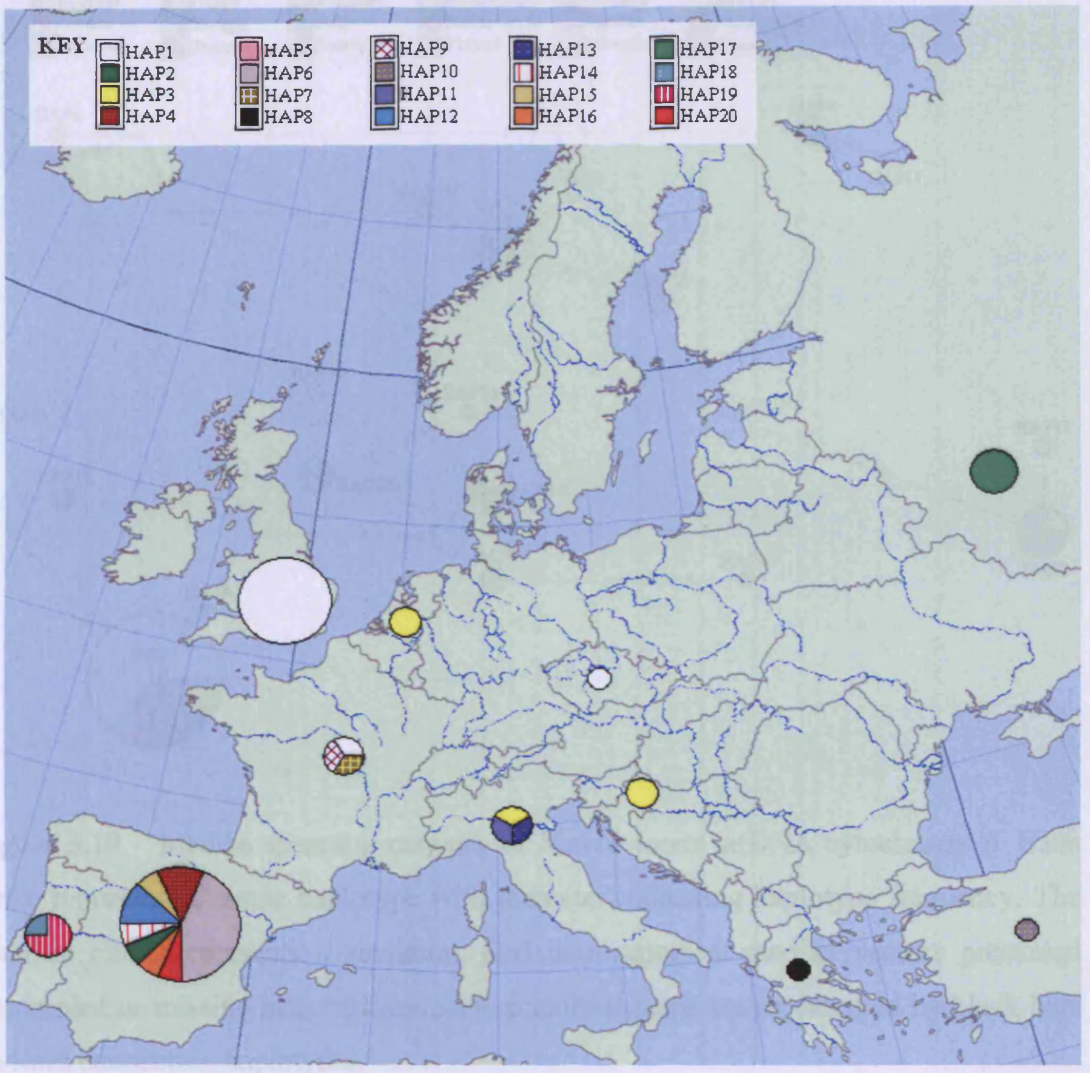

Figure 3.9 Diagram displaying haplotype incidence across Europe for *Coronella austriaca* from mtDNA cytochrome *b* sequences (50 sequences; 141 bp). Haplotypes are represented by a single pie diagram per country with diameter indicating haplotype incidence.

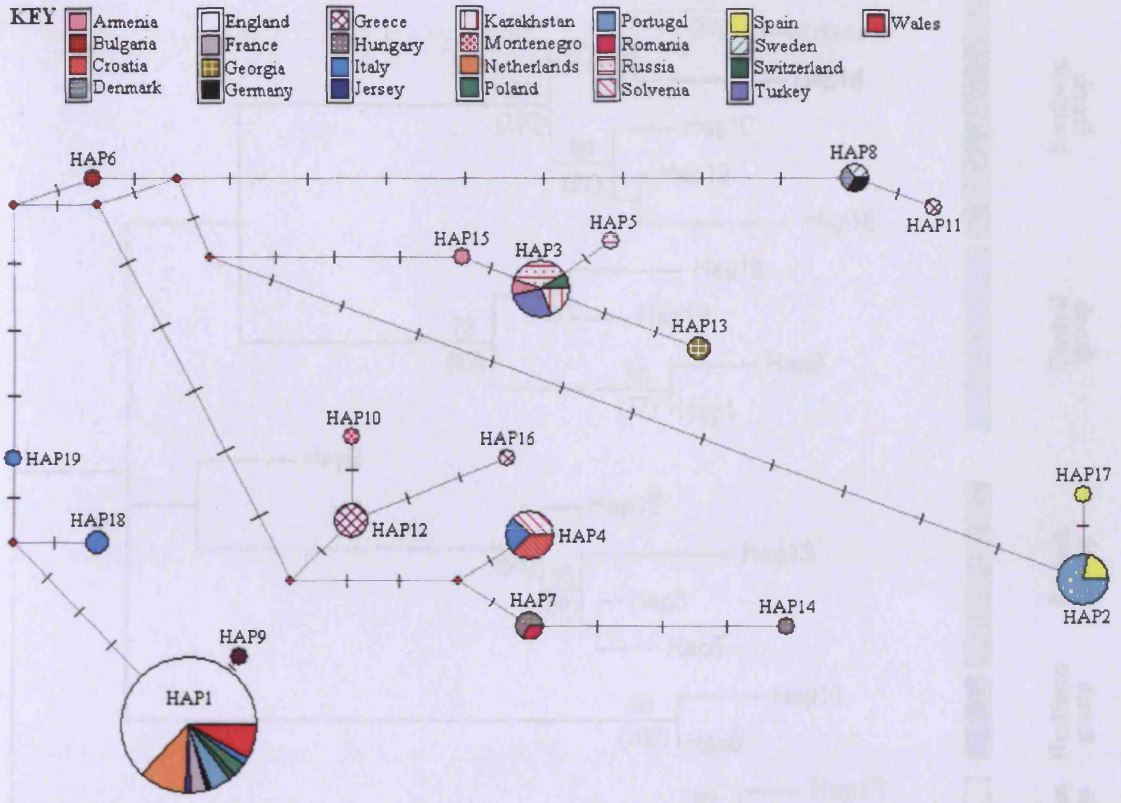

Figure 3.10 Median spanning network of *Natrix natrix* mtDNA cytochrome *b*. Each circle represents a single haplotype with diameter indicating haplotype frequency. The smallest circle represents a singleton. Red dots represent median vectors presumed unsampled or missing intermediates and mutational steps are represented by black bars on lines connecting haplotypes.

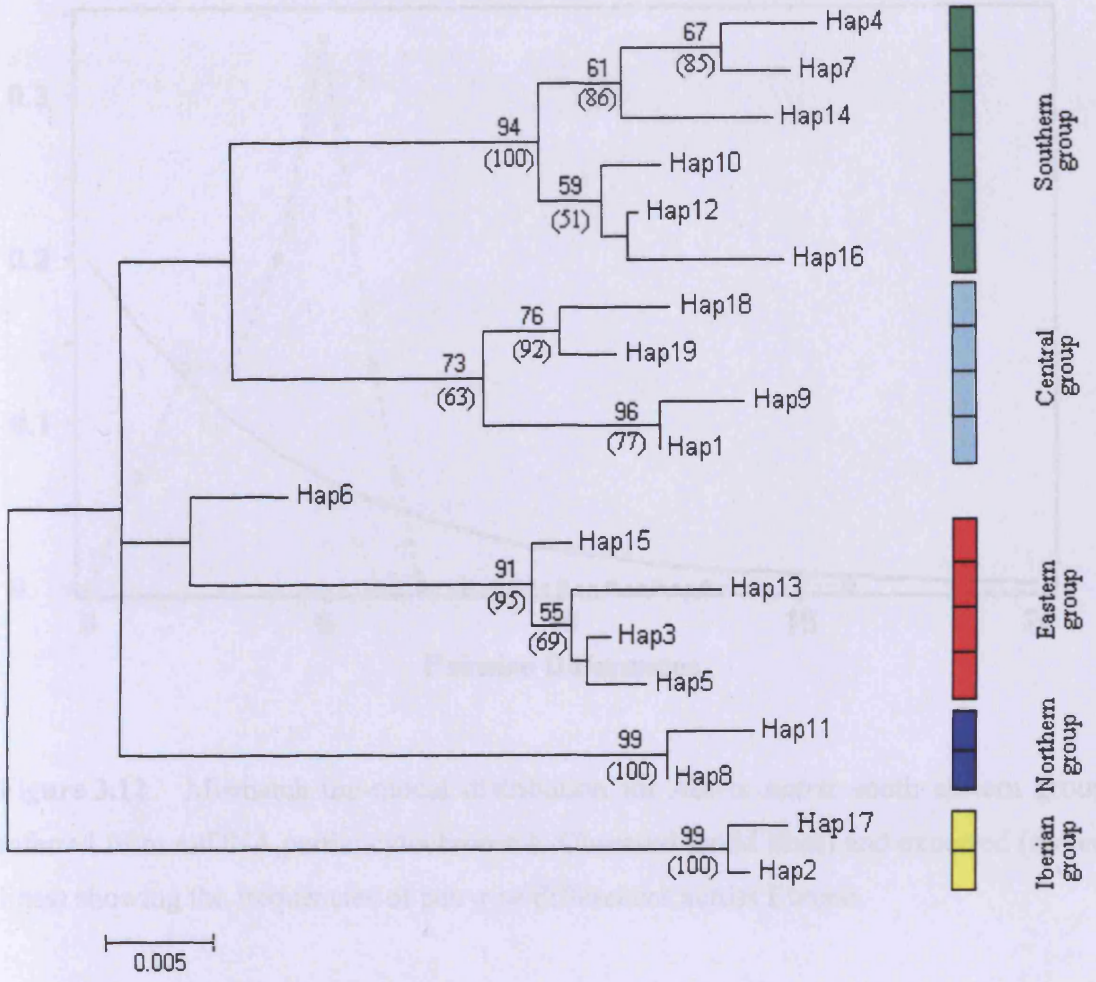

Figure 3.11 NJ tree from mtDNA cytochrome *b* sequences (114 sequences; 265 bp) for *Natrix natrix*. Values for bootstraps support are shown for nodes found in more than 50% of 100,000 trees for NJ (Kimura 2-parameter; top) and Bayesian analysis (bottom in parenthesis). Haplotypes are listed in Appendix II.

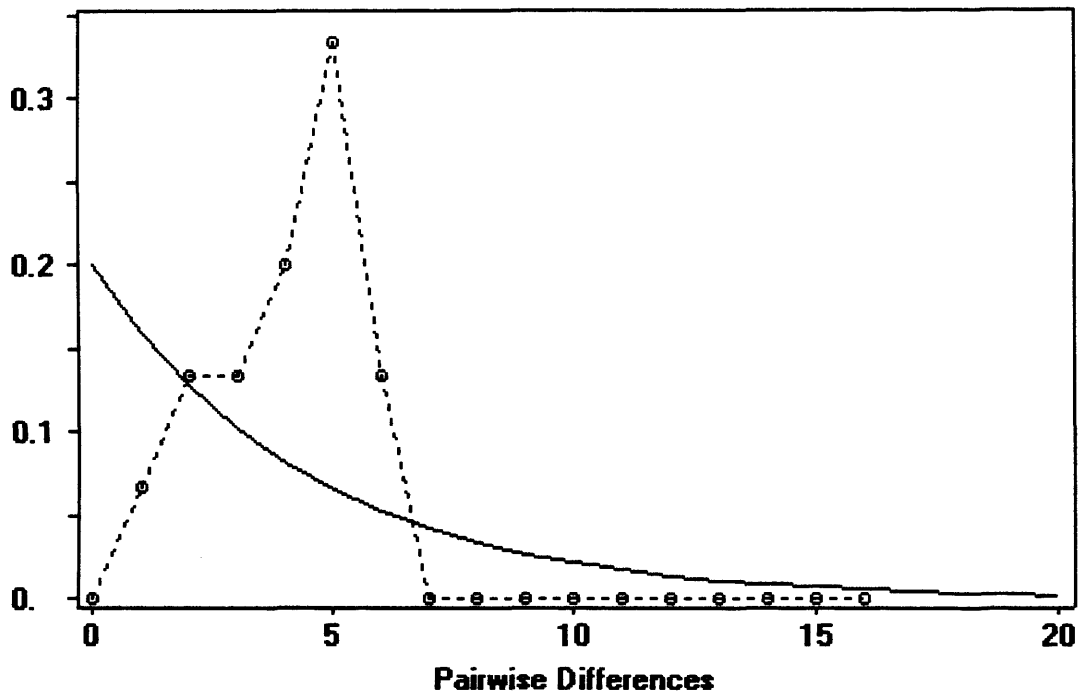

Figure 3.12 Mismatch uni-modal distribution for *Natrix natrix* south eastern group inferred from mtDNA partial cytochrome *b*. Observed (solid lines) and expected (dotted lines) showing the frequencies of pairwise differences across Europe.

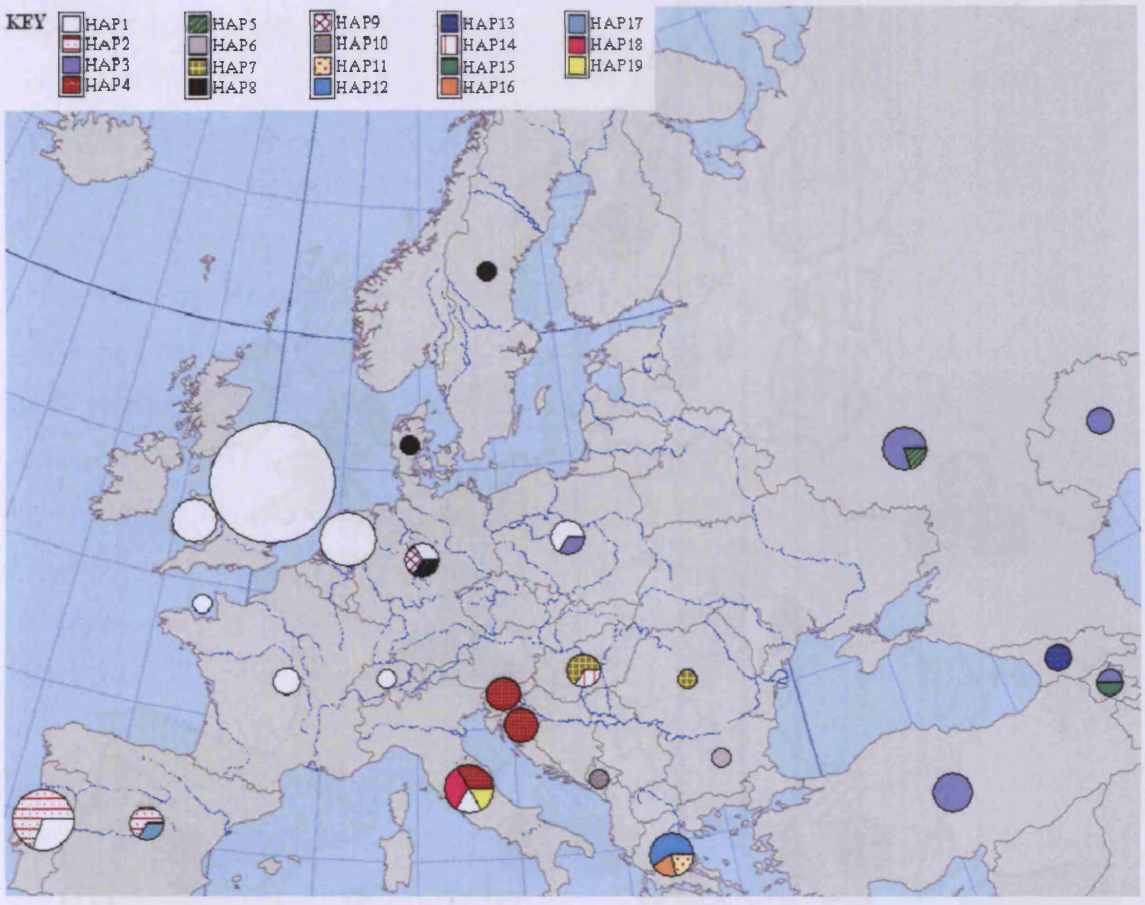

Figure 3.13 Diagram displaying haplotype incidence across Europe for *Natrix natrix* from mtDNA cytochrome *b* sequences (114 sequences; 265 bp). Haplotypes are represented by a single pie diagram per country with diameter indicating haplotype incidence.

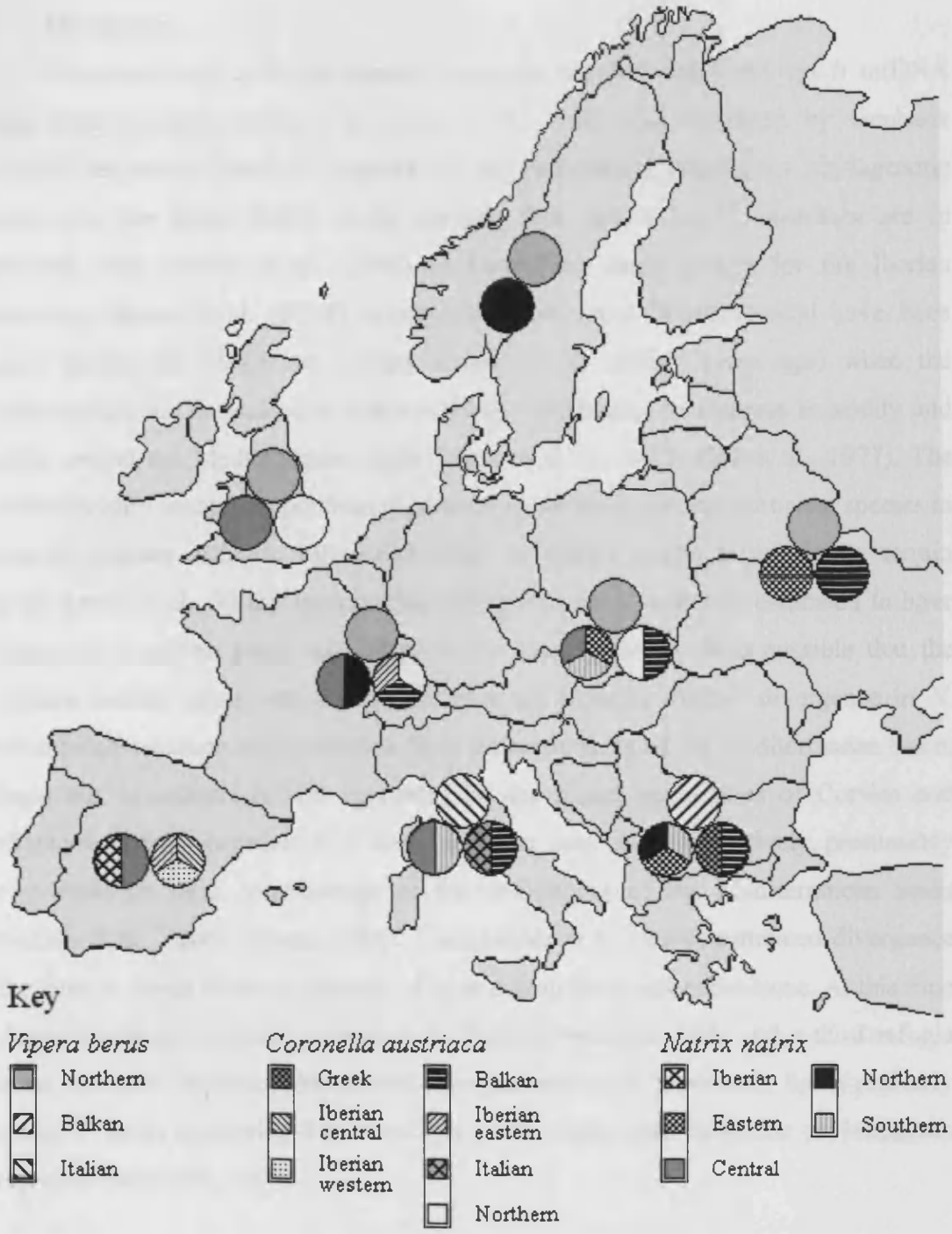

Figure 3.14 Relative lineage incidence across Europe for *Vipera berus* (top centre), *Natrix natrix* (bottom left) and *Coronella austriaca* (bottom right) from mtDNA partial cytochrome *b* sequences. Pie diagrams show the occurrence, but not the frequency, of lineages. Each lineage consists of closely related haplotypes that are geographically connected.

3.4 Discussion

Non-invasively collected sample materials supplied sufficient cyt *b* mtDNA (using methodologies outlined in Jones et al., 2008 and supported by Genbank published sequences listed in Appendix I) to successfully reconstruct phylogenetic histories for the three British snake species. Our results for *C. austriaca* are in agreement with Santos et al. (2008) in identifying three groups for the Iberian Peninsular. Santos et al. (2008) hypothesized that these lineages could have been formed during the Messinian Salinity Crisis (~5.33 million years ago) when the Mediterranean Sea desiccated almost completely producing an increase in aridity and salinity around the Mediterranean basin (Duggen et al., 2003; Hsü et al., 1977). The increased aridity caused evaporation of internal lakes forcing many European species to retreat to moister Atlantic influenced areas or higher cooler mountainous terrain (García-Antón et al., 2002). Intraspecific divergence for *N. natrix* is estimated to have commenced 6 million years ago (Mya) in the late Miocence. It is possible that the Messinian salinity crisis was also responsible for inducing further divergence in *N. natrix* through isolation of populations from the re-flooding of the Mediterranean basin. Perhaps this hypothesis is best supported by the island populations of Corsica and Sardinia where the subspecies *N. n. corsa* and *N. n. cetti* occur respectively, presumably due to isolation as a consequence of the re-flooding of the Mediterranean basin (Guicking et al., 2006; Thorpe, 1984). Ursenbacher et al. (2006) estimated divergence of the three *V. berus* clades to around 1.4 Mya during the lower Pleistocene. At this time *V. berus* would have already occupied the Balkan Peninsula, Italy and a third refugia situated near the Carpathian Mountains. The presence of *V. seoanei* in Spain probably excluded *V. berus* occupying Iberian refugia as this snake inhabits similar environments (Kalyabina-Hauf et al., 2004).

3.4.1 *Structure across Europe*

Bayesian, NJ and MSN analyses for *V. berus* presented the simplest population structure of the three study species revealing three geographically distinct groups. Firstly the Italian group, which was restricted to northern Italy, south eastern Switzerland, Austria and Slovenia. Secondly the Balkan group comprised of Croatia, Bosnia, Montenegro and Serbia; all countries within the distribution range of *V. b. bosinensis* (Ursenbacher et al., 2006). Finally the Northern group, which was the largest and most widely distributed, included Great Britain to the west, Russia to the east and

Fennoscandia to the north. The Northern group is split into two, a basal lineage consisting of Poland, Slovakia and Romaina and the rest of the northern European expanse. This structure is largely in agreement with both Ursenbacher at al.'s (2006) and Carlsson et al.'s (2004) studies of *V. berus* mtDNA. Both Carlsson and Ursenbacher et al. (2006) suggested that the basal subgroup in the north could present an eastern European refugium, situated close to the Carpathian Mountains. In addition, Ursenbacher et al. (2006) proposed that the remaining northern expanse (from France to Russia) was composed of two further groups, albeit of low bootstrap values. Our data supports the Carpathian refugia hypothesis but due to low bootstrap values we cannot confidently support the two additional groups, preferring instead to recognise one. Also, although only limited sampling was undertaken for Sweden (n=10) a clear definition could be made between samples acquired from southern (64th parallel) and northern (59th parallel) Sweden. This observation is supported by previous studies of *V. berus* inferred from RAPD and mtDNA analysis (Carlsson et al., 2004) suggestive of a contact zone between an eastern and western lineage within Fennoscandian adders stemming from migration out of two separate glacial refugia.

In contrast, due to insufficient sampling and short sequence length only limited phylogenetic inference could be recovered from *C. austriaca*. Bayesian, NJ and MSN analyses revealed seven groups, three of which were within the Iberian Peninsular; Iberian eastern which included southern France and both north eastern and eastern Spain; a Spanish Iberian central group that included snakes ranging from Segovia in the north west to Albacete in the south east; and an Iberian western group incorporating northern western Spain and Portugal. The latter grouping is recognised as *C. a. acutirostris* (see Malkmus, 1995). Despite a wide distribution only one other subspecies of *C. austriaca* is recognised (*C. a. fitzingeri*) forming the Italian group in the current data set and consisting of samples from Sicily in the extreme south of Italy. However, the legitimacy of *C. a. fitzingeri* as a subspecies is questioned by Tortonese and Lanza (1968). The remainder of the northern European expanse is occupied by the nominal subspecies *C. a. austriaca* and includes the Greek group which consists of sequences from both southern Greece and Turkey. The Balkan group comprised of Croatian, north eastern Italian and Russian samples and samples collected from the Netherlands. Finally, the Northern group consisted of samples ranging from Austria to northern France and southern England. Although data obtained from the current study is largely in agreement with previous studies of *C. austriaca* (see Santos et al., 2008) only limited data exists for comparison across Europe and here too we offer only a limited European

data set. However, we also present the first mtDNA data set across *C. austriaca*'s entire southern English range.

Phylogeographic analyses for *Natrix natrix* revealed five groups. The Iberian group included of all the Spanish and the majority of the Portuguese samples. The Southern group consisted of two subgroups incorporating both the Greek subgroup which includes Montenegro, Turkey and Greece and the Balkan subclade which includes Slovenia, Romania, Croatia and Hungary. The Eastern group unites eastern Poland, Russia, Kazakhstan, Georgia and Armenia. The Northern group comprised of southern Sweden, Denmark and Germany to the north and Greece to the south. Finally, the Central group comprised of two subgroups; the Italian consisting of all Italian samples and the north western group which included England, Scotland, Wales, Jersey, France, Netherlands, western Poland, Switzerland and samples from Portugal. In addition, Bulgaria (n=1) loosely grouped with the Eastern samples but could present a possible sixth group as it exhibited a reasonably high genetic distance to all other samples. Data obtained from the current study is largely in agreement with both Thorpe's (1984) morphometric study which also identifies Italy as the ice age refuge of the British lineage of the grass snake. In addition, our results agree with Guicking et al.'s (2006) mtDNA work of *N. natrix*, albeit that bootstrap support was slightly lower in the current study when compared to Guicking's study due to shorter sample sequence length.

3.4.2 *Populations in the UK*

During the last glacial maxima (18,000-20,000 yrs ago) in Europe, populations of the native UK snake species withdrew to warmer refugia in southern Europe. As the ice retreated reptiles would have once again expanded from refugia, introgressing in northern Europe. It is generally believed that the study species would have retreated again south during the Younger Dryas; a brief period that saw a rapid return to glacial conditions in the higher latitudes of the northern hemisphere, 12,900-11,500 years ago (Alley, 2000). Reptiles would have again resided in warmer southerly refugia but would have not necessarily retreated as far south as previously (Ursenbacher et al., 2006).

According to Thorpe (1984), *N. natrix* expanded northwards from Iberian refugia some 20,000 year ago and, as the climate got warmer populated northern Europe. The current study has identified the UK *N. natrix* lineage also in the Italian Peninsular. Residence in Italy could have occurred from *N. natrix* migrating out of Spain along the Mediterranean coastal edge of southern France east of the Pyrenees.

This route has been taken by other herptiles, most notably the natterjack toad (*Epidalea calamita*) and presents the only feasible migration route in and out of Iberia where amphibian prey would be in abundance (Rowe et al., 2006). The lineage that now populates the UK expanded out of Italy but would have retreated to warmer southern climates during the Younger Dryas. Possible Younger Dryas refugia include the south of France and an area surrounding the Carpathian Mountains; two refugia thought to have been also important for *V. berus* (see Ursenbacher at al., 2006).

Due to insufficient sampling, the current data set is unable to confidently determine Younger Dryas refugia for *C. austriaca*. However, there is weak evidence for Iberian, Italian and Balkan refugia. The lineage currently populating the UK would have originally migrated out of Italy and currently occupies areas surrounding southern France and the Carpathian Mountains, both important Younger Dryas refugia for *V. berus* and *N. natrix*.

The cold Younger Dryas climate was in sharp contrast to the warm preceding interstadial deglaciation (the Bölling-Allerod) when summer temperatures were similar to present day averages (Alley, 2000). The adder requires a low mean body temperature and the heat of the preceding interstadial would have driven this snake species to cooler mountain ranges. This hypothesis would go some way to explain the identified Carpathian, Tantra and Alpine mountain refugia occupied by *V. berus* during the Younger Dryas (Ursenbacher et al., 2006; Carlsson and Tegelström, 2002). In addition, Ursenbacher et al. (2006) suggests that adders may have retreated to mountain ranges in response to competition from other snake species better adapted to warmer temperatures, such as *Vipera aspis*.

The Younger Dryas cooling was most pronounced in Britain compared to southern Europe with mean summer temperatures dropping to 10 and 12°C in northern and southern Britain, respectively, in less than 20 years (Isarim and Bohncke, 1999). Mountain range refugia in southern France and the Carpathians would have provided a platform for *V. berus* to rapidly migrate north during the colder Younger Dryas. Although British Younger Dryas temperatures would have certainly encouraged *C. austriaca* and *N. natrix* to migrate south (Wisler et al., 2008; Beebee and Griffiths, 2000; Mertens, 1994; Spellerberg and Phelps, 1975), it is likely that *V. berus* could have survived under these cooler conditions in Britain.

In addition to temperature, prey availability in the UK during the Younger Dryas might have influenced snake distributions. *C. austriaca* relies heavily on saurians (82.1% of diet; Galán, 1988) and *N. natrix* on anuran prey (63% of diet; Gregory and

Isaac, 2004). In contrast, the almost exclusive warm blooded mammalian prey of *V. berus* would have given it an advantage in populating the UK in advance of the northerly migration of the smooth and grass snakes. Therefore, *V. berus* may have remained in southern Britain during the Younger Dryas. There is evidence of other similar species existing in more northerly areas than the traditionally accepted southern European refugia most notably the natterjack toad, the Asp viper (*Vipera aspis*), the pool frog (*Rana lessonae*), and common lizard (*Lacetra* (*Zootoca*) *vivipara*) which is currently sympatric with *V. berus* (see Rowe et al., 2006; Ursenbacher, 2006; Snell et al., 2005; Guillaume et al., 2000). However, there is insufficient data available to establish the population density of these species to know whether they could sustain snake populations residing in the UK.

The hypothesis that *V. berus* was the first snake species to populate the British Isles following the last ice age is further supported from the current study. Four additional unique British haplotypes were identified in addition to the common northern European haplotype for *V. berus.* This is in direct contrast to the monophyly of the other two snake species within Britain. The greater haplotype diversity for *V. berus* indicates that the viper either undertook multiple migratory events or that it has been present in the UK long enough to diversify prior to the warmer temperatures of the Bölling-Allerod and the separation of Britain from mainland Europe. As these haplotypes are situated in North and South Wales, Sherwood Forest and the New Forest, the latter explanation seems more plausible. This is particularly interesting as these haplotypes are allopatric (although sympatric to the common European haplotype) and highly localised to areas associated with dense forest at the time of the Younger Dryas. Such forests would not only have insulated against the extremes of Younger Dryas winter but would have offered shelter during the warmer summers of the Bölling-Allerod (see Spellerberg and Phelps, 1977).

In contrast to British adder populations, *C. austriaca* now only occupy heathland in three southern English counties; Dorset, Hampshire and Surrey. There is particular interest in the genetic diversity between the sympatric populations of Hampshire and Dorset and the allopatric population in Surrey (Bond et al., 2005; Pernetta, 2005). Unlike *V. berus*, *C. austriaca* and *N. natrix* would have had further to migrate from their Younger Dryas refugia to populate the UK and only limited opportunity to access Britain via the land bridge connection to Europe. It is then not surprising that *C. austriaca* and *N. natrix* are both monophyletic in the UK and represented by a single haplotype common to a northern European group.

V. berus is likely to have established a presence in the UK earlier, and continues to maintain a higher population density and greater geographical distribution throughout Britain and Europe, than the other study species (Santos et al., 2008; Ursenbacher et al., 2006; Beebee and Griffiths, 2000; Thorpe, 1984). However, future climate change could see the adder retracting its distribution to cooler mountain clines throughout its range (see Ursenbacher et al., 2006). In contrast, *C. austriaca* is restricted in its British range to southern English lowland heath and is experiencing extinctions throughout its European range (Santos et al., 2009; Bond et al., 2005; Reading, 1997). With 20% of the remaining European lowland heath still located within the UK the importance of smooth snake conservation in Britain has never been so high on the agenda (Britton et al., 2000; Farrell, 1989) but paradoxically, global warming is likely to increase this species' range in the UK. Perhaps *N. natrix* will be presented with the greatest challenges to its continued population densities and range throughout the UK. Climate change compounded by anthropogenic habitat loss will reduce available wildlife corridors throughout the UK (Hulme, 2005; Halpin, 1997). Unlike its viviparous compatriots, such corridors are vital to access the range of habitats essential to the life cycle of the oviparous grass snake (Shine et al., 2002; Bonnet et al., 1999) and could see a decline in British populations without the institution of counterbalancing mitigation measures.

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CHAPTER 4: Reptile translocation: potential impacts of parasitism on slow worm (*Anguis fragilis*) populations in the UK

Abstract

Although removal of protected species from sites threatened by development is a common mitigation procedure, post-translocation monitoring is often neglected. The majority of British reptiles are only partially protected against intentional harming, killing or trading, and little consideration is given to the extended welfare of these animals following translocation. Reptiles are host to parasites that are potentially easily transferred to source populations during translocation, but this has not been previously investigated in the UK. For the first time, we identified an apparently common nematode, *Neoxysomatium brevicaudatum* (83% prevalence, n=100), in British slow worms (*Anguis fragilis*). We translocated a further 315 slow worms from three UK populations, monitored their growth and investigated the impact of *N. brevicaudatum* on these hosts. We found an average receptor site recapture rate of 24% with all monitored animals maintaining or increasing body condition in the first year following translocation. However, increased parasite load negatively affected slow worm body condition and, although not significant, parasitic load varied amongst host populations. We discuss the value of post-translocation monitoring and disease surveillance as an important conservation tool in preserving threatened reptile species.

4.1 Introduction

Translocation, the deliberate movement of wild individuals or populations from one part of their range to another (IUCN, 1996) is a common mitigation response for protected species when threatened by development (Young et al., 2006; Alberts and Gerber, 2004; Cunningham, 1996; Reinert, 1991). IUCN (1996) describes translocation exercises as aiming to secure the long-term survival of a species, re-establishing keystone species in an ecosystem, maintaining and/or restoring natural biodiversity, providing long-term economic benefits to local and/or national economies, and promoting conservation awareness. However, to date translocation has generally been regarded as a high risk mitigation measure and should only be carried out as a last resort (Wendelberger et al., 2007; Cosgrove and Hastie, 2001; Letty et al., 2000). Establishing the effectiveness of translocation is made all the more difficult since data are, at best, scarce due to poor documentation (Fischer and Lindenmayer, 2000; Falk et al., 1996;

Hall, 1987). However, the actual number of global translocations per year is high and many successful translocations have been documented (Zeisset and Beebee, 2003).

Understanding the role of genetic diversity is pivotal to the conservation of translocated populations as this, in conjunction with other biotic factors, influence the survival and effectiveness of animal translocation (Alberts, 2007; Jusaitis, 2005; Miller et al., 1999; Storfer, 1999). Populations can exhibit inbreeding depression, genetic drift or fixation of alleles due to small population size or isolation (Keller and Waller, 2002; Bryant, 1999; Ellstrand and Elam, 1993). The introduction of genetic variation through translocation to resident populations can be beneficial increasing genetic diversity and supplementing or creating new populations within the metapopulation (Madson et al., 1999; Willis and Wiese, 1993). However, introductions can also cause negative effects (Storfer, 1999; Lynch, 1991), leading to an increase in intra-specific competition and aggression (e.g. Goossens et al., 2005). In addition, mating between native and translocated animals can lead to a loss of intraspecific genetic diversity and potential outbreeding depression (Ficetola and De Bernardi, 2005) with translocated populations genetically altering locally adapted populations, possibly even increasing the frequency of deleterious traits (Frankham, 2007; Ficetola and De Bernardi, 2005; Lynch, 1991).

Within the first year following animal relocation, high mortality and low reproductive success have been reported from a wide range of species, such as the Starling (*Sturnus vulgaris*), otter (*Lutra lutra*), European Lynx (*Lynx lynx*) and mandrills (*Mandrillus sphinx*) (see Peignot et al., 2008; Beck et al., 1994; Shepherdson, 1994). These factors are common even amongst the most successful translocations (Moore and Smith, 1991), and have been linked to capture, transport and release protocols (Saltz and Rubenstein, 1995). Age at time of release is an important factor influencing translocation success. High juvenile survival rates are commonly recorded for many species during the first year following their re-location (Armstrong and Ewen, 2001). For example, Massot (1994) showed evidence of a higher juvenile survival rate amongst a population of introduced common lizards (*Lacerta* (*Zootoca*) *vivipara*) immediately following translocation. In contrast, Sarrazin and Legendre (2000) argued that adult Griffin vultures (*Gyps fulvus fulvus*) displayed higher survival rates than juvenile birds. However, these data were reanalysed by Roberts et al. (2004) incorporating both demographic parameters and genetic considerations, and they concluded there was actually better long-term efficiency in the release of juveniles.

As translocations can have a negative impact on resident prey populations, receptor sites should be examined for the abundance of appropriate food resources prior

to translocation. In addition, ideal receptor sites should be in areas with a suitable annual climate and without excessive competition (Teixeira et al., 2007; Kvitek, 2006; Berman and Li, 2002; Armstrong and Ewen, 2001; Hodder, and Bullock, 1997; Bain, 1993).

Often translocations are deemed successful without anything more than confirmed presence at a receptor site, but success should only be measured in terms of long term population viability (Watts et al., 2008; Tuberville et al., 2005; Tenhumberg et al., 2004; Imam et al., 2002; Fischer and Lindenmayer, 2000; Griffith et al., 1989; Dodd and Seigel, 1991;). This requires substantial post translocation study (Watts et al., 2008; Hochkirch et al., 2006), ten or more years in the case of UK reptiles (Strum 2005; Platenberg and Griffiths, 1999), which are long lived animals (Nelson et al., 2002; Towns and Ferreira, 2001). Not surprisingly given the lack of data on reptile relocation, reported successful translocations for reptiles and amphibians are lower than those of birds and mammals (Tuberville et al., 2005; Dodd and Seigel, 1991). In their review of 24 British translocations, Bullock (1998) found post translocation changes ubiquitous for both animal and plant communities alike. These changes were linked to poor management and aftercare with communities settling in poorly to their receptor environments. A successful translocation exercise was reported by Burke (1989) when gopher tortoises (*Gopherus polyphemus*) showed evidence of reproduction after two years. However, Dodd et al. (1991) suggested that this claim of success was premature. Translocation success rates have been shown to decline as post-translocation periods are extended. For example, Griffith et al.'s (1989) study of avian and mammal translocations originally recorded a 44% translocation success rate over 3 years. This data was reviewed by Wolf et al. (1996) and amended to 38% following an extended 6 year post translocation monitoring. A higher survival success rate of 57% was recorded by Nelson et al. (2002) on their 5 year study following the translocation of Tuatara (*Sphenodon* spp.) to a rodent-free island in New Zealand. Nelson's efforts indicate the potential for a successful reptile translocation made possible through good pre and post translocation monitoring. However, Tuataras are long-lived, late-maturing reptiles with slow reproduction; hence it will take decades of monitoring to confirm a self-sustaining population.

Parasites are a commonly neglected biotic factor of stress (Marcogliese, 2004). Many translocated wild animals carry substantial parasitic loads and when transported to a receptor site potentially generate new host-parasite interactions (Woodroffe, 1999; Cunningham, 1996; Holmes, 1996; Snyder, 1996; McCallum and Dobson, 1995;

Viggers et al., 1993). Reptiles can be heavily infected with a range of macro- and microparasites (Dobson et al., 1992; Schall, 1992), which may regulate host population dynamics and influence community structure by affecting behaviour, growth, fecundity and mortality (Marcogliese, 2004; Schall, 1992). This can inevitably result in declines or extinctions of host populations (McCallum and Dobson, 1995; Viggers et al., 1993). Parasites can also act as agents of competition (Dobson et al., 1992) with resistant host species excluding other more parasitized species from their range. Equally, this can work the other way. Schall (1992) demonstrated that a malarial parasite, *Plasmodium azurophilum*, permitted coexistence of two highly competitive *Anolis* lizard species on St. Maarten, a small island in the Caribbean. The stronger competitor species, *A. gingivinus* is found almost exclusively throughout the island and is prone to parasitization by *P. azurophilum*, where as *A. wattsi* is rarely infected. Where *A. gingivinus* is parasitized in the central hills of St. Maarten, it coexists with *A. wattsi*, but in other regions where *A. gingivinus* is uninfected, it excludes *A. wattsi.* Schall and Vogt (1993) found further evidence of parasite mediated competition between *Anolis* lizard species in Puerto Rico. Additional sampling of these lizard species (n=4859) in the Lesser Antilles was able to determine the ancient association between *P. floridense*, *P. azurophilum* and their *Anolis* lizard hosts (Staats and Schall, 1996).

In general, parasites are detrimental to host fitness, although some have been shown to enhance beneficial traits in reptiles, at least in the short term. These include enhanced host fecundity or reduced time to maturity (Minchella, 1985; Lafferty, 1993). It has been suggested that where local parasitic infection is encountered, maternal effects could be a mechanism to pre-adapt lizards to such an environment (Meylan and Clobert, 2004). Sorci et al. (1994) revealed poor maternal condition, caused by ectoparasites during gestation of common lizards (*Lacerta* (*Zootoca*) *vivipara*), affected offspring dispersal and 'preadapted' neonates to sprint faster. Sprint speed in reptiles is generally considered to be a fitness-linked trait (Bennett and Huey, 1990) and would be a selective advantage countering the negative effects of the parasitic infection and therefore increasing the survival rate of young parasitized lizards. To date, there has not been any study into the implications of parasitic load during reptile translocations across the UK. However, implementation of parasite screening programmes could decrease the probability of parasite transmission rates between native and translocated populations (Hein, 1997; Viggers et al., 1993).

In addition to biotic factors, abiotic factors such as human disturbance can seriously impact on translocation success (Watson and Thirgood, 2001; Beebee and

Griffiths, 2000). Reptilian translocations often provoke negative publicity whereas equivalent avian or mammalian projects generally receive greater public support (Sullivan et al., 2004; Shine and Koenig, 2001). Anthropogenic habitat alteration often introduces new predator relationships, particularly domestic cats, introducing unnecessary stress and fatalities to reptile populations (Hardman and Moro, 2006). Most importantly, the effects of successive abiotic and/or biotic stressors can be additive or accumulative, impacting on an animal's health and all aspects of its fitness (Teixeira et al., 2007).

Where land development projects enforce conservationists to undertake mitigation these translocations are more accurately described as rescue operations as they rarely have a scientific basis. Following successful development applications, a short time frame often only allows a small proportion of the population to be recovered before building commences (Webster, 2007; Beebee and Griffiths 2000). Under such circumstances it appears that translocation is carried out purely for commercial purposes (Dodd and Seigel, 1991) with little conservation value and, despite protective legislation, is still undertaken for the rarest of species to facilitate human expansion (Beebee and Griffiths, 2000). In the UK, three options are recognised for the mitigation of reptiles: translocation, compensation and reduction. True translocation involves the removal of a population to a site of equal or improved habitat suitability. Compensation involves the purchase of suitable land to substitute that which is lost to development and the subsequent translocation of animals, whereas reduction seeks to secure a portion of the development site for housing threatened animals (Platenberg and Griffiths, 1999). Suitable assessment of the receptor site habitat is key to the success of all the aforementioned options (Armstrong and Seddon, 2008; Beebee and Griffiths 2000; Griffith et al., 1989).

Development schemes exhibiting suitable reptile habitat are subjected to ecological surveys and, if reptiles are located on site, planning permission will only be granted with appropriate mitigation in place. Should mitigation involve translocation, then reptiles should not be subject to further disturbance following their relocation (Dodd and Seigel, 1991). The receptor site should provide ideal habitat, be protected against future development and present a location where reptiles are less at risk compared to their original donor site. Guidelines for carrying out such exercises exist (e.g. JNCC, 1998; Hodder, and Bullock, 1997; Falk et al., 1996; IUCN, 1996; Dodd and Seigel, 1991; Griffith et al., 1989). However, within the current UK legislation post-

translocation monitoring of non European protected species has been largely neglected (but see Webster, 2007; Platenberg and Griffiths, 1999).

The slow worm (*Anguis fragilis*) is one of the three native lizard species of the British Isles; the others being the common lizard (*Lacerta* (*Zootoca*) *vivipara*) and the sand lizard (*Lacerta agilis*). Of these, *A. fragilis* is the only species that could be practically used to assess the feasibility of translocations due to its ease of capture, non-invasive identification, limited vagility and, most importantly, comparatively high population density. The slow worm is a viviparous legless lizard (up to 40 cm in length) with a wide distribution throughout Europe. Although little is known of their ecology (Capula et al., 1996; Capula and Luiselli, 1993), it is the most common British reptile and occupies herbaceous microhabitats with high vegetation cover (Ferreiro et al., 2004; Capula et al., 1998; Stumpel, 1985). These anguid lizards are highly camouflaged within their environment and both morphologically and functionally adapted to a semi-subterranean life (Cabido et al., 2004). As a thigmotherm, this semi-fossorial lizard prefers to thermoregulate under refugia and is rarely observed in open ground (Platenberg and Griffiths, 1999). The slow worm is most frequently encountered in spring and autumn, brumating over winter and preferring its semi-subterranean existence, and aestivation, during the warmer summer months. Slow worms are under high risk of predation from reptilian, avian and mammalian predators (Salvador, 1998). They regularly exhibit scarring and up to 70% found in the field have shed tails (Beebee and Griffiths, 2000). Slow worms are part protected under Wildlife and Countryside Act (1981, Section 9, as amended) requiring developers to avoid death or injury to the reptile within the timescale of the translocation exercise and making trade of the animal illegal (JNCC, 1998). However, with only partial protection under the W&C Act and not being a European protected species, slow worm translocations are not subject to the conditions of monitoring required for licenced translocations.

This study aims to evaluate the success of slow worm translocations in the UK using non-invasive individual identification and to examine the potential impacts of pathogens on host growth.

4.2 Material and methods

4.2.1 *Donor sites*

Donor Site 1 consisted of 2.5 km^2 of urban environment running adjacent to an active intercity train track that was designated for retail development. Only around a third of the site was judged suitable for slow worms with many inhospitable areas consisting of short improved grassland or being too heavily trafficked by pedestrians. The remainder consisted of allotments (managed for 82 years) and railway wagon yards (concrete/ railway ballast/ railway structure and buildings). The site was littered with numerous *in situ* refugia (ground sheeting, water collectors and engineered areas) and unmanaged vegetation. Before it was possible to survey, 535 m^3 of waste material was removed including 60 tonnes of metal. Construction of a network of safe paths throughout the site enabled the supervised removal of refuse by light plant. Natural dispersal of reptiles was only possible via the train track embankment to the east of the site as a heavily urbanised main road lay to the south with a town centre to the north and west.

Donor Site 2 was located 1 km west of Site 1 and consisted of a corridor of fringe land scrub heavily trafficked by pedestrians. Both sites were scheduled for development by the same supermarket chain with Site 2 compensating for the loss of allotment ground at Site 1. On the site was a disused World War II ammunitions delivery rail line that had lain as wasteland since 1970 (38 years). Most of the 4925 m^2 site was deemed unsuitable reptile habitat being heavily shaded by bramble (*Rubus fruticosus*), birch scrub (*Betula* sp.) and Japanese knotweed (*Fallopia japonica*). However, a large fringe edge (to the south) supplied ample cover, plentiful basking locations for reptiles and an abundance of invertebrate food, such as ant eggs, earthworms, slugs and snails. Due to public access, this was the most disturbed donor site with considerable human damage to refugia, therefore a 2.5 m tall chain-link steel fence was erected to secure the site and minimise human disturbance. Reptile migration was limited to adjacent gardens to the north and a footpath to the east. This footpath (1 m wide) posed no barrier to migration and led into a 0.5 km strip of land providing identical habitat to that of the donor site. At the time of translocation this continuation of habitat had also been earmarked for development and did not constitute a suitable receptor site.

Donor Site 3 consisted of a small (1500 m^2) plot of south facing hillside grassland with natural reptile refugia, but planning permission had been granted for the

construction of two houses. Reptile emigration was possible via gardens to the west, north and east of the site, but was limited by a 6 m sheer drop to a road to the south. This site provided near perfect conditions for slow worms.

4.2.2 *Receptor sites*

Receptor Site 1 consisted of a 5000 m^2 island in the middle of an 18 hole golf course 4 km from Donor site 1. Thirty years (1978) prior to its construction, the course had been the site of a working quarry but had been progressively colonised by mixed woodland, and there was a south facing calcareous grassland slope. Public access to the course was restricted with the receptor island rarely disturbed. Natural colonisation of the island by slow worms would be restricted by expansive managed greens.

Receptor Site 2 covered an area of 5000 m^2 surrounded by cliffs on three sides with a small road and stream to the east 6.9 km from Donor site 2. The site had previously been a working quarry and was designated a geological SSSI status. The island clearing, lain disused for 17 years (1991) consisted of bare rock, rubble and ephemeral/short perennial vegetation with sediment grading towards calcareous grasslands surrounded by dense woodland. Rocky outcrops surrounding the basin limited natural dispersal corridors to the site.

Receptor Site 3 was a 150 m^2 section of Donor Site 3 divided by an 8 m long section of 60 cm high plastic half pipe sunk to 40 cm and backfilled to produce a one-way reptile resistant fence. The site was privately owned semi-improved grassland surrounded by thick privet hedges on three sides with access only available from donor Site 3 to the east.

Maps detailing the topography of donor and receptor sites are presented in Appendix III.

4.2.3 *Translocation and monitoring*

All donor and receptor sites were subjected to an initial 2 week study to establish slow worm presence and population density. Five existing *in situ* shelters were selected and 25 artificial refugia (roofing felt sections (aka felts, tins), 50×50 cm) were laid at each site. All 30 refugia were checked six times over a two week period under ideal weather conditions (see Appendix IV). Native slow worms were present at all sites, with the exception of receptor Site 1. To maximize capture rates at donor sites, felts were increased to 200, 170 and 100 for Sites 1, 2 and 3, respectively, bringing suitable habitat to a density of 350 refugia ha^{-1}. A fortnight after the felts were laid,

intensive collections were undertaken at each site. Collection visits were performed twice daily during suitable weather conditions in the early morning or late afternoon between March and June 2007 for Site 1 (n=40) and between July and September 2007 for Sites 2 (n=32) and 3 (n=20) until captures declined to zero. After ten sequential 'zero capture' days, each site was subjected to a supervised removal of refugia, ground level vegetation strimming and top soil scrape to eliminate the possibility of immigration and to encourage dispersal of any remaining subterranean reptiles not previously recovered.

Over a 90 day period, slow worms were released to the correspondingly numbered receptor site on the same day that they were captured from their donor site. Individuals were released directly to purpose built hibernacula at each site (see Appendix V). Monitoring commenced 7 days after the last animal was released. Sites were surveyed 4 times pre-brumation and 10 times post-brumation (n=14) over a 12 month period to record reptile data and eventual evidence of reproduction.

Data recorded for all translocated slow worms included age, sex, weight (g) and length (snout to vent and vent to tail, mm). Lizards were also examined for ectoparasites, but none were detected. All slow worms were photographed for both dorsal and ventral head profiles providing a unique identification for each animal (see Appendix VI. Previous to this study, this identification method had been reserved for adult slow worms as juveniles were not consistently tracked over time (Platenberg and Griffiths, 1999; Smith 1990). However, both adult and juveniles were consistently identified here, this being confirmed by the correct identification of 10 morphologically similar slow worms by five independent researchers in a blind trial (100% success for two separate tests). Insuring correct lighting for photography and comparative corresponding data on weight, length and scarring, all slow worms were able to be tracked throughout the duration of this study. Individual slow worms recaptured at the receptor sites were identified, weighed and measured in the same fashion, as were data for a naturally occurring population found at receptor Site 2. Monitoring of populations continued for up to a month after the main study to brumation 2008.

No slow worms were harmed during the translocation exercises and no deaths were recorded.

4.2.4 *Stool sample collection and parasite screening*

To assess slow worm gastrointestinal helminth fauna, 100 faecal samples from *Anguis fragilis* were collected in early 2007 prior to the translocation study at 10

locations across Wales, UK (see Table 4.1). Animals recovered from artificial refugia were photographed, measured and weighed. During this brief handling period, approximately one third of lizards naturally defecated (cf. Jones et al., 2008) and these samples were either temporarily stored in sterile faecal vials (Starstedt stool sample tubes) and examined fresh on return to the laboratory or fixed in 90% ethanol for subsequent screening.

During the translocation study, 76 slow worm stool samples were collected that included eight native samples from donor Site 2 immediately before and six months after translocation, eight samples from each of the three translocated populations 6 months following translocation, and additional samples collected randomly from Wales again at 6 months post-translocation to control for potential temporal variation in parasite loads. One year post-translocation, a further 36 faecal samples were collected from translocated animals at Sites 1 (n=2), 2 (n=16) and 3 (n=2), and from natives at Site 2 (n=16).

Each faecal sample was screened for the presence of parasitic worms by diluting the sample in water, clearing in beechwood creosote and scanning for parasites using a stereo-dissecting microscope (up to ×30 magnification) with fibre optic illumination. Nematode samples were prepared in duplicate both in 90% ethanol or fixed in 10% formalin and sent to the Natural History Museum, London, for identification.

4.2.5 *Statistical analyses*

Body condition index per day $^{-1}$ was calculated modifying Platenberg and Griffiths's (1999) formula to CL= (mass per day $^{-1}$/ (SVL per day $^{-1}$) ^{3}) $\times 10^{6}$. Gravid females and slow worms that had shed their tails at any point during the study (Site 1, n=1; Site 2, n=2; Site 3, n=5) were omitted from this analysis. Multiple recaptures of individuals allowed comparative body condition indices to be constructed. Principal Component Analysis (PCA) and General Linear Modelling were used to compare the body condition at the three sites and the native population at receptor Site 2. General Linear Modelling (GLM) supports the analysis of both categorical and continuous variables within the same model. Lizards were categorised as male, female or juvenile (the latter including neonates), and effect of age was assessed by comparing adults versus juveniles. Comparisons between groups (age, sex and site) were performed using a chi square test. All statistical tests were performed in Minitab vs.15.

4.3 Results

4.3.1 *Donor sites*

A total of 272 slow worms (Site 1, 40; Site 2, 139; Site 3, 93) were caught and removed from three donor sites. During this 90 day collection period, the last 10 visits from each site resulted in zero captures confirming that the majority of animals had been recovered (see Appendix VII). From the initial two week study of receptor sites, Site 2 was found to have a small native population of slow worms (n=8). However, during the post-translocation monitoring, new individuals were captured at Receptor Site 2 bringing the total native population to 43.

4.3.2 *Receptor sites*

Following translocation, 14 visits were made to each of the three receptor sites over a 34 week period, September-October 2007 pre-brumation (4 visits) and February-October 2008 during the slow worm yearly active period (10 visits; Fig. 4.1). Mating, gravid individuals and neonates (indicating new births) were observed amongst the translocated animals at Sites 1 and 3, and for both the native and translocated animals at Site 2.

4.3.3 *Recapture rates*

All animals at receptor Sites 1 and 3 were individually identified confirming no slow worms were present prior to translocation and that no immigration had occurred. From the 14 visits to receptor sites, 148 slow worms were recaptured (64 individuals recaptured once and 16 individuals recaptured >1, range 2-5, mean=3) (Fig. 4.1). Thus, 24% of translocated animals were recaptured at the three receptor sites (Table 4.2).

Average slow worm encounter rate was 2.0 individuals per site/d with total encounter rates of 43% Site 1 (17/40), 40% Site 2 (55/139) and 42% Site 3 (39/93). Native (58 captures: 11/43 individual recaptures) and translocated slow worms (53 captures: 37/139 individual recaptures) were encountered at a similar frequency at Site 2 (Chi-sq=0.0134, d.f. =1, p=0.908). Encounter rates were comparable at Sites 1 and 2, but lower for Site 3 due to a land slide in early spring 2008. This was a consequence of substantial rainfall over the winter period which resulted in considerable damage to the site. The area was condemned as unsafe and with no maintenance undertaken it gradually became overgrown with dense vegetation. Only juveniles (n=14) and adult males (n=2) were recaptured. No carcasses were found immediately after the land slide (both surface and subterranean) so mass migration might have occurred, probably

driven by lack of food resources and increased exposure to predation. Evidence for increased predation came from observing a noticeable increase in scarring and tail loss from this population post land-slide compared to the other study sites (50% scarring at Site 3 compared to <2% for other sites; and 13% tail loss compared to <1.5% others).

Adult slow worm recapture rate from all translocated populations was 23% (35% males and 16% females), but increased to 40% (45% males and 35% females) when omitting Site 3. More males than females were recaptured, but this was not significantly different (Chi-Sq=0.037, d.f. =1, p=0.847). This observation could be explained by sampling bias with males being active on average a month earlier than females throughout all sites. The earliest recorded slow worm activity on 12/02/2008 was of an adult male. There was no significant difference between adult (23%) and juvenile (32%) recapture rates (Chi-Sq =0.157, d.f. =1, p=0.691).

Table 4.3 shows the mean yearly growth rates for each population. None of the animals lost weight, in fact 70% increased in body condition within the first year following translocation (Table 4.4). No significant differences were found between the increase in body condition, sex, age and/or site (GLM $F_{1,4}$=0.95, p=0.515).

4.3.4 *Parasite distribution*

The initial survey of slow worm faecal samples identified a single nematode species, *Neoxysomatium brevicaudatum* (Zeder, 1800). Larval, female and male *N. brevicaudatum* were common at an overall prevalence of 83% (mean intensity 135, range 1- 2012; see Table 4.1), but no other parasites were detected. Quantitatively, we would expect much higher parasitic loads from dissected animals, but, non-invasive sampling has provided an estimate of parasitic load, providing useful data for our translocation experiments.

Analysis from the translocated slow worm faecal samples (from eight slow worms from each receptor site and eight natives, n=32) also revealed the presence of *N. brevicaudatum*. Overall, 80% of slow worms were found to be shedding these parasites in their faeces (mean intensity 89, range 1-2000). Parasite recovery was significantly higher from male compared to female slow worms (GLM $F_{1, 30}$=4.46, p=0.045). There were also significantly more parasites recovered from Site 2 native slow worms compared to all translocated animals (GLM $F_{1,30}$=4.72, p=0.041; see Table 4.5). Two distinct age categories of nematodes were apparent: adults (~3 mm long, n=16, 21% prevalence, mean intensity =0.5, range =1-11) and the more frequently observed juveniles (~1.3 mm, n=60, 78% prevalence, mean intensity =91, range =1-2000).

Significant temporal variation was observed between the Site 2 native population at 6 and 12 months post-translocation with a decrease in juvenile *N. brevicaudatum* recovered ($F_{1,24}$ =4.86; p=0.046), but no decrease for adult nematodes and no differences observed between parasitic load and the sex or age of slow worms (p>0.05 for both). Parasite load varied between receptor sites (6 months post-translocation) for adult *N. brevicaudatum* (GLM $F_{1,23}$=5.51, p=0.018) but was not significant for juvenile nematodes (GLM $F_{3,32}$=2.73, p=0.063).

Table 4.1 *Neoxysomatium brevicaudatum* prevalence, mean intensity and range in slow worms (*Anguis fragilis*) collected from Wales, UK.

Site	Infected hosts (Prevalence)	Mean intensity	Range
1. Aberamen	3/4 (75%)	26	18 - 39
2. Llandysul	3/4 (75%)	23	20 - 28
3. Llysdinam	2/2 (100%)	4	1 - 5
4. Machen	2/2 (100%)	23	22 - 24
5. Pentwyn	3/3 (100%)	9	2 - 20
6. Monmouth	2/3 (33%)	11	1 - 20
7. Pencoed	8/9 (89%)	48	2 - 253
8. Pontypool	9/12 (75%)	15	1 - 44
9. St Athan	11/11 (100%)	345	4 - 1399
10. St Bride's Major	40/50 (80%)	167	1 - 2012
Total	**83/100 (83%)**	**61**	**1 - 2012**

Table 4.2 Slow worm capture rates from three donor sites and the corresponding three receptor sites (14 visits per site over a 12 month period). Native population data are included for the Site 2 receptor site and total percentage recapture rates for all sites.

		Female Adult : Juveniles	**Male** Adult : Juveniles	**Neonate**	**Total**	**% recapture** Based on all age/sex classes
Site 1	Donor site capture / introduced	21:1	14:0	4	40	
	Receptor site recapture	5:0	7:0	0	12	30.0%
Site 2	Donor site capture / introduced	16:9	17:4	75	139	
	Receptor site recapture	8:1	7:4	17	37	26.6%
Site 2	Native population	7:2	20:8	6	43	
	Native population recapture	2:0	3:2	4	11	25.6%
Site 3	Donor site capture / introduced	44:3	20:5	11	93	
	Receptor site recapture	0:0	1:5	9	15	16.1%*

*Lower than predicted recapture rate due to post-brumation landslide event at this site.

Table 4.3 Average growth rates from four slow worm populations monitored over 34 weeks following translocation.

	Average weight gain Log_{10} g / day^{-1}	**Average length gain** mm / day^{-1}
Site 1	1.72	0.04
Site 2	1.23	0.01
Site 2 (natives)	0.82	0.02
Site 3	1.63	0.04

Table 4.4 Percentage of slow worm population (n=38) that increased in body condition index at each study site in the year following translocation. Gravid females and animals that had shed their tails were omitted from this analysis.

	Adult Male	**Adult Female**	**Total Adult**	**Juvenile**	**Total Population**
Site 1	86%	75%	81%	-	81%
Site 2	75%	80%	78%	43%	66%
Site 2 (natives)	80%	100%	90%	75%	85%
Site 3	50%	-	50%	67%	59%

Table 4.5 *Neoxysomatium brevicaudatum* prevalence, mean intensity and range from slow worm faecal samples (n=76). Hosts collected from Site 2 (translocated animals 0, 6 and 12 months, and natives 6 and 12 months after translocation), Sites 1 and 3 (6 months post translocation) and a random sample of hosts from Wales.

	Site 2 0 months	**Site 2** 6 months	**Site 2** 12 months	**Native2** 6 months	**Native 2** 12 months	**Site 1** 6 months	**Site 3** 6 months	**Welsh** 6 months
Juvenile nematodes								
Prevalence	75%	75%	75%	75%	88%	88%	63%	75%
Mean intensity	21	21	17	392	5	46	6	13
Range	2-117	2-103	2-56	20-2000	2-12	2-253	1-41	3-22
Adult nematodes								
Prevalence	0%	13%	38%	38%	38%	13%	0%	17%
Mean intensity	0	0.3	0.5	2	1.1	0.1	0	0.2
Range	0	2	1	2-11	1-8	1	0	1
Total nematodes								
Prevalence	75%	75%	75%	88%	88%	88%	63%	92%
Mean intensity	21	21	17	392	6	47	6	13
Range	2-117	2-103	1-56	1-2000	1-12	2-254	1-41	1-22
N	8	8	8	8	16	8	8	12

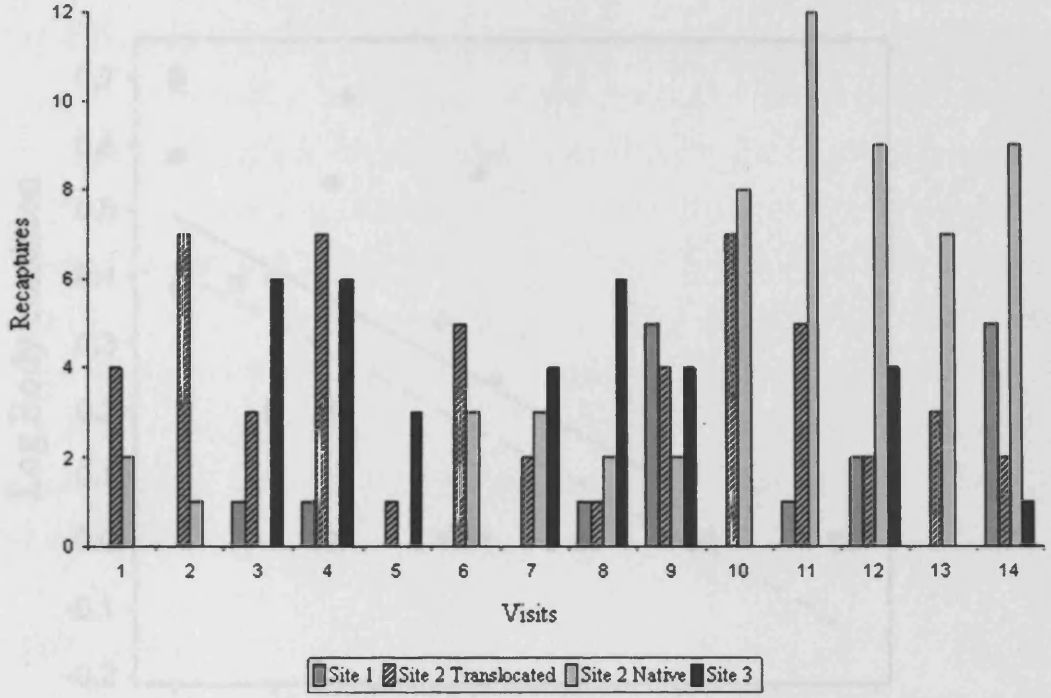

Figure 4.1 Total number of recaptures of slow worms (n=148) following translocation to Receptor Sites 1, 2 and 3 plus Site 2 natives over 14 visits. Visits 1-4 pre-brumation 2007 and visits 5-14 are post-brumation 2008.

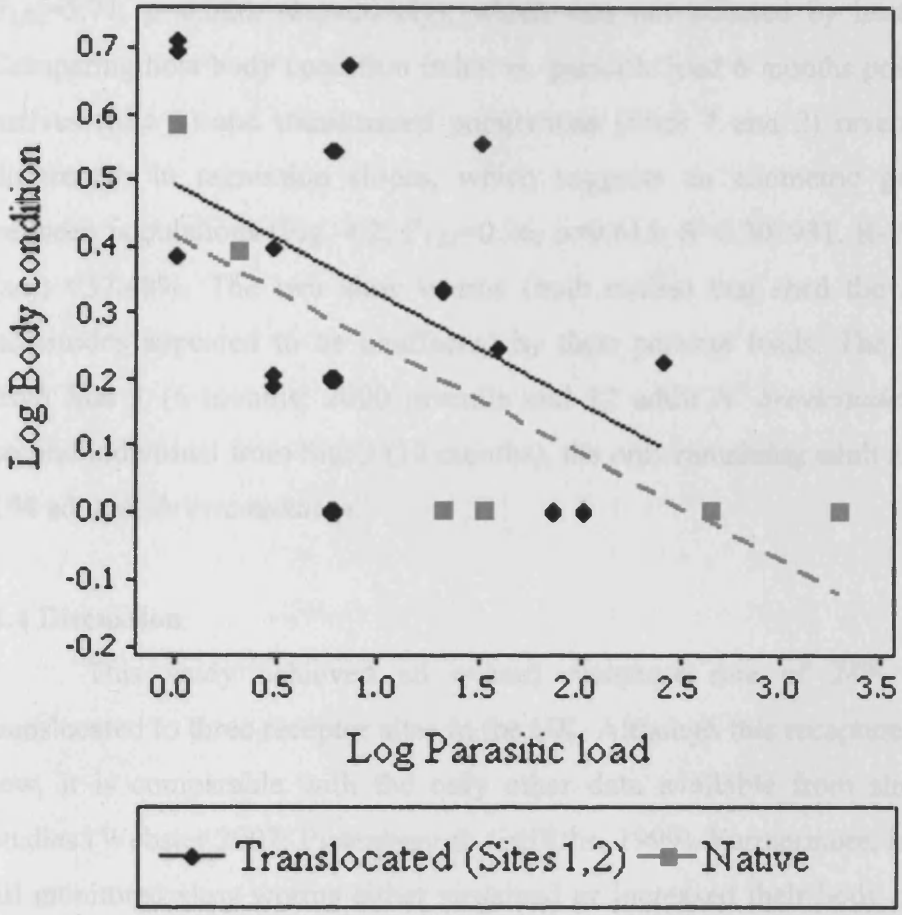

Figure 4.2 Comparison of body condition (log 10) vs. parasite load (log 10 Small) for Site 1 (n=8), Site 2 (n=8) (black circle) and Site 2 natives (n=8) (grey square) showing parallel relationships between regression lines for translocated and native animals ($F_{1,23}$=0.26; P=0.615 ; S=0.202931, R- Sq =46.01%, R-Sq (adj) =37.48%).

4.3.5 *Impact of parasites on host body condition*

When examining receptor populations at 6 months post-translocation, there was a significant negative relationship between parasite load for combined juvenile and adult *N. brevicaudatum* and host body condition (GLM, $F_{1,32}$=7.73; p=0.009 and $F_{1,23}$=5.71, p=0.026, respectively), which was not effected by host sex or maturity. Comparing host body condition index vs. parasitic load 6 months post-translocation for natives (Site 2) and translocated populations (Sites 1 and 2) revealed no significant differences in regression slopes, which suggests an allometric growth relationship between populations (Fig. 4.2; $F_{1,23}$=0.26, p=0.615; S=0.202931, R- Sq =46.01%, R-Sq (adj) =37.48%. The two slow worms (both males) that shed the largest number of nematodes appeared to be unaffected by their parasite loads. The first was captured from Site 1 (6 months; 2000 juvenile and 12 adult *N. brevicaudatum*), whereas the second individual from Site 3 (12 months), the only remaining adult at the site, had shed 194 adult *N. brevicaudatum*.

4.4 Discussion

This study achieved an overall recapture rate of 24% for slow worms translocated to three receptor sites in the UK. Although this recapture rate might appear low, it is comparable with the only other data available from similar translocation studies (Webster 2007; Platenberg & Griffiths, 1999). Furthermore, in the current study all monitored slow worms either sustained or increased their body condition one year post-translocation, and we observed slow worm mating, reproduction and neonates at all sites. We also present the first evidence that infection by the common gastrointestinal nematode *Neoxysomatium brevicaudatum* affects the growth rate of lizards.

Each receptor site was surveyed prior to translocation revealing native slow worms only to the extreme west of Site 2. The initial survey only established the presence of a small slow worm population (n=8), however, 8 months post-translocation monitoring revealed the native population to be 43. If the actual size of the native population had been accurately estimated this would have prevented its use as a receptor site so the survey guidelines (JNCC, 1998) used in this study should be reviewed and consideration given to extending receptor site surveys. Receptor sites should ideally be situated close to the donor sites, but not at the detriment of habitat quality. However, with the increased demand for sites driven by development ideal translocation habitat is becoming rarer. Furthermore, land owners aware of the complications arising should they later seek to develop their land are less amenable towards reptile translocations.

Consequently, many animals are relocated to substandard sites due to time constraints and demands from developers.

Prior to the current study, the only comprehensive study of slow worm post-translocation was undertaken by Platenberg and Griffiths (1999) who performed a 2 year study in south east England. This was followed up by Webster (2007) a decade later who examined the same slow worm population for a further 2 years. We undertook relatively few post-translocation receptor site survey visits (n=14), compared to 140 by Platenberg and Griffiths (1999) and 26 site visits per year by Webster (2007). We also used only 41-66% of the receptor site refugia used by Webster (2007) and Platenberg and Griffiths (1999), and yet achieved comparable yearly individual recapture rates (+4% recapture cf. Platenberg and Griffiths 1999 and -2% recapture cf. Webster 2007). However, this method should only be employed by experienced reptile ecologists and should in no way be seen as a substitute to established mitigation guidelines. Our adult female recapture rate (34%) was identical to Platenberg and Griffiths (1999), but our yearly capture rate was 16% higher for adult males and 10% lower for juveniles. Recapturing more males than females in the current study could be explained by observing slow worms directly exiting brumation in contrast to Platenberg and Griffiths (1999) who only monitored slow worm activity from March to September. Although we captured more juveniles than adults, this reflected the higher proportion of younger animals in our populations. Due to the similar recapture rates at all three studies, the current work indicates less intensive sampling is effective and this potentially reduces stress to the translocated animal and cost of the mitigation strategy.

Beginning the post-translocation monitoring pre-brumation, allowed us to examine slow worm weight change and mortality after the following winter period. Surprisingly, there were no significant weight losses recorded directly following brumation and recapture levels remained high with no obvious declines in population density. During the study, all slow worms at least retained their original weight and 70% increased in body condition. In contrast, Platenberg and Griffiths (1999) recorded weight loss for all their animals in the two years following translocation. However, 10 years after translocation body condition had improved although the population density had reduced by 30% (Webster, 2007). Slow worm weight naturally fluctuates due to infrequent, large meals or tail loss. This is best illustrated by a small 3 g translocated male slow worm that regurgitated a 1 g earthworm on response to being captured. In another example, a 30 cm long male slow worm at time of translocation was recovered 6 months later having shed 10 cm of its tail, but still retained its initial capture weight of

10 g. Weight also varies seasonally for female slow worms with rapid increases and decreases in body weight during and after pregnancy, with many females highly emaciated following birth (Gregory et al., 2006; Filippi et al., 2005; Gregory and Isaac, 2004).

We observed mating, gravid females and neonates at all receptor sites and within the Site 2 native population. In contrast, Platenberg and Griffith (1999) recorded reduced population density and no evidence of reproduction in the first year followed by reduced breeding in the second year following translocation. Preparation and appropriate mitigation for receptor site over wintering facilities, including confirmation of suitable food resources, prior to translocation might explain the success of the current translocations.

The relationship between parasites and their reptile hosts has been poorly investigated in the UK and is not currently regarded as a translocation issue. We recorded negative consequences of parasitic infection amongst both the native and translocated populations. Parasites can affect reptiles in a number of ways through intra- and interspecific competition (Brinkman et al., 2005). These include lowering host body condition, reduction of fat deposition, reduction in fecundity and reducing locomotive capability (Heideman, 1997). All these effects could prove detrimental to a reptile population. Temperate reptiles commonly reproduce biannually due to an inability to produce sufficient fat reserves to breed annually. Further reduction of fat deposition coupled by increased predation from limited locomotion associated with occupancy of colder climates would have a direct effect on population density. In addition, increased parasite load can delay sexual maturity and prolong life expectancy of animals, which in turn may affect population dynamics (Bonnet et al., 2002).

For the first time *Neoxysomatium brevicaudatum* was recorded from UK slow worms at a prevalence of 83% and mean intensity of 61. This nematode is a common pathogen of herpetofauna throughout Europe (Saglam and Arikan, 2006; Borkovcová and Kopřiva, 2005; Kirin and Buchvaov, 2002; Sharpilo, 1974; Yamaguti, 1961). It infects the intestine of amphibians (e.g. *Bombina, Bufo, Hyla, Rana, Triturus*) but previously has only been found occasionally in reptiles (e.g. *Anguis*, *Natrix*; see Saglam and Arikan, 2006; Karadenız et al., 2005; Kirin, 2002; Shimalov and Shimalov, 2000; Shimalov et al., 2000). It has a direct life-cycle, the eggs hatching outside the host and the first stage larvae developing and moulting twice to the infective third stage. The final host probably becomes infected orally and larvae are often found in tissues (Saeed et al., 2007; Vashetko et al., 1999). Male *N. brevicaudatum* range from 3-4 mm in

length while the ovoviviparous females are slightly larger at 4-5 mm (Ryzikov et al., 1980). In the current study juvenile *N. brevicaudatum* were more abundant than adults in all slow worm populations. Adult nematodes were only common among the native Site 2 population, which shed the most parasites throughout the study. However, only two of the native males at Site 2 were heavily infected and if these were removed from the data, the results were comparable for all hosts. These larger older males probably had accumulated a greater number of parasites from increased exposure over time. However, it is unknown how parasite counts from stool samples correlate with actual worm burdens.

As observed in other studies, nematodes were significantly more common in male than female hosts (Silva et al., 2007; Leinwand et al., 2005; Salkeld and Schwarzkopf, 2005; Heideman, 1997), which could be explained by the immunocompetence hypothesis (ICHH; Folstad and Karter 1992). As testosterone is responsible for secondary production of sexual traits and is simultaneously immunosuppressive (Cox and John-Alder, 2007), the cost of expressing sexual traits could lead to decreased immune function. As a consequence reptiles are burdened with increased parasitic loads, with only the fittest males able to express sexual characteristics fully (Cox and John-Alder, 2007; Roberts et al 2004; Veiga et al., 1998; Folstad and Karter, 1992).

Both juvenile and adult *N. brevicaudatum* had significant negative effects on slow worm body condition reducing growth rate. Lower body condition and increased mortality due to parasitic infection has been recorded for many reptile species including common garter snakes (*Thamnophis sirtalis*), rough green snakes (*Opheodrys aestivus*; see Brower and Cranfield, 2001), water pythons (*Liasis fuscus*; see Madsen et al., 2005), *Anolis* lizards (Schall, 1992), geckos (*Lepidodactylus lugubris*; see Hanley et al., 1998), Eastern water skinks (*Eulamprus quoyii*; see Salkeld and Schwarzkopf, 2005) and in highly parasitized common lizards (*Lacerta* (*Zootoca*) *vivipara*; see Uller et al., 2003 and Clobert et al., 2000). Studies of the common lizard (Sanchis et al., 2000) and other anguid species, such as the alligator lizard (*Gerrhonotus multicarinatus*; see Goldberg and Bursey, 1990), have also reported parasitism by a single helminth species similar to the observed infection of slow worms in the current study. However, higher multiple helminth infections have been observed (e.g., Rocha and Vrcibradic, 2003).

The potential for new host parasite relationships is high given that slow worms are frequently being introduced. The spread of directly transmitted disease increases with an increased density of susceptible hosts (Anderson and May, 1986). Within small

endangered populations it is assumed that the risk of disease outbreak should be limited and an unlikely agent of extinction (Dobson & May 1987). However, van Oosterhout et al. (2007) found the opposite to be true whilst supplementing wild guppy populations with naïve captive-breed hosts experiencing high parasite-induced mortality of captive-bred fish. This has important consequences for conservation biology with supplementation programs increasing the density of susceptible hosts and increasing the risk of parasite outbreak. The gradual release of animals is preferred to *en masse* translocation as this will limit the density of susceptible individuals and hence lower the probability of parasite outbreak (Faria et al., in press). With suitable knowledge of native parasites at the receptor sites, translocated animals could potentially be exposed to these pathogens prior to release in order to increase their resistance (van Oosterhout et al., 2007) but further analysis of British macroparasite communities is needed to establish the extent of the threat posed.

In the current study, we have demonstrated that the previously recommended 60-90 days for reptile translocation (Herpetofauna groups of Britain and Ireland, 1998) is sufficient time to remove the majority of animals from donor sites providing suitable guidelines are followed. However, no time restraints were imposed allowing us to achieve 10 collection days of zero captures at each donor site. In practice, this option is not often afforded amid considerable time pressure from developers. However, even within the current study the cryptic subterranean nature of slow worms makes it highly unlikely that the entire population was removed from the donor sites. Initial survey work to census reptile populations is vital, as illustrated by an independent assessment that was undertaken at the three donor sites used in the current study. These assessors employed a ten visit, refugia-search survey method (see Smith, 1990) and estimated donor site population density at 1000 individuals per hectare at each site. This population estimate at Site 2 was comparable with our own, with both surveys underestimating the population by 21%. However, Site 3 population was underestimated by 97% and Site 1 overestimated by 6250% (Blanket assessment extrapolated from population density per m^2).

When estimating population size, comprehensive knowledge of the interaction between beneficial ecological features and slow worm population density is essential. What can firstly be perceived as good reptile habitat may be free of animals, due to food availability, water table level, disturbance or high predation. In contrast, some disturbed brown field sites can sustain healthy populations.

Our 12 month post-translocation study illustrated a potentially successful exercise, although longer term monitoring is needed to determine population fitness. As temperate reptiles commonly breed on a biannual basis and with slow worms not reaching sexual maturity for 4 to 5 years, evidence of a viable self sustaining population is unlikely to be achieved in less than 10 to 20 years (Webster 2007; Platenberg and Griffiths, 1999). Generally, such time periods are impractical for both commercial developers and herpetologists; however, we recommend a minimum of 5 year post-translocation monitoring of receptor site with preferably four site visits per year. This number of visits is a compromise between ideal recapture rate and costs implications imposed by clients of environmental consultants.

Unfortunately, many translocations are performed to unrealistic deadlines conducted in sub-standard weather conditions, at unfavourable times of the year by ecologists with limited reptile knowledge. Saving an animal from the initial impact of construction only to relocate it at a substandard receptor site experiencing high rates of mortality is of little value other than to satisfy current legal obligations. However, as illustrated in the current study, translocations can be conducted successfully providing all the potential variables influencing translocation success are considered. Only through collective endorsement of reptile mitigation standards will we generate positive conservation measures for British reptiles.

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5.0 General discussion

Genetic studies of native herpetofauna populations are important for the conservation of European biodiversity; however, previous studies had been largely dependent on invasive sample collection (see Feldman & Spicer, 2002; Voris et al., 2002; Keogh, 1998). The first part of this study (Chapter 2) set out to demonstrate the efficiency of non-invasive sampling (NIS) for molecular studies and to review the various potential sources of such samples, including sloughed skin, faeces, eggs, aborted embryos, along with road kills, predated specimens and museum samples. Faecal material is now one of the most commonly used animal by products for DNA extraction (e.g. Goossens and Bruford, 2009). Unfortunately, such samples from reptiles are often difficult to detect in the field. However, during faecal sample collection for this study, a new method was devised for obtaining snake faeces in the field. As this method is semi-invasive (i.e., the animal is handled), stress to the reptile was limited by scooping and immediately releasing the reptile following a natural defecation threat response. This procedure was most reliable with grass snakes (about 95% defecation) compared to smooth snakes (about 75%) and adders (about 70%). If the animal had not defecated within a minute it was immediately released.

Using mitochondrial cytochrome *b* primers, 500 and 758 bp amplicons were successfully sequenced from a variety of snake tissues collected by NIS. The availability and degradation of these materials differed greatly, and both DNA extraction and PCR success appeared dependent upon sample origin and storage. This work demonstrated for the first time that reptile faecal, egg and foetal tissues, as well as sloughed skin and carcasses, represent valuable NIS source material permitting genetic studies with minimal disturbance to the individual and its population.

There are several protocols for the extraction of DNA from bone (e.g. Rohland et al., 2004; Ye et al., 2004; Wandeler et al., 2003; Götherström, 2002; Kalmár et al., 2000) but all methods are time consuming and therefore not competitive with other sources of NIS. Therefore, the bones of British reptiles, which are particularly small, were not explored in this project as a potential source of NIS. However, advancements in DNA extraction protocol should be examined to re-assess the viability of native reptile bone as a source of NIS in the future (see Odile et al., 2007; Rohland and Hofreiter, 2007).

Chapter 2 provided the methodology that formed the basis of Chapter 3, which for the first time assessed the phylogeography of the three native snake species in the UK through phylogenetic analysis of cytochrome *b* sequences. This study looked at the

historic distribution and origins of *Vipera berus*, *Natrix natrix* and *Coronella austriaca* across their entire European range. Although *V.berus*, *N. natrix* and *C. austriaca* have been identified as occupying at least three refugia during last glacial maximum (LGM), snake lineages now occupying the UK were all identified as originating from Italy. This finding is in agreement with morphometric studies conducted by Thorpe (1984) who found a clear relationship between latitude and the extent of differentiation between 41 characteristics (e.g., colour pattern, scalation and internal morphology) of *N. natrix*. Thorpe (1984) was able to demonstrate the migration of a *N. natrix* lineage originating at the Italian Peninsular that had extended north to populate the UK. During the course of this PhD, three other research groups independently investigated the phylogeographic structure of *V. berus*, *N. natrix* and *C. austriaca.* Guicking et al. (2006) closely grouped Italian and British populations together in their phylogenetic analysis of *N. natrix* cytochrome *b*, ND1, ND2 and ND4. The LGM Italian refugia for *V. berus* were identified by Ursenbacher et al. (2006) through phylogenetic analysis of cytochrome *b* and the D-loop. These authors were also able to establish colonisation routes from Italy through to Britain using samples collected by W. Wüster (North Wales) and the author (RJ; South Wales). Finally, Santos et al. (2008) was able to demonstrate *C. austriaca*'s expansion from the Iberian Peninsular into mainland Europe through phylogenetic analysis of cytochrome *b* and 16S rRNA. As well as the LGM ice age refugia, Rowe et al. (2006), Ursenbacher et al. (2006) and Guillaume et al. (2000) identified possible Younger Dryas refugia surrounding the Carpathian mountain range and southern France. These areas have been previously identified as important Younger Dryas refugia for many European species (see Chapter 3).

In addition to confirming the European phylogenetic structure of *N. natrix, V. berus* and *C. austriaca*, the current study offered the first comprehensive overview of their current population structure within the UK. *V. berus* displayed greater genetic diversity (five UK haplotypes) than the other two study species, which predictably were monophyletic. Although the common northern European haplotype of *V. berus* was ubiquitous throughout the UK, four additional haplotypes existed in allopatric populations in north and south Wales, Nottingham and the New Forest that are historically associated with dense canopy forest. Such forested areas would have insulated *V. berus* from extreme cold of the Younger Dryas. As the viper is a cold tolerant specialist and displays greater genetic diversity in Britain than *N. natrix* or *C. austriaca*, it is probable that *V. berus* took refuge in Britain during the younger Dryas or at the very least migrated into Britain in advance of the other two British snake species.

Moreover, the same deciduous canopied forest would have offered shaded refuge to the viper as temperatures rose during the Bölling-Allerod (see Spellerberg and Phelps, 1977).

Although cytochrome *b* has been informative for phylogeographic structure the data would have been more robust with additional genetic markers, perhaps of the control region or even a nuclear gene. Unfortunately, this was beyond the funding and time constraints of the current project. In addition, large areas of Europe were under represented and many existing haplotypes for all three study species will have remained cryptic. Although reasonable sample sizes were obtained for *V. berus* and *N. natrix*, more extensive sampling and longer sequence length for *C. austriaca* is required to accurately identify the colonisation routes for mainland Europe. In particular, it would be interesting to more intensely sample refugia situated in France and the Carpathians to better examine genetic diversity and more robustly identify Younger Dryas colonisation routes for all three study species.

Sampling revealed the extent of anthropogenic effects on UK reptiles with large areas devoid of suitable reptile habitat. This emphasises the need to maintain corridors of connectivity between the mainland metapopulations. Increasingly, land development causes habitat loss and translocation of these reptiles is increasingly common practice (Dodd and Seigel, 1991). However, relatively few studies have investigated the effectiveness of these mitigation exercises due to the vagility and relatively small population densities of British snakes (Beebee and Griffiths, 2000). However, another UK reptile, the slow worm *Anguis fragilis*, is relatively common throughout mainland UK and has a more localised, high density distribution making it an ideal model to assess translocation success (Webster, 2007; Platenberg and Griffiths, 1999).

During the current study, 315 slow worms were monitored for two years following their translocation to three sites in South Wales. This work while achieving similar recapture rates to previous studies (Webster, 2007; Platenberg and Griffiths, 1999) also recorded evidence of reproduction in all three translocated populations. In practical terms, external head markings proved 100% efficient at recognising individual animals, and methods were identified for improving mitigation by reducing frequency sampling and density of refugia. However, two years is insufficient time to establish population sustainability therefore permission has been granted (from Countryside Council for Wales) to undertake a 20 year post translocation monitoring programme at two sites in South Wales. It is hoped that this long term monitoring will help to better understand the value of translocation but also identify other ways of improving

mitigation to increase relocation success. Further studies are planned to identify key habitat features and ideal refugia placement. Also, it would be interesting to investigate ideal relocated population size and the effects of introduced lizards to population genetic diversity.

Understanding the role of genetic diversity is pivotal to the conservation of translocated populations as this, in conjunction with other biotic factors, influences the survival and effectiveness of animal translocation (Alberts, 2007; Jusaitis, 2005; Miller et al., 1999; Storfer, 1999). Populations can exhibit inbreeding depression, genetic drift or fixation of alleles due to small population size or isolation (Keller and Waller, 2002; Bryant, 1999; Ellstrand and Elam, 1993). The introduction of genetic variation through translocation to resident populations can be beneficial, increasing genetic diversity and supplementing or creating new populations within the metapopulation (Madsen et al., 2004, 1999; Willis and Wiese, 1993). However, introductions can also cause negative effects (Storfer, 1999; Lynch, 1991), leading to an increase in intra-specific competition and aggression (e.g. Goossens et al., 2005). In addition, mating between native and translocated animals can lead to a loss of intraspecific genetic diversity and potential outbreeding depression (Ficetola and De Bernardi, 2005) with translocated populations genetically altering locally adapted populations, possibly even increasing the frequency of deleterious traits (Frankham, 2007; Ficetola and De Bernardi, 2005; Lynch, 1991).

The current study has highlighted the need to include disease surveillance in post-translocation monitoring and its importance as a conservation tool in preserving threatened reptile species. During translocation studies, an apparently common nematode, *Neoxysomatium brevicaudatum* was identified from slow worms (83% prevalence, n=100; Fig. 5.1). This is both the first recorded infection of a slow worm by *N. brevicaudatum* and the first record of this parasite in the UK. *N. brevicaudatum* negatively affected slow worm body condition and, although not significant, parasitic load varied amongst the host populations (mean intensity 135, range 1-2012).

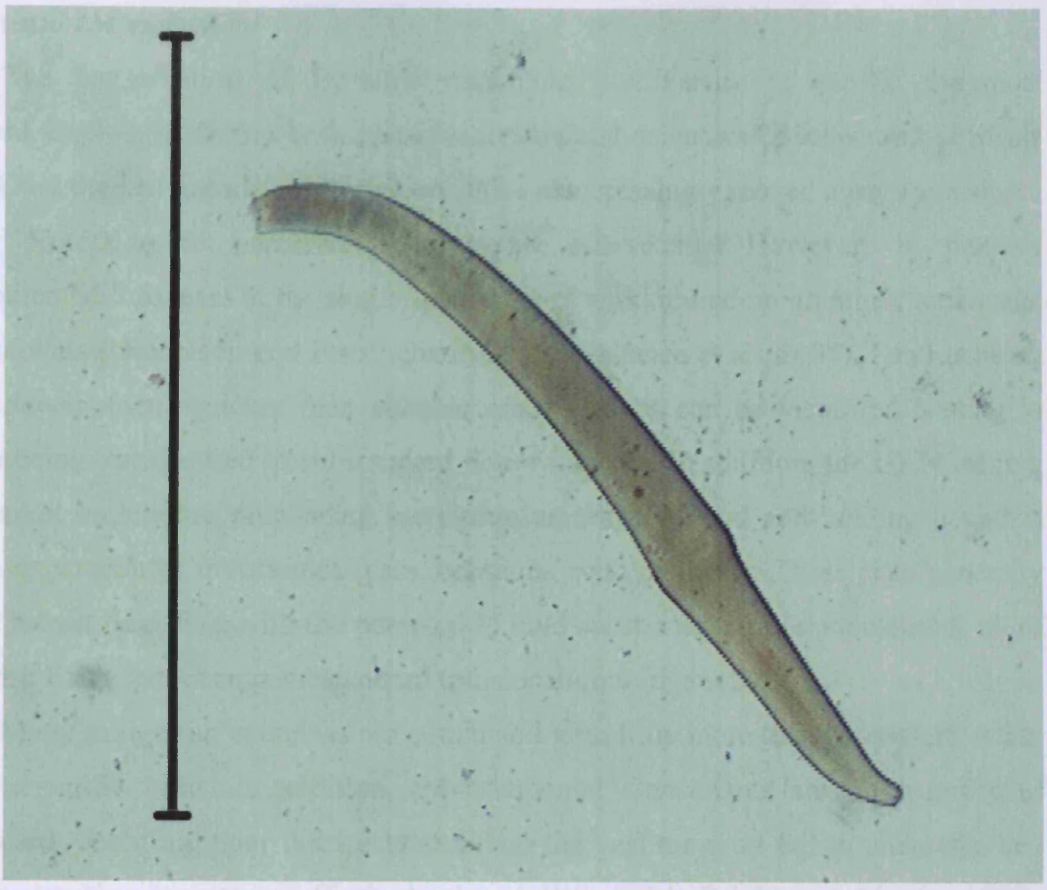

Figure 5.1 Juvenile *Neoxysomatium brevicaudatum* recovered from a slow worm (*Anguis fragilis*) stool sample. Scale bar =1 mm.

Although *N. brevicaudatum* is now known throughout Wales, further study is planned to assess its distribution in the UK. This would include comparing parasite prevalence in Southern England to that of Scotland to examine the effects of altitude and range on host parasitic load within the UK. Ideally further samples could be collected from other British reptiles to investigate the host range and specificity of *N. brevicaudatum*. Specific future research planned includes the collection of stool samples from alien species such as the Aesculapian snake (*Elaphe longissima*) from north Wales and red sliders from Cardiff (*Trachemys scripta elegans*). Ultimately, research conducted in western Europe will aim to show connectivity between British *N. brevicaudatum* and populations previously recorded in Eastern Europe (Saglam and Arikan, 2006; Borkovcová and Kopřiva, 2005; Kirin and Buchvaov, 2002; Sharpilo, 1974; Yamaguti, 1961).

Conservation Management

The conservation of Britain's remaining biodiversity is one of the most important challenges facing both conservationists and scientists (Beebee and Griffiths 2000). Over the last decade, conservationists have increasingly moved away from single species protection to preservation of entire ecosystems. However, in practice management still focuses at the single species level with limited programmes to manage entire habitats (Nicholson and Possingham, 2006; Moilanen et al., 2005). Land is being lost to development quicker than suitable receptor sites can be identified leading to animals being translocated to substandard donor habitats. In addition, the UK's leading supermarket outlets are purchasing increasing amounts of land and holding it with a view to expansion or investment (pers. commun. with retailers). These sites generally exist as brown field sites with the potential to hold substantial reptile populations; all of which risk being lost during substandard translocation mitigation.

Many mitigation exercises are conducted with little more than guesswork rather than a scientific basis. In addition, Environmental Consultants are often guilty of substandard recording, poor documentation and the vast majority fail to undertake any post translocation monitoring (Sutherland and Hill, 2004; Bright and Morris, 1993). Mitigation is driven by the available resources generated though limited client contribution and endorsed by the various statutory bodies. However, mitigation should be designed through successful survey effort, sufficient monitoring, incorporating site assessment and increasing the quality of reporting. Without exception, management should always be followed by monitoring (Sutherland and Hill, 2004). Finally, there should be a central record centre that maintains documentation of all translocation events.

Presently, reptile population densities are categorised according to the Herpetofauna workers guide or Froglife Fact Sheet 10 (Froglife, 2000; 1999 respectively) from the total number of animals recovered by a single consultant on a single day. This method offers no distinction between translocated, native or multiple recaptures of the same animal. Not only is there little effort to monitor our native populations long term but there is generally little understanding as to why this is required. It is incorrect to assume that substantial costs would be required to extend monitoring. Through appropriate targeting and efficient surveying translocational monitoring could be incorporated into the overall relocation mitigation without necessarily increasing costs. Post translocational monitoring can indicate the effectiveness of a programme and provide feedback on how to improve management

practices (White et al., 2003; Cromwell et al., 1999). The non-invasive tracking and survey recovery methods outlined in Chapter 3 offer the basis for credible mitigation that, with limited training to the consultant ecologist, could become commonplace and endorsed by statutory bodies.

There is a great need for increased collaboration between consultants and researchers. The scientific community needs to be resourceful in its employment and training of ecological consultants, who often have limited in-depth knowledge. For example, genetic sampling of native populations essential for understanding population dynamics and facilitating appropriate mitigation measures (Sunnucks, 2000) is rarely considered by consultants. With minimal additional effort, field surveyors could easily collect non-invasive samples (see Chapter 2) that would be available for subsequent genetic analyses. The current void that exists between sound scientific population studies and the inadequate, financially driven mitigation measures currently endorsed by statutory bodies is less than progressive. In short, greater influence from the scientific community must be endorsed if we are to generate meaningful data and maintain healthy, sustainable reptile populations in the UK.

Ultimately the future of reptile conservation lies in education and research. Many of our reptile populations lie outside the protection of nature reserves and SSSI's and are subject to 'in vogue' policies and the need to accommodate an ever increasing human population. Conservation management aims to counterbalance the unchecked exploitative management of natural resources through sustainable development. However during economic downturns, funding for ecological mitigation is often one of the first cut backs.

Connectivity between remaining reptile populations is pivotal in safeguarding biodiversity. Additional research is needed to investigate the success of migratory individuals that breed with isolated populations (e.g. *V. berus* study by Madsen et al., 1999). Ultimately, we need to recognise that single species protection is acceptable providing habitat connectivity is also examined. If we ignore this warning and continue to isolate reptile populations then their long term future in the British Isles remains uncertain.

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Appendix I

The following appendix lists spreadsheet records of non-invasively collected sample material for *Vipera berus*, *Natrix natrix* and *Coronella austriaca* sequenced for use within this project. Spreadsheet data includes CODE (laboratory code), HAP (haplotype codes for each species), SEQ (a record of amplification success), SEX (the gender of animal, where known), LOCATION (sample location), REGION (greater sample location), COUNTRY (country of origin), GRID REFERENCE (grid location of sample), COLLECTOR (name of collector), DATE (date of sample collection), YEAR (year of sample collection) and AGE (age of sampled animal categorised as either adult or juvenile, where identified).

In addition to collected samples, a number of Gen bank sequences for *Vipera berus*, *Natrix natrix* and *Coronella austriaca* were included in analysis. Spreadsheets list sequence information by CODE, HAP, ACCESSION (Gen bank accession number), LOCATION, GRID REFERENCE and finally PAPER (the publication reference for samples included from previously published works). Publications are referenced at the end of this Appendix.

CODE	MAP	SEQ	SEX	LOCATION	REGION	COUNTRY	GRID REFERENCE	COLLECTOR	DATE	YEAR	AGE
Vipera berus											
VB1	1	Y		LLYSDINAM FIELD CENTRE, NEWBRIDGE ON WYE	POWYS	WALES	52°12'57.84"N, 3°27'15.98"W	FRED SLATER		2001	A
VB2				ST. DAVID'S POINT	PEMBROKESHIRE	WALES		FRED SLATER		2002	A
VB3	1	Y			DORSET	ENGLAND	50°43'54.07"N, 1°58'21.23"W	TOM LANGFORD	01-Apr	2002	A
VB4	1	Y			DORSET	ENGLAND	50°43'20.06"N, 1°58'45.44"W	TOM LANGFORD		2002	A
VB5				MONMOUTH	MONMOUTHSHIRE	WALES		JERRY LEWIS		2002	A
VB6	1	Y		TOWN COMMON	DORSET	ENGLAND	50°36'32.97"N, 1°57'41.53"W	CHRIS GLEED-OWEN	23-May	2002	A
VB7				CREEDON HEATH	DORSET	ENGLAND		CHRIS GLEED-OWEN	25-Aug	2002	A
VB8		Y		TURBARY COMMON, BOURNEMOUTH	HAMPSHIRE	ENGLAND	50°45'10.76"N, 1°55'21.28"W	CHRIS GLEED-OWEN	03-Aug	2002	A
VB9	1	Y	F	TURBARY COMMON, BOURNEMOUTH	HAMPSHIRE	ENGLAND	50°45'10.76"N, 1°55'21.28"W	CHRIS GLEED-OWEN	15-Sep	2002	A
VB10	1	Y		POOLE	DORSET	ENGLAND	50°43'20.06"N, 1°58'45.44"W	CHRIS GLEED-OWEN		2002	A
VB11	1	Y	F	TRIGON EAST	DORSET	ENGLAND	50°41'34.79"N, 2° 3'0.56"W	CHRIS GLEED-OWEN	14-Aug	2002	A
VB12				MASTER'S PIT	DORSET	ENGLAND		DAVE BIRD		2002	A
VB13	1	Y		HARTLAND MOOR	DORSET	ENGLAND	50°58'27.87"N, 2°19'42.61"W	CHRIS GLEED-OWEN		2002	A
VB14	10	Y		TOWN COMMON	DORSET	ENGLAND	50°36'32.97"N, 1°57'41.53"W	CHRIS GLEED-OWEN	10-Aug	2002	A
VB15				STONE HOUSE FARM, WEST HERLING	NORFOLK	ENGLAND				2002	A
VB16				WEST BURTON POWER STATION	NOTTINGHAMSHIRE	ENGLAND		ERIC PALMER	02-Aug	2005	A
VB17	1	Y	M	THORNE MOORS	SOUTH YORKSHIRE	ENGLAND	53°37'44.06"N, 0°57'3.02"W			1950	A
VB18	10	Y		FALL BAY, NR ROSSILI	GOWER	WALES	51°33'57.83"N, 4°17'11.05"W	KEVIN DUPÉ	09-Sep	1997	A
VB19	1	Y		BASILDON, NR A127	ESSEX	ENGLAND	51°34'19.85"N, 0°27'44.86"E	JON CRANFIELD	01-Sep	2002	A
VB20				GOLDCLIFF, NEWPORT	GWENT	WALES		KEVIN DUPÉ	05-Sep	2002	A
VB22	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	15-Apr	2002	A
VB23	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	15-Apr	2002	A
VB24	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	15-Apr	2002	A
VB25	1	Y	F	WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	01-Jul	1991	A
VB26	1	Y	F	WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	11-Jun	1994	A
VB27	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS		2002	A
VB28	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS		2002	A
VB29	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS		2002	A
VB30	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	22-Apr	1992	A
VB31	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	12-Apr	1995	A
VB32	1		M	WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND		TONY PHELPS	18-Apr	1998	A
VB33	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS	03-Jun	1989	A
VB34				WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND		TONY PHELPS		2002	A
VB35	1	Y		WYRE FOREST, NR KIDDIMINSTER	WORCESTERSHIRE	ENGLAND	52°23'6.19"N, 2°16'40.32"W	TONY PHELPS		2002	J
VB36				CASTLEMARTIN	PEMBROKESHIRE	WALES		N. MUSEUM WALES		1934	A
VB37				ROATH PARK	CARDIFF	WALES		N. MUSEUM WALES		1920	A
VB38				HAVERFORDWEST	PEMBROKESHIRE	WALES		N. MUSEUM WALES		1913	A
VB39				HAVERFORDWEST	PEMBROKESHIRE	WALES		N. MUSEUM WALES		1913	A
VB40				COWBRIDGE	VALE OF GLAMORGAN	WALES		N. MUSEUM WALES		1908	A
VB41				PENDOYLAN	VALE OF GLAMORGAN	WALES		N. MUSEUM WALES		1910	A
VB42				THE GARTH	CARDIFF	WALES		N. MUSEUM WALES		1907	A
VB43				HORTON	GOWER	WALES		N. MUSEUM WALES		1910	A
VB44				TONGWYNLAIS	CARDIFF	WALES		N. MUSEUM WALES		1927	A
VB45				WELSH ST.DONATS	VALE OF GLAMORGAN	WALES		N. MUSEUM WALES	27-May	1912	A
VB46				REDBROOKS, FOREST OF DEAN	MONMOUTHSHIRE	WALES		N. MUSEUM WALES		1956	A
VB47				KENFIG	BRIDGEND	WALES		N. MUSEUM WALES		1906	A
VB48				KENFIG	BRIDGEND	WALES		N. MUSEUM WALES		1906	A
VB49				FRESHWATER EAST	PEMBROKESHIRE	WALES		N. MUSEUM WALES	24-Aug	1960	J
VB50				FRESHWATER EAST	PEMBROKESHIRE	WALES		N. MUSEUM WALES	24-Aug	1960	J

CODE	MAP	SEQ	SEX	LOCATION	REGION	COUNTRY	GRID REFERENCE	COLLECTOR	DATE	YEAR	AGE
VB51				REDBROOKS, FOREST OF DEAN	MONMOUTHSHIRE	WALES		N. MUSEUM WALES	18-Apr	1960	A
VB52				REDBROOKS, FOREST OF DEAN	MONMOUTHSHIRE	WALES		N. MUSEUM WALES	18-Apr	1960	A
VB53			M	HIGH TOR, SOUTH GATE	GOWER	WALES		JULIAN WOODMAN	02-Aug	2002	A
VB54			M	NR WESTLETON,	SUFFOLK	ENGLAND		JOHN BAKER		2002	A
VB55			M	NR SNAPE	SUFFOLK	ENGLAND		JOHN BAKER		2002	A
VB56				LOCH LUICHART, CORRIEMOILLIE FOREST, A832	WESTER ROSS	SCOTLAND		ALAN HARDWICK	22-Jan	2003	A
VB57				BARDEN MOOR, WHARFEDALE	YORKSHIRE	ENGLAND		KEVIN SUNDERLAND	13-Apr	2003	A
VB66				WENCH HILL, NR SCAR HOUSE RR, UPPER NIDDERDALE	YORKSHIRE	ENGLAND		KEVIN SUNDERLAND	13-Sep	2002	A
VB67				HOODSTORCH, UPPER WASHBURN VALLEY	YORKSHIRE	ENGLAND		KEVIN SUNDERLAND	05-May	2003	A
VB68				ALLERCHORPE COMMON, NR YORK	YORKSHIRE	ENGLAND		KEVIN SUNDERLAND	03-May	2003	A
VB69				HOODSTOURCH LANE, THRUSCROSS RESERVOIR	YORKSHIRE	ENGLAND		KEVIN SUNDERLAND	25-Aug	1986	A
VB70				HATFIELD HOUSE GARDENS,	HERTFORDSHIRE	ENGLAND		RORY DIAMOND		2003	A
VB71				A43, DUDDINGTON	NORTHAMPTONSHIRE	ENGLAND		RUPERT PAUL	12-Apr	2003	A
VB72			M	FURZEBROOK	DORSET	ENGLAND		TONY PHELPS		2002	A
VB73	1	Y	M	TURBARY COMMON, BOURNEMOUTH	HAMPSHIRE	ENGLAND		TONY PHELPS		2002	A
VB74			F	EAST CREECH	DORSET	ENGLAND		TONY PHELPS		2002	A
VB75			M	TURBARY COMMON, BOURNEMOUTH	HAMPSHIRE	ENGLAND		TONY PHELPS		2002	A
VB76			F	TURBARY COMMON, BOURNEMOUTH	HAMPSHIRE	ENGLAND		TONY PHELPS		2002	A
VB77			F	NOON HILL, VERWOOD	DORSET	ENGLAND		TONY PHELPS		2002	J (MEL)
VB78			F	FURZEBROOK	DORSET	ENGLAND		TONY PHELPS		2002	J
VB79			F	FURZEBROOK	DORSET	ENGLAND		TONY PHELPS		2002	J
VB80				TUNSTALL FOREST	SUFFOLK	ENGLAND		JOHN BAKER		2003	A
VB81	1	Y	F	CRANBORNE COMMON	DORSET	ENGLAND	50°54'56.47"N, 1°55'18.53"W	TONY PHELPS		2004	A
VB82				TUNSTALL FOREST	SUFFOLK	ENGLAND		JOHN BAKER		2004	A
VB83				CHATLEY HEATH	SURREY	ENGLAND		TONY PHELPS		2004	A
VB84				NOON HILL, VERWOOD	DORSET	ENGLAND		TONY PHELPS		2004	A
VB85				TOWN COMMON, Nr CHRISTCHURCH	DORSET	ENGLAND		TONY PHELPS		2004	A
VB86	1	Y		LAMMERMUIR HILLS, S.E. EDINBURGH		SCOTLAND	55°57'8.65"N, 2°58'33.23"W	DAVE BRADLEY		2004	A
VB87				MASTER'S PIT	DORSET	ENGLAND		RHYS JONES		2004	A
VB88	10	Y		MASTER'S PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	RHYS JONES		2004	A
VB89	1	Y		FOULSHAW MOSS	CUMBRIA	ENGLAND	54°14'18.10"N, 2°48'36.83"W	JOHN DUNGAVIN	11-Aug	2003	A
VB90				THORPE	EAST ANGLEA	ENGLAND		ANGUS CARPENTER	27-Apr	2004	A
VB91				THORPE	EAST ANGLEA	ENGLAND		ANGUS CARPENTER	27-Apr	2004	A
VB92	1	Y		THORPE	EAST ANGLEA	ENGLAND	53°18'52.82"N, 0°14'50.23"E	ANGUS CARPENTER	27-Apr	2004	A
VB93	1	Y		BRISTOL	BRISTOL	ENGLAND	51°27'19.13"N, 2°35'30.85"W	HYDER		2004	A
VB94				MOLD	GWENYDD	WALES		RHYS JONES		2005	A
VB95					NORFOLK	ENGLAND		JOHN BAKER		2002	A
VB96					NORFOLK	ENGLAND		JOHN BAKER		2002	A
VB97					NORFOLK	ENGLAND		JOHN BAKER		2002	A
VB98					SUFFOLK	ENGLAND		JOHN BAKER		2002	A
VB99	1	Y		SUFFOLK	SUFFOLK	ENGLAND	52°14'27.13"N, 1°3'20.55"E	JOHN BAKER		2002	A
VB100					SUFFOLK	ENGLAND		JOHN BAKER		2002	A
VB101	1	Y		SUFFOLK	SUFFOLK	ENGLAND	52°14'27.13"N, 1°3'20.55"E	JOHN BAKER		2002	A
VB102					SUFFOLK	ENGLAND		JOHN BAKER		2002	A
VB103					SUFFOLK	ENGLAND		JOHN BAKER		2002	A
VB104					ANGELSEY (BLOOD 1)	WALES		WOLFGANG WUSTER	JUNE	2004	A
VB105	1	Y		E309	VITTANGIA	SWEDEN	67°41'32.18"N, 21°39'44.04"E	THOMAS MADSEN	MAY	2002	A
VB106	1	Y		E311	VITTANGIA	SWEDEN	67°41'32.18"N, 21°39'44.04"E	THOMAS MADSEN	MAY	2002	A
VB107	1	Y		E312	VITTANGIA	SWEDEN	67°41'32.18"N, 21°39'44.04"E	THOMAS MADSEN	MAY	2002	A
VB108	1	Y		F7	STORA FJÄDERÄGG	SWEDEN	63°46'33.02"N, 21°0'14.84"E	THOMAS MADSEN	MAY	2002	A
VB109	1	Y		F9	STORA FJÄDERÄGG	SWEDEN	63°46'33.02"N, 21°0'14.84"E	THOMAS MADSEN	MAY	2002	A
VB110	1	Y		HV155	HALLANDS VÄDERÖ	SWEDEN	56°26'37.57"N, 12°33'43.04"E	THOMAS MADSEN	MAY	2002	A

CODE	MAP	SEQ	SEX	LOCATION	REGION	COUNTRY	GRID REFERENCE	COLLECTOR	DATE	YEAR	AGE
VB111	1	Y		HV310	HALLANDS VÄDERÖ	SWEDEN	56°26'37.57"N, 12°33'49.04"E	THOMAS MADSEN	MAY	2002	A
VB112	11	Y		J110 JUKKASJÄRVI	KIRUNA	SWEDEN	67°51'8.02"N, 20°35'51.57"E	THOMAS MADSEN	MAY	2002	A
VB113	11	Y		J119 JUKKASJÄRVI	KIRUNA	SWEDEN	67°51'8.02"N, 20°35'51.57"E	THOMAS MADSEN	MAY	2002	A
VB114	1	Y		S32 SMYGEHUK	SMYGEHAMN	SWEDEN	55°20'36.47"N, 13°22'1.85"E	THOMAS MADSEN	MAY	2002	A
VB115				S34		SWEDEN		THOMAS MADSEN	MAY	2002	A
VB116	1	Y	F	WOKING	SURREY	ENGLAND	51°19'0.55"N, 0°33'32.71"W	TONY PHELPS		2003	A
VB117	1	Y	M	CORFE CASTLE	DORSET	ENGLAND	50°38'19.01"N, 2° 3'24.65"W	TONY PHELPS		2003	A
VB118	1	Y	M	CHOBHAM	SURREY	ENGLAND	51°20'41.70"N, 0°36'11.34"W	TONY PHELPS		2003	A
VB119			F	MENDIP HILLS	SOMERSET	ENGLAND		TONY PHELPS		2003	A
VB120	1	Y	M	BOURNEMOUTH	DORSET	ENGLAND	50°43'4.11"N, 1°52'45.94"W	TONY PHELPS		2003	A
VB121			M	CHAPMANS FOREST	DORSET	ENGLAND		TONY PHELPS		2003	A
VB122	1	Y	M	ABERAMAN	RHONDDA CYNON TAF	WALES	51°42'11.42"N, 3°25'40.32"W	RHYS JONES		2005	A
VB123	1	Y	F	ABERAMAN (SLOUGH)	RHONDDA CYNON TAF	WALES	51°42'11.42"N, 3°25'40.32"W	RHYS JONES		2005	A
VB124	1	Y	F	ABERAMAN (PRETTY)	RHONDDA CYNON TAF	WALES	51°42'11.42"N, 3°25'40.32"W	RHYS JONES		2005	A
VB125	1	Y	F	ABERAMAN (DARK)	RHONDDA CYNON TAF	WALES	51°42'11.42"N, 3°25'40.32"W	RHYS JONES		2005	A
VB126	1	Y		ANGLESEY (BLOOD 2)		WALES		WOLFGANG WUSTER		2004	A
VB127	1	Y		ANGLESEY		WALES	53°16'57.56"N, 4°20'45.38"W	WOLFGANG WUSTER		2004	A
VB128	1	Y		ANGLESEY		WALES	53°16'57.56"N, 4°20'45.38"W	WOLFGANG WUSTER		2004	A
VB129	1	Y		ANGLESEY		WALES	53°16'57.56"N, 4°20'45.38"W	WOLFGANG WUSTER		2004	A
VB130	23	Y		NEW FOREST	HAMPSHIRE	ENGLAND	50°51'26.90"N, 1°37'45.41"W	TIM CARTER		2005	A
VB131	1	Y		SHREWSBURY	SHROPSHIRE	ENGLAND	52°42'29.31"N, 2°45'15.55"W	VIV GREEN		2004	A
VB132		Y		SHREWSBURY	SHROPSHIRE	ENGLAND	52°42'29.31"N, 2°45'15.55"W	VIV GREEN		2004	A
VB133	1	Y		BARBADOES HILL	MONMOUTHSHIRE	WALES	51°42'7.05"N, 2°41'26.65"W	MATT PICKARD		2006	A
VB134	1	Y		HINDHEAD COMMON	SURREY	ENGLAND	51° 6'1.65"N, 0°44'13.19"W	GEMMA JANE FAIRCHILD	26-May	2003	A
VB135				HINDHEAD COMMON	SURREY	ENGLAND	45° 5'1.75"N, 14°54'6.90"E	GEMMA JANE FAIRCHILD	08-Jul	2003	A
VB150	26	Y		SLAVONIJA	nr SLAVONSKI BROD	CROATIA	45°10'4.29"N, 18° 2'6.20"E	ZORAN TADIC		2005	A
VB151	26	Y		SLAVONIJA	nr SLAVONSKI BROD	CROATIA	45°10'4.29"N, 18° 2'6.20"E	ZORAN TADIC		2005	A
VB152	26	Y		SLAVONIJA	nr SLAVONSKI BROD	CROATIA	45°10'4.29"N, 18° 2'6.20"E	ZORAN TADIC		2005	A
VB153	25	Y		SLAVONIJA	nr SLAVONSKI BROD	CROATIA	45°10'4.29"N, 18° 2'6.20"E	ZORAN TADIC		2005	A
VB154	26	Y		SLAVONIJA	nr SLAVONSKI BROD	CROATIA	45°10'4.29"N, 18° 2'6.20"E	ZORAN TADIC		2005	A
VB155	24	Y		NOTTINGHAM	NOTTINGHAMSHIRE	ENGLAND	52°57'18.38"N, 1° 8'57.48"W	SHEILA WRIGHT	7TH JUL	2005	A
VB166	7	Y		SRBIJA I CRNA GORA		MONTENEGRO	43°12'34.97"N, 19° 0'38.87"E	LIDIJA POLOVIC		2006	A

CODE	SEQ	SEX	LOCATION	REGION	COUNTRY	GRID REFERENCE	COLLECTOR	DATE	YEAR	AGE
Matrix	*matrix*									
NN1			TOWN COMMON, Nr CHRISTCHURCH	DORSET	ENGLAND		CHRIS GLEED-OWEN	23-May	2002	A
NN2	Y		WAREHAM COMMON	DORSET	ENGLAND	50°40'49.54"N, 2° 6'35.28"W	CHRIS GLEED-OWEN		2002	A
NN3	Y		WAREHAM COMMON	DORSET	ENGLAND	50°40'49.54"N, 2° 6'35.28"W	CHRIS GLEED-OWEN		2002	A
NN4	Y		CORFE BLUFF, PURBECK	DORSET	ENGLAND	50°42'44.98"N, 2° 2'13.85"W	CHRIS GLEED-OWEN		2002	A
NN5	Y	M	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN6	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN7	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN8	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN9	Y		FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN10	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN11	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN12		F	FORDWICH	KENT	ENGLAND		LEIGH ANNE ISSAC	27-Sep	2002	A
NN13	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN14	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN15		M	FORDWICH	KENT	ENGLAND		LEIGH ANNE ISSAC	27-Jan	2002	A
NN16	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN17	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN18	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN19	Y	F	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN20	Y	M	FORDWICH	KENT	ENGLAND	51°17'36.02"N, 1° 7'34.03"E	LEIGH ANNE ISSAC	27-Sep	2002	A
NN21	Y		TOWN COMMON, Nr CHRISTCHURCH	DORSET	ENGLAND	50°46'10.60"N, 1°50'3.18"W	CHRIS GLEED-OWEN		2002	A
NN22	Y		MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	DAVE BIRD		2002	A
NN23			BATH RUGBY CLUB GROUND		ENGLAND		SHARON PILINGTON		2002	A
NN24			ANGSKAR		SWEDEN		JOHN BAKER		2002	A
NN25	Y		DUDDLE HEATH	DORSET	ENGLAND	50°43'19.02"N, 2°22'6.93"W	CHRIS GLEED-OWEN	17-Sep	2002	A
NN26	Y		MONMOUTH	MONMOUTHSHIRE	WALES	51°48'51.13"N, 2°42'46.52"W	JERRY LEWIS		2002	A
NN27			MASTERS PIT	DORSET	ENGLAND		DAVE BIRD		2002	A
NN28	Y		WAREHAM COMMON	DORSET	ENGLAND	50°41'16.16"N, 2° 7'26.78"W	CHRIS GLEED-OWEN	06-Jul	2002	A
NN29	Y		SOUTH PARLEY	DORSET	ENGLAND	50°46'43.09"N, 1°51'30.62"W	TONY PHELPS	03-Jul	2002	A
NN30			DONCASTER	YORKSHIRE	ENGLAND				1960	A
NN31			STUDLAND HEATH, PURBECK	DORSET	ENGLAND		CHRIS GLEED-OWEN		2003	A
NN32	Y		CAMP HEATH	SUFFOLK	ENGLAND	52°12'52.33"N, 0°54'25.91"E	STEPH R. YOUELL	09-Jul	2002	A
NN33	Y		TATCHELLS QUARRY	DORSET	ENGLAND	50°42'39.47"N, 2° 6'35.12"W	CHRIS GLEED-OWEN		2004	A
NN34	Y		STUDLAND HEATH, PURBECK	DORSET	ENGLAND	50°38'30.37"N, 1°57'10.13"W	RHYS JONES		2004	A
NN35	Y		SOLIHULL	BIRMINGHAM	ENGLAND	52°24'52.95"N, 1°46'27.47"W	DAVID BRADLEY		2004	A
NN36	Y		KIRBY MOOR	LINCOLNSHIRE	ENGLAND	53° 3'2.20"N, 0°10'16.77"W	DAVE BROMWICH	27-Jul	2002	A
NN37	Y		OUISNÉ COMMON		JERSEY	49°10'25.65"N, 2°11'1.66"W	RICHARD GRIFFITHS		2004	A
NN38	Y		HINDHEAD COMMON	SURREY	ENGLAND	51° 6'1.64"N, 0°44'13.16"W	JEMMA FAIRCHILD		2002	A
NN39	Y		NOON HILL, VERWOOD	DORSET	ENGLAND	50°52'43.10"N, 1°51'37.75"W	CHRIS GLEED-OWEN		2002	A
NN40			WENTWOOD FOREST	NEWPORT	WALES		RHYS JONES	11-Aug	2002	A
NN41	Y	M	DONCASTER	YORKSHIRE	ENGLAND	53°31'22.95"N, 1° 8'1.54"W			1960	A
NN42			TOWN COMMON, Nr CHRISTCHURCH	DORSET	ENGLAND		CHRIS GLEED-OWEN		2002	A
NN43	Y	J	FURZEBROOK	DORSET	ENGLAND	50°39'13.79"N, 2° 5'55.01"W	TONY PHELPS		2002	J
NN44	Y	M	FURZEBROOK	DORSET	ENGLAND	50°39'13.79"N, 2° 5'55.01"W	TONY PHELPS		2002	A
NN45	Y	F	FURZEBROOK	DORSET	ENGLAND	50°39'13.79"N, 2° 5'55.01"W	TONY PHELPS		2002	A

CODE	SEQ	SEX	LOCATION	REGION	COUNTRY	GRID REFERENCE	COLLECTOR	DATE	YEAR	AGE
NN47	Y		STAMFORD	YORKSHIRE	ENGLAND	53°59'26.09"N, 0°54'49.31"W				
NN48	Y		STAMFORD	YORKSHIRE	ENGLAND	53°59'26.09"N, 0°54'49.31"W				
NN50			MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	TONY PHELPS		2003	
NN51			MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	TONY PHELPS		2003	
NN52			MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	TONY PHELPS		2003	
NN53	Y		LLANFOIST, Nr ABERGAVENNY		WALES	51°48'46.91"N, 3°2'12.46"W	RHYS JONES		2005	
NN54			MASTERS PIT	DORSET	ENGLAND		TONY PHELPS		2003	
NN55	Y		MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	TONY PHELPS		2003	
NN56			HALLEN, Nr BATH		ENGLAND		HYDER		2005	
NN57	Y		HALLEN, Nr BATH		ENGLAND	51°31'6.90"N, 2°38'56.31"W	RHYS JONES		2005	
NN58		EGG	DURHAM		ENGLAND		TERRY COULT		2002	
NN59	Y		LLANISHAN, CARDIFF	S.GLAM	WALES	51°31'45.47"N, 3°11'21.87"W	RICHARD COWIE		2005	
NN60		F	FLEET	HAMPSHIRE	ENGLAND		TONY PHELPS		2003	
NN61			ST MELLONS, CARDIFF	S. GLAM	WALES		MATT PICARD		2005	
NN62			BRIDGEND	S.GLAM	WALES		RHYS JONES		2006	
NN63			ST MELLONS, CARDIFF	S. GLAM	WALES		MATT PICARD		2005	
NN64			LLANISHAN, CARDIFF	S.GLAM	WALES		RHYS JONES		2004	
NN66			ALGERIA		ALGERIA		DAVID DONAIRE		2004	
NN67			ANDALUTHIA		SPAIN		DAVID DONAIRE		2004	
NN68			DURHAM (1)		ENGLAND				2002	
NN69			DURHAM (2)		ENGLAND				2002	
NN70	Y		NN02/0035 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
NN71	Y		NN02/0081 SERRA DO GERÊS		PORTUGAL		ARMANDO LOUREIRO		2002	A
NN72			NN02/0097 SERRA DO GERÊS		PORTUGAL		ARMANDO LOUREIRO		2002	A
NN73	Y		NN02/0108 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
NN74	Y		NN02/0263 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
NN75	Y		NN03/0144 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
NN76	Y		NN03/0164 SERRA DO GERÊS		PORTUGAL		ARMANDO LOUREIRO		2002	A
NN77	Y		NN98/009 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
NN78	Y		NN99/0011 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
NN80I	Y		RE00006 MONTIANO	MONTIANO	ITALY	42°38'45.68"N, 11°13'28.92"E	STEFANO MAZZOTTI		2002	A
NN81I			RE00105 AIRPORT, FERRARA	FERRARA	ITALY		STEFANO MAZZOTTI		2002	A
NN82I			RE00121 RAVENNATE	RAVENNA	ITALY		STEFANO MAZZOTTI		2002	A
NN83I	Y		RE00125 FERRARA	FERRARA	ITALY	44°50'16.42"N, 11°37'13.56"E	STEFANO MAZZOTTI		2002	A
NN84I	Y		RE00150 FERRARESE	FERRARA	ITALY	44°58'9.40"N, 11°41'59.16"E	STEFANO MAZZOTTI		2002	A
NN85I			RE00160 LAGO BARGETANA	LIGONCHIO	ITALY		STEFANO MAZZOTTI		2002	A
NN1927A	Y		CARDIFF	S.GLAM	WALES	51°31'45.47"N, 3°11'21.87"W	RHYS JONES		1927	A
NND	Y		STUDLAND HEATH, PURBECK	DORSET	ENGLAND	50°38'30.37"N, 1°57'10.13"W	RHYS JONES		2003	A
NN90G	Y			ATHENS	GREECE	37°58'45.05"N, 23°42'59.93"E	DEMITRIS POSANTZIS		2005	A
NN96C	Y		SLAVONIJA		CROATIA	45° 5'1.75"N, 14°54'6.90"E	ZORAN TADIC		2006	A
NN97C	Y		SLAVONIJA		CROATIA	45° 5'1.75"N, 14°54'6.90"E	ZORAN TADIC		2006	A
NN98C	Y		SLAVONIJA		CROATIA	45° 5'1.75"N, 14°54'6.90"E	ZORAN TADIC		2006	A
NN100M	Y		SRBIJA I CRNA GORA		MONTENEGRO	43°12'34.97"N, 19° 0'36.87"E	LIDIJA POLOVIC		2006	A
NNAMS1	Y		AMSTERDAM		NETHERLANDS	52°21'58.86"N, 4°53'51.78"E	PIETER DE WIJER		2007	A
NNAMS2	Y		AMSTERDAM		NETHERLANDS	52°21'58.86"N, 4°53'51.78"E	PIETER DE WIJER		2007	A
NNAMS4	Y		AMSTERDAM		NETHERLANDS	52°21'58.86"N, 4°53'51.78"E	PIETER DE WIJER		2007	A
NNAMS5	Y		AMSTERDAM		NETHERLANDS	52°21'58.86"N, 4°53'51.78"E	PIETER DE WIJER		2007	A
NNAMS6	Y		AMSTERDAM		NETHERLANDS	52°21'58.86"N, 4°53'51.78"E	PIETER DE WIJER		2007	A
NNAMS7	Y		AMSTERDAM		NETHERLANDS	52°21'58.86"N, 4°53'51.78"E	PIETER DE WIJER		2007	A

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NNAMS8	Y		AMSTERDAM		NETHERLANDS	52°21'58.86"N, 4°53'51.78"E	PIETER DE WIJER		2007	A
NNA1POL	Y		GRZEDY VILLAGE, BIEBRZA RIVER	BIEBRZA	POLAND	53°29'7.91"N, 22°36'48.08"E	JAN WOJCIK		2005	A
NNA3POL	Y		GRZEDY VILLAGE, BIEBRZA RIVER	BIEBRZA	POLAND	53 30 N, 22 36 E	JAN WOJCIK		2005	A
NNA4POL	Y		GRZEDY VILLAGE, BIEBRZA RIVER	BIEBRZA	POLAND	53 29 N, 22 35 E	JAN WOJCIK		2005	A
NNASWIS	Y		FMNH 1602 BASEL		SWITZERLAND	47°33'34.61"N, 7°34'50.20"E	ALAN RESETAR FMNH		2004	A
NNAFRANCE	Y		FMNH 25025 PARIS		FRANCE	46°51'24.00"N, 2°21'3.55"E	ALAN RESETAR FMNH		2004	A
NNGWYN	Y		CRICCIETH	GWYNEDD	WALES	52°55'10.27"N, 4°14'2.86"W	DOREEN LINDSEY		2006	A

CODE	HAP	SEQ	SEX	LOCATION	REGION	COUNTRY	GRID REFERENCE	COLLECTOR	DATE	YEAR	AGE
Coronella austriaca											
CA1											
CA2	1	Y		MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	DAVE BIRD		2002	A
CA3				TOWN COMMON	DORSET	ENGLAND	50°36'32.97"N, 1°57'41.53"W	CHRIS GLEED-OWEN	22-Aug	2002	A
CA4	1	Y		NORTH WEST WOOLSBARROW	DORSET	ENGLAND	50°44'24.35"N, 2°10'28.09"W	CHRIS GLEED-OWEN	12-Aug	2002	A
CA5	1	Y	M	DUDDLE HEATH	DORSET	ENGLAND	50°43'19.02"N, 2°22'6.93"W	CHRIS GLEED-OWEN	17-Sep	2002	A
CA6				MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	TONY PHELPS	11-Jul	2002	A
CA7	1	Y		MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	TONY PHELPS		2002	A
CA8	1	Y		TOWN COMMON	DORSET	ENGLAND	50°36'32.97"N, 1°57'41.53"W	CHRIS GLEED-OWEN	10-Aug	2002	A
CA9				GALLOWS HILL	DORSET	ENGLAND		CHRIS GLEED-OWEN	16-Aug	2002	A
CA10	1	Y		DUDDLE HEATH	DORSET	ENGLAND	50°43'19.02"N, 2°22'6.93"W	CHRIS GLEED-OWEN	17-Sep	2002	A
CA11				DUDDLE HEATH	DORSET	ENGLAND		CHRIS GLEED-OWEN		2002	J
CA12				MASTERS PIT	DORSET	ENGLAND		TONY PHELPS		2002	A
CA13				NOON HILL, VERWOOD	DORSET	ENGLAND		TONY PHELPS	23-May	2002	A
CA14			F	FURZEBROOK	DORSET	ENGLAND		TONY PHELPS		2002	A
CA15			F	FURZEBROOK	DORSET	ENGLAND		TONY PHELPS		2002	A
CA16	1	Y	F	FURZEBROOK	DORSET	ENGLAND	50°39'13.79"N, 2°5'55.01"W	TONY PHELPS		2002	A
CA17			M	FURZEBROOK	DORSET	ENGLAND	50°39'13.79"N, 2°5'55.01"W	TONY PHELPS		2002	A
CA18			F	FURZEBROOK	DORSET	ENGLAND		TONY PHELPS		2002	A
CA19				CRANSBORNE COMMON	DORSET	ENGLAND		TONY PHELPS		2003	A
CA20	1	Y		MASTERS PIT	DORSET	ENGLAND	50°42'43.94"N, 2°13'4.33"W	TONY PHELPS		2003	A
CA21				CRANSBORNE COMMON	DORSET	ENGLAND		TONY PHELPS		2003	A
CA22	1	Y		WHITLEY HEATH	SURREY	ENGLAND	51°23'16.69"N, 0°37'44.96"W	TONY PHELPS		2004	A
CA23				CADFUR (FURZEBROOK)	DORSET	ENGLAND		TONY PHELPS		2002	J
CA24	1	Y		CADSTO	DORSET	ENGLAND	50°38'30.37"N, 1°57'10.14"W	TONY PHELPS		2002	J
CA25				BILBOA		SPAIN	43°15'25.07"N, 2°55'24.39"W	DAVID DONAIRE		2004	A
CA26				SEOUA DE NOROBNUDO	MENOZ DE TOUROO	SPAIN		DAVID DONAIRE		2004	A
CA27				SUR AL PICO DEL ESCUDO,	CANTABRIA	SPAIN	43°6'32.27"N, 4°0'52.69"W	DAVID DONAIRE		2004	A
CA28		SCAT	M	WAREHAM FOREST	DORSET	ENGLAND		TONYPHELPS		2003	A
CA29			F	HARTLAND, PURBECK	DORSET	ENGLAND	50°39'30.71"N, 2°5'5.17"W	RHYS JONES		2003	A
CA30I				FMNH		BOHEIMIA	49°51'55.98"N, 13°34'11.33"E	ALAN RESETAR FMNH		2003	A
CA31I				RE0007 SALA	CESENATICO	ITALY		STEFANO MAZZOTTI		2002	A
CA32I				RE0046 MIRANDOLA	MIRANDOLA	ITALY		STEFANO MAZZOTTI		2002	A
CA33I				RE00147 SUCCISO INFERIORE	RAMISETO	ITALY	44°22'5.50"N, 10°11'55.55"E	STEFANO MAZZOTTI		2002	A
CA34				RE00158 BOLOGNESE	BOLOGNA	ITALY	44°28'58.42"N, 11°20'17.00"E	STEFANO MAZZOTTI		2002	A
CA35				CS 02-0388 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
CA36				CS 03-0137 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
CA37				CS 03-0138 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
CA38				CS 03-0148 SERRA DO GERÊS		PORTUGAL	41°39'1.21"N, 8°11'3.57"W	ARMANDO LOUREIRO		2002	A
CA39	3	Y		ZEGREB		CROATIA	45°48'25.50"N, 15°57'52.69"E	ZORAN TADIC		2005	A
CA40	3	Y		ZEGREB		CROATIA	45°48'25.50"N, 15°57'52.69"E	ZORAN TADIC		2005	A
CA41	3	Y		HEATHLAND	DAAZ WOLFHESE	NETHERLANDS	52°20'22.64"N, 5°41'9.92"E	ANNEMARIE VAN DIEPENBEEK	24-Aug	2007	A
CA42	3	Y		HEATHLAND	DAAZ WOLFHESE	NETHERLANDS	52°20'22.64"N, 5°41'9.92"E	ANNEMARIE VAN DIEPENBEEK	24-Aug	2007	J
CA43				HEATHLAND	DAAZ WOLFHESE	NETHERLANDS	52°20'22.64"N, 5°41'9.92"E	ANNEMARIE VAN DIEPENBEEK	06-Aug	2007	J
CA44	3	Y		ZEGREB		CROATIA	45°48'25.50"N, 15°57'52.69"E	ZORAN TADIC		2005	A

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Vipera berus						
GB j10 Czech republic	1	(DQ186057)	Hradec Králové	Czech Republic	50°12'33.93"N, 15°49'57.45"E	B
GB j14 Czech republic	1	(DQ186058)	Hraničná	Czech Republic	50°21'7.71"N, 12°28'18.53"E	B
GB j12 Switzerland	1	(DQ186043)	Lngalma, Uppsala Län	Sweden	60° 6'37.44"N, 17°38'32.85"E	B
GB j13 Germany	1	(DQ186037)	Fulda	Germany	50°33'14.63"N, 9°40'28.17"E	B
GB j16 Norway	1	(DQ186047)	Ertvågøy	Norway	63°11'19.85"N, 8°26'52.46"E	B
GB j2 France	1	(DQ186034)	Massif Central	France	47°46'57.01"N, 7°30'55.27"E	B
GB J6 France	1	(DQ186035)	Paimpont	France	48° 1'16.96"N, 2°10'17.45"W	B
GB j17 Sweden	1	(DQ186041)	Mora	Sweden	61° 0'23.59"N, 14°32'15.19"E	B
GB j11 Sweden	1	(DQ186046)	Grebbestad	Sweden	58°41'28.30"N, 11°15'34.95"E	B
GB Germany	1	(AJ275719)		Germany	51° 9'56.77"N, 10°27'5.52"E	D
GB j23 Russia	2	(DQ186050)	St Petersburg Prov	Russia	59°57'6.84"N, 30°27'17.32"E	B
GB j25 Russia	2	(DQ186052)	Altai	Russia	52°32'46.62"N, 82°31'33.90"E	B
GB j20 Finland	2	(DQ186048)	Karhujrvi	Finland	66°40'47.11"N, 27°14'18.22"E	B
GB j21 Finland	2	(DQ186049),	Hmeenlinna	Finland	60°59'45.65"N, 24°27'51.32"E	B
GB j22 Finland	2	(DQ186044)	Polvijärvi	Finland	62°51'17.18"N, 29°21'50.32"E	B
GB j29 Romania	3	(DQ186061),	Oradea	Romania	47° 3'39.38"N, 21°55'39.59"E	B
GB j30 Romania	3	(DQ186062)	Chirui	Romania	46° 16' 0" N, 25° 29' 0" E	B
GB j3 Austria	4	(DQ186036)	Vorarlberg	Austria	47°13'5.04"N, 9°53'3.29"E	B
GB j5 Swis	4	(DQ186032)	Uri	Switzerland	46°45'37.16"N, 8°40'39.46"E	B
GB j32 Swis	5	(DQ186067)	Pontresina	Switzerland	46°29'40.30"N, 9°54'3.44"E	B
GB j33 Italy	5	(DQ186069)	Pontebba	Italy	46°30'15.67"N, 13°18'20.66"E	B
GB j40 Austria	6	(DQ186064)	Puchberg a. Schneeberg	Austria	47°47'13.94"N, 15°54'48.06"E	B
GB j39 Austria	6	(DQ186065),	Zederhaus	Austria	47° 9'12.12"N, 13°30'23.27"E	B
GB j36 Slovenia	6	(DQ186071	Krma	Slovenia	46°25'48.50"N, 13°56'25.28"E	B

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GB j41Bosnia	7	(DQ186076),	Treskavica	Bosnia	43°34'2.34"N, 18°20'41.87"E	B
GB j44 Montenegro	7	(DQ186073),	Bjelasica mountain	Montenegro	42°53'23.28"N, 19°38'1.57"E	B
GB j45 Serbia	8	(DQ186074),	Kopaonik mountain	Serbia	43°14'58.93"N, 20°14'57.03"E	B
GB j46 Serbia	8	(DQ186075)	Stara Planina mt."	Serbia	44°21'47.90"N, 19°15'10.39"E	B
GB j49 Russia	9	(DQ186055),	Khabarovsky Kroy	Russia	54°23'22.71"N, 136°37'21.00"E	B
GB j50 Russia	9	(DQ186054)	Yuzhno-Sakhalinsk	Russia	46°57'40.54"N, 142°43'55.13"E	B
GB j1 Swis	11	(DQ186031)	Vallee de Joux	Switzerland	46°36'43.97"N, 6°14'9.08"E	B
GB j15 Denmark	13	(DQ186045)	Fyns Hoved	Denmark	55°23'20.86"N, 10°22'42.71"E	B
GB j19 Estonia	14	(DQ186042)	Hiiumaa	Estonia	58°53'24.59"N, 22°33'13.92"E	B
GB j27 Poland	15	(DQ186059)	Ustrzyki Dolne	Poland	49°25'49.17"N, 22°35'39.25"E	B
GB j28 Slovakia	16	(DQ186060)	Vihorlat Mountain	Slovakia	49°16'25.29"N, 21°54'12.15"E	B
GB j35 Italy	17	(DQ186066)	Ugorizza	Italy	46°13'1.74"N, 12°59'17.77"E	B
GB j37 Italy	18	(DQ186070)	Val Cimoliana	Italy	46°11'34.51"N, 12°45'14.58"E	B
GB j38 Solvenia	19	(DQ186072)	Jelendol	Solvenia	45°40'5.68"N, 14°45'46.42"E	B
GB j8 Wales A	20	(DQ186039)	Bangor	Wales	53°13'34.81"N, 4° 8'2.05"W	B
GBFrance	21	(AY321091)		France	46°13'41.66"N, 2°12'47.64"E	E
GB Russia	22	(AJ275728)	Sachalin	Russia	50° 8'4.96"N, 142°42'42.54"E	D

CODE	HAP	ACCESSION	REGION	COUNTRY	GRID REFERENCE	PAPER
Natrix natrix						
GBGermany	1	(AY487745)		Germany	51° 9'56.49"N, 10°27'5.49"E	C
GBFrance	1	(AY866537)		France	46°13'39.50"N, 2°12'49.50"E	C
GBKent	1	(AY866544)	Kent	England	52°50'48.27"N, 2°17'52.36"W	C
GB NSpain	2	(AY866536)	north eastern	Spain	41°40'9.49"N, 2°11'39.66"W	C
GB SSpain	2	(AY866535)	Southern	Spain	38°15'39.15"N, 4°18'13.03"W	C
GB Portugal 1	2	(AY487728)		Portugal	39°46'34.22"N, 8° 7'12.06"W	C
GB Portugal 2	2	(AY487724)		Portugal	39°46'34.22"N, 8° 7'12.06"W	C
GB Russia Penza	3	(AY487754)	Penza Region	Russia	53°12'3.35"N, 45° 0'37.52"E	C
GB Russia Rybachy 1	3	(AY487740)	Rybachy	Russia	55° 9'47.75"N, 20°50'23.22"E	C
GB Russia Rybachy 2	3	(AY487741)	Rybachy	Russia	55° 9'47.75"N, 20°50'23.22"E	C
GB Russia Samara	3	(AY487753)	Samara Region	Russia	53° 8'21.43"N, 50° 5'52.53"E	C
GB Kazakhstan1	3	(AY487750)	Emba River	Kazakhstan	48°50'19.21"N, 58° 7'52.60"E	C
GB Kazakhstan2	3	(AY487749)	Emba River	Kazakhstan	48°50'19.21"N, 58° 7'52.60"E	C
GB Turkey 1	3	(AY487730)	Yenicaga	Turkey	40°46'19.56"N, 32° 1'58.44"E	C
GB Turkey 2	3	(AY487726)	Sarkale	Turkey	39°58'42.79"N, 32°47'56.17"E	C
GB Turkey 4	3	(AY487723)	Sarkale	Turkey	39°58'42.79"N, 32°47'56.17"E	C
GB Armenia	3	(AY866543)		Armenia	40° 4'8.76"N, 45° 2'17.48"E	C
GB Slovenia 1	4	(AY487738)		Slovenia	46° 9'4.47"N, 14°59'43.67"E	C
GB Solvenia 2	4	(AY487739)		Slovenia	46° 9'4.47"N, 14°59'43.67"E	C
GB Tula Russia	5	(AF471059)	Tula Region	Russia	54°12'43.39"N, 37°36'32.35"E	F
GB Bulgaria	6	(AY866542)		Bulgaria	42°44'1.98"N, 25°29'8.99"E	C
GB Romaina	7	(AY866540)		Romania	45°54'9.55"N, 24°58'24.59"E	C
GB Hungary 1	7	(AY487752)		Hungary	47° 9'43.64"N, 19°30'17.96"E	C
GB Hungary 2	7	(AY487732)		Hungary	47° 9'43.64"N, 19°30'17.96"E	C
GB Denmark	8	(AY866539)		Denmark	56°15'50.11"N, 9°30'6.43"E	C
GB Sweden	8	(AY487755)	Smaland	Sweden	57° 4'17.24"N, 14°54'43.47"E	C
GB Germany 5	8	(AY487727)	Lake Constance	Germany	47°46'24.08"N, 8°50'18.78"E	C

CODE	HAP	ACCESSION	REGION	COUNTRY	GRID REFERENCE	PAPER
GB Germany (W)	9	(AY866538)	Western	Germany	50°53'6.38"N, 7°25'35.88"E	C
GB Greece 1	12	(AY487747)	Peloponnes	Greece	37°39'4.80"N, 21°34'31.53"E	C
GB Greece 3	12	(AY487743)	Golf of Arta	Greece	39° 9'39.43"N, 20°59'31.51"E	C
GB Greece 4	12	(AY487742)	Golf of Arta	Greece	39° 9'39.43"N, 20°59'31.51"E	C
GB Greece 5	12	(AY487725.)	Ioannina	Greece	39°39'21.44"N, 20°50'3.21"E	C
GB Georgia1	13	(AY487736)	Batumi	Georgia	41°39'6.27"N, 41°39'22.28"E	C
GB Georgia 2	13	(AY487737)	Batumi	Georgia	41°39'6.27"N, 41°39'22.28"E	C
GB Hungary 3	14	(AY487731)	Szeged	Hungary	46°15'11.52"N, 20° 8'43.70"E	C
GB Armenia 1	15	(AY487735)		Armenia	40° 4'8.76"N, 45° 2'17.48"E	C
GB Greece 2	16	(AY487746)	Peloponnes	Greece	37°39'4.80"N, 21°34'31.53"E	C
GB Spain1	17	(AY487734)	Extremadura	Spain	39°12'48.43"N, 6° 5'40.99"W	C
GB Italy 2	18	(AY487733)		Italy	41°52'18.26"N, 12°33'57.32"E	C
GB Italy 3	18	(AY487729)		Italy	41°52'18.26"N, 12°33'57.32"E	C
GB Italy1	19	(AY487744)		Italy	41°52'18.26"N, 12°33'57.32"E	C

Coronella austriaca

CODE	HAP	ACCESSION	REGION	COUNTRY	GRID REFERENCE	PAPER
GBTURKEY	10	(AY486930)		Turkey	39°58'42.79"N, 32°47'56.17"E	A
GBSP1	2	(EU022665)	Tendi Valley, Asturias	Spain		G
GBPO1	5	(EU022669)	Pitoes	Portugal		G
GBPO2	19	(EU022662)	Serra da Estrela	Portugal		G
GBPO3	19	(EU022663)		Portugal		G
GBPO4	18	(EU022664)	Serra da Estrela	Portugal		G
GBPO5	19	(EU022668)		Portugal		G
GBSP2	4	(EU022666)	Covanera, Burgos	Spain		G
GBSP3	4	(EU022667)	Sedano, Burgos	Spain		G
GBSP4	15	(EU022675)	Riopar, Albacete	Spain		G
GBSP5	16	(EU022674)	Montes de Toledo, Toledo	Spain		G
GBSP6	20	(EU022672)	Espinero, Segovia	Spain		G
GBSP7	14	(EU022673)	Sierra de Guadarrama, Mad	Spain		G
GBFR1	9	(EU022649)	Tigouleix, Creuse	France		G
GBGB1	1	(EU022648)	Hampshire	England		G
GBFR2	1	(EU022650)	Merindal, Creuse	France		G
GBA1	1	(EU022651)	Au, Vorarlberg	Austria		G
GBG1	8	(EU022647)	Peak Kazarma, Kardhista	Greece		G
GBI1	3	(EU022646)	Treviso	Italy		G
GBR1	17	(EU022642)	Kalininskiy, Rostov	Russia		G
GBR2	17	(EU022643)	Volkonschino, Penza	Russia		G
GBR3	17	(EU022644)	Matveevskiy, Rostov	Russia		G
GBR4	17	(EU022645)	Vysha, Republic of Mordovia	Russia		G
GBI2	11	(EU022670)	Etna, Adrano, Sicily	Italy		G
GBI3	13	(EU022671)	Etna, Saifio, Sicily	Italy		G
GBFR3	7	(EU022654)	Canigou	France		G

CODE	HAP	ACCESSION	REGION	COUNTRY	GRID REFERENCE	PAPER
GBSP8	12	(EU022652)	Sierra Nevada, Granada	Spain		G
GBSP9	12	(EU022653)	Sierra Nevada, Granada	Spain		G
GBSP10	6	(EU022655)	Canejan, Lleida	Spain		G
GBSP11	6	(EU022656)	Arguijo, Soria	Spain		G
GBSP12	6	(EU022657)	Puerto Piqueras, Soria	Spain		G
GBSP13	6	(EU022658)	Puertos de Beceite, Castell	Spain		G
GBSP14	6	(EU022659)	Valle de Anso, Navarra	Spain		G
GBSP15	6	(EU022660)	Lasarte, Alava	Spain		G
GBSP16	6	(EU022661)	Escoriaza, Alava	Spain		G

Publications supplying mitochondrial cytochrome *b* gene sequences used in Chapter 3.

A Nagy, Z.T., Lawson, R., Joger, U. and Wink, M. (2004). Molecular systematics of racers, whipsnakes and relatives (Reptilia: Colubridae) using mitochondrial and nuclear markers. *Journal of Zoological Systematics and Evolutionary Research* 42, 223-233.

B Ursenbacher, S., Carlsson, M., Helfer, V., Tegelstrom, H. and Fumagalli, L. (2006). Phylogeography and Pleistocene refugia of the adder (*Vipera berus*) as inferred from mitochondrial DNA sequence data. *Molecular Ecology* 15, 3425-3437.

C Guicking, D., Lawson, R., Joger, U. and Wink, M. (2006). Evolution and phylogeny of the genus *Natrix* (Serpentes: Colubridae). *Biological Journal of the Linnean Society of London* 87, 127-143.

D Lenk, P., Kalyabina, S., Wink, M. and Joger, U. (2001). Evolutionary relationships among the true vipers (Reptilia: Viperidae) inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 19, 94-104.

E Garrigues, T., Dauga, C., Ferquel, E., Choumet, V. and Failloux, A.B. (2005). Molecular phylogeny of *Vipera Laurenti*, 1768 and the related gene *Macrovipera* (Reuss, 1927) and Daboia (Gray, 1842), with about neurotoxic *Vipera aspis aspis* populations. *Molecular Phylogenetics and Evolution* 35, 35-47.

F Lawson, R., Slowinski, J.B., Crother, B.I. and Burbrink, F.T. (2005). Phylogeny of the Colubroidea (Serpentes): New evidence from mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution* 37, 581-601.

G Santos, X., Roca, J., Pleguezuelos, J.M., Donaire, D., Carranza, S. (2008). Biogeography and evolution of the Smooth snake *Coronella austriaca* (Serpentes: Colubridae) in the Iberian Peninsula: evidence for Messinian refuges and Pleistocenic range expansions. *Amphibia-Reptilia* 29, 35-47.

Appendix II

Appendix II lists sequences (as haplotypes) used to construct phylogenies for *Vipera berus*, *Natrix natrix* and *Coronella austriaca* in Chapter 3 of this project.

Natrix natrix

(265bp cyt *b*; 114 sequences, 19 Haplotypes; 2 unique to this project)

NN= Project sequence GB=Gen Bank sequence

Haplotype 1 (62)

ATTTTTTATCTGTATCTATACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCGGGAACCACCTTACTAATTATCCTTAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACAGTAATTACAAATCTCCTAACTGCCGTACCCTACCTAGGAAAC
ACCCTCACAACCTGACTCTGGGGAGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

GBGermany (AY487745), GBFrance (AY866537), GBKent (AY866544), NNAFRANCE (Chicago Field Museum 25025); Switzerland (1) NNASWIS (Chicago Field Museum 1602); Wales (5) NN1927, NNGWYN, NN26, NN53, NN59; England (39) NND, NN2, NN3, NN4, NN5, NN6, NN7, NN8, NN9, NN10, NN11, NN13, NN14, NN16, NN17, NN18, NN19, NN20, NN21, NN22, NN25, NN28, NN29, NN32, NN33, NN34, NN35, NN36, NN38, NN39, NN41, NN43, NN44, NN45, NN47, NN48, NN55, NN57; Netherlands (7) NNAMS1, NNAMS2, NNAMS4, NNAMS5, NNAMS6, NNAMS7, NNAMS8; Jersey (1) NN37JERSEY; Italy (1) NN80I; Poland (2) NNPOL1, NNPOL3; Portugal (3) NN70P, NN77P, NN78P

Haplotype 2 (9)

ATTCTTCATCTGCATTTACACCCACATTGCACGTGGACTTTATTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCAGGAACCACCCTACTAATTATCCTTAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCATTTTGA
GCAGCAACAGTAATTACTAACCTCCTAACGGCCGTACCTTACCTAGGAAAC
ACCCTCACAACCTGACTCTGGGGAGGATTCTCAATTAATGACCCTACCTTAA
CCCGA

Spain (2) GB NSpain(AY866536), GB SSpain (AY866535); Portugal (7) GB Portugal 1 (AY487728), GB Portugal 2 (AY487724), Portugal NN71P, NN73P, NN74P, NN75P, NN76P

Haplotype 3 (11)

ATTTTTCATCTGCATCTACACCCACATCGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCAGGAACCACCCTACTAATCATCCTCA
TAGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTG
AGCAGCAACAGTAATTACAAACCTCCTAACTGCCGTACCCTATCTAGGAAA
CACCCTCACAACCTGACTCTGAGGGGGGTTCTCAATTAATGACCCAACCTTA
ACCCGA

Russia (4) GB Russia Penza (AY487754), GB Russia Rybachy 1 (AY487740), GB Russia Rybachy 2 (AY487741), GB Russia Samara (AY487753); Kazakstan (2) GB Kazakhstan1 (AY487750), GB Kazakhstan 2 (AY487749), Turkey (3) GB Turkey 1 (AY487730), GB Turkey 2 (AY487726), GB Turkey 4 (AY487723); Armenia (1) GBArmenia (AY866543); Poland (1) NNPoland4

Haplotype 4 (7)

ATTTTTTATCTGCATCTACACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCAGGAACCACCTTACTCATTATCCTAAT
AGCCACGGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACCGTAATTACAAACCTCCTAACTGCCGTACCCTACCTAGGAAAT
ACCCTCACAACCTGACTCTGGGGGGGATTCTCAATCAATGACCCAACCTTAA
CCCGA

Slovenia (2) GB Slovenia 1 (AY487738), GB Slovenia 2 (AY487739); Italy (2) NN83I, NN84I; Croatia (3) NN96C, NN97C, NN98C

Haplotype 5 (1)

ATTTTTCATCTGCATCTACACCCACATCGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCAGGAACCACCCTACTAATCATCCTCA
TAGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTG
AGCAGCAACAGTAATTACAAATCTCCTAACTGCCGTACCCTATCTAGGAAA
CACCCTCACAACCTGACTCTGAGGGGGGTTCTCAATTAATGACCCAACCTTA
ACCCGA

Russia (1) GB Tula Russia (AF471059)

Haplotype 6 (1)

ATTTTTTATCTGCATCTACACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCGGGAACCACCCTACTAATTATCCTCA
TAGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTG
AGCAGCAACAGTAATTACAAACCTCCTAACTGCCGTACCCTATCTAGGAAA
CACCCTCACAACCTGACTCTGGGGGGGATTCTCAATTAATGACCCAACCTTA
ACCCGA

Bulgaria (1) GB Bulgaria (AY866542)

Haplotype 7 (3)

ATTTTTTATCTGCATCTACACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCAGGAACCACCTTACTCATTATCCTAAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACCGTAATTACAAACCTCCTAACTGCCGTACCATACCTAGGAAAT
ACCCTCACAACCTGACTCTGGGGGGGATTCTCAATCAATGACCCAACCTTAA
CCCGA

Romaina (1) GB Romaina (AY866540); Hungary (2) GB Hungary 1 (AY487752), GB Hungary 2 (AY487732)

Haplotype 8 (3)

ATTTTTTATCTGCATCTACACCCATATTGCACGTGGACTTTACTATGGCTCCT
ACCTTAACAAAGAAGTATGACTATCAGGAACAACCCTACTAATTATCCTTAT
AGCCACAGCATTCTTCGGCTATGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCGACAGTAATTACAAACCTCCTAACTGCCGTACCCTACCTAGGAAAC
ACCCTCACAACCTGACTCTGAGGGGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Denmark (1) GB Denmark (AY866539); Sweden (1) GB Sweden (AY487755); Germany (1) GB Germany 5(AY487727)

Haplotype 9 (1)

ATTTTTTATCTGTATCTATACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCGGGAACCACCTTACTAATTATCCTTAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACAGTAATTACAAATCTCCTAACTGCCGTACCCTACCTAGGAAAC
ACCCTCACAACCTGACTCTGGGGAGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Germany (1) GB Germany (W) (AY866538)

Haplotype 10 (1)

ATTTTTTATCTGCATCTACACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCAGGAACCACCTTACTCATTATCCTAAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACCGTAATTACAAACCTCCTGACTGCCGTACCCTACCTAGGAAAC
ACCCTCACAACCTGACTCTGAGGGGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Montenegro (1) NN100M

Haplotype 11 (1)

ATTTTTTATCTGCATCTACACCCATATTGCACGTGGACTTTACTATGGCTCCT
ACCTTAACAAAGAAGTATGACTATCAGGAACAACCCTACTAATTATCCTTAT
AGCCACAGCATTCTTCGGCTATGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCGACAGTAATTACAAACCTCCTAACTGCCGTACCCTACCTGGGAAAC
ACCCTCACAACCTGACTCTGAGGGGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Greece (1) NN90G

Haplotype 12 (4)

ATTTTTTATCTGCATCTACACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCAGGAACCACCTTACTCATTATCCTAAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACCGTAATTACAAACCTCCTGACTGCCGTACCCTACCTAGGAAAC
ACCCTCACAACCTGACTCTGGGGGGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Greece (4) GB Greece 1 (AY487747), GB Greece 3 (AY487743), GB Greece 4 (AY487742), GB Greece3 (AY487725.)

Haplotype 13 (2)

ATTTTTCATCTGCATCTACACCCACATCGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTTTGACTATCAGGAACCACCCTACTAATCATCCTCAT
AGCCACAGCATTCTTCGGCTATGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACAGTAATTACAAACCTCCTAACTGCCGTACCCTATCTAGGAAAC
ACCCTCACAACCTGACTCTGAGGGGGGTTCTCAATTAATGACCCAACCTTAA
CCCGA

Georgia (2) GB Georgia1 (AY487736), GB Georgia 2 (AY487737)

Haplotype 14 (1)

ATTTTTTATCTGCATCTACACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCAGGAACCACCTTACTCATTATCCTAAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACCGTAATTACAAACCTCCTAACTGCCGTACCATACCTAGGAAAT
ACCCTCACAACCTGACTCTGAGGAGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Hungary (1) GB Hungary 3 (AY487731)

Haplotype 15 (2)

ATTTTTCATCTGCATCTACACCCACATCGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCAGGAACCACCCTACTAATCATCCTCA
TAGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTG
AGCAGCAACAGTAATTACAAACCTCCTAACTGCCGTACCCTATCTAGGAAA
CACCCTCACAACCTGACTCTGGGGGGGGTTCTCAATTAATGACCCAACCTTA
ACCCGA

Armenia (2) GB Armenia1 (AY487735), GB Armenia2 (AY866543)

Haplotype 16 (1)

ATTTTTTATCTGCATCTACACTCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTGTGACTATCAGGAACCACCTTACTCATTATCCTAAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACCGTAATTACAAACCTCCTGACTGCCGTACCTTACCTAGGAAAC
ACCCTCACAACCTGACTCTGGGGGGGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Greece (1) GB Greece 2 (AY487746)

Haplotype 17 (1)

ATTCTTCATCTGCATTTACACCCACATTGCACGTGGACTTTATTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCAGGAACCACCCTACTAATTATCCTTAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCATTTTGA
GCAGCAACAGTAATTACTAATCTCCTAACGGCCGTACCTTACCTAGGAAAC
ACCCTCACAACCTGACTCTGGGGAGGATTCTCAATTAATGACCCTACCTTAA
CCCGA

Spain (1) GBSpain1 (Extremadura) (AY487734)

Haplotype 18 (2)

ATTTTTTATCTGTATCTATACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCGGGAACCACCTTACTAATTACCCTCA
TAGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTG
AGCAGCAACAGTAATTACAAATCTCCTAACTGCCGTACCCTACCTAGGAAA
CACCCTCACAACCTGACTCTGGGGGGGATTCTCAATTAATGACCCAACCTTA
ACCCGA

Italy (2) GB Italy 2 (AY487733), GB Italy 3 (AY487729)

Haplotype 19 (1)

ATTTTTTATCTGTATCTATACCCACATTGCACGTGGACTTTACTATGGCTCCT
ACCTAAACAAAGAAGTATGACTATCGGGAACCACCTTACTAATTATCCTCAT
AGCCACAGCATTCTTCGGCTACGTCCTCCCATGAGGACAAATATCCTTTTGA
GCAGCAACAGTAATTACAAATCTCCTAACTGCCGTACCCTATCTAGGAAAC
ACCCTCACAACCTGACTCTGGGGGGGATTCTCAATTAATGACCCAACCTTAA
CCCGA

Italy (1) GB Italy1 (AY487744)

Appendices

Vipera berus

(434bp cyt b; 112 sequences; 26 Haplotypes; 7 unique to this project)

VB= Project sequence GB= Gen Bank sequence

Haplotype 1 (68)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Netherlands (1) GB j9 (DQ186038.); Slovakia (1) GB j18 (DQ186056.); Czech republic (2) GB j10 (DQ186057), GB j14 (DQ186058); Switzerland (1) GB j12 (DQ186043); Germany (2) GB j13 (DQ186037), GBGermany (AJ275719); Norway (1) GB j16 (DQ186047); France (2) GB j6 (DQ186035), GB j2 (DQ186034); Sweden (10) GB j17 (DQ186041), GB j11 Sweden (DQ186046), VB105, VB106, VB107, VB108, VB109, VB110, VB111, VB114 Sweden; Wales (10) VB1, VB122, VB123, VB124, VB125, VB126, VB127, VB128, VB129, VB133; England (37) VB3, VB4, VB6, VB8, VB9, VB10, VB11, VB13, VB17, VB19,VB22, VB23, VB24, VB25, VB26, VB27, VB28, VB29, VB30, VB31, VB32, VB33, VB35, VB73, VB81, VB89, VB92, VB93, VB99, VB101, VB116, VB117, VB118, VB120, VB131, VB132, VB134; Scotland (1) VB86

Haplotype 2 (5)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTCGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Russia (2) GB j23 Russia (St Petersburg Prov) (DQ186050), GB j25 Russia (Altai) (DQ186052); Finland (3) GB j20 Finland (DQ186048), GB j21 Finland (DQ186049), GB j22 Finland (DQ186044)

Haplotype 3 (2)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTAACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Romaina (2) GB j29 Romania (DQ186061), GB j30 Romania (DQ186062)

Haplotype 4 (2)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCTTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Austria (1) GB j3 Austria (DQ186036); Switzerland (1) GB j5 Swis (DQ186032)

Haplotype 5 (2)

CTTAGCCTTCTCATCCATTGTCCACATCACCCGGGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTACTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAATTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACTAACCTCCTAACAGCAGTCCCATACCTTGGGACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAATGATCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Switzerland (1) GB j32 Swis (DQ186067); Itlay (1) GB j33 Italy (Pontebba) (DQ186069)

Haplotype 6 (3)

CCTAGCCTTCTCATCCATTGTCCACATCACCCGGGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTATTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTACTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAATTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACTAACCTCCTAACAGCAGTCCCATACCTTGGGACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAACGATCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Austria (2) GB j40 Austria (DQ186064), GB j39 Austria (DQ186065); Slovenia (1) GB j36 Slovenia (DQ186071)

Haplotype 7 (3)

CTTAGCTTTCTCATCCATTGTCCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTACTTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGAGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTGACAGCAATCCCATACCTTGGAACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Bosnia (1) GB j41Bosnia (DQ186076); Montenegro (2) GB j44 Montenegro (DQ186073), Montenegro VB166

Haplotype 8 (2)

CTTAGCTTTCTCATCCATTGTCCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTACTTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGAGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTGACAGCAATCCCATACCTTGGAACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATGTCCTTC
TCCTTCACAACGAAGGCT

Serbia (2) GB j45 Serbia (DQ186074), GB j46 Serbia (DQ186075)

Haplotype 9 (2)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTTGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Russia (2) GB j49 Russia (Khabarovsky Kroy) (DQ186055), GB j50 Russia (Yuzhno-Sakhalinsk) (DQ186054)

Haplotype 10 (4)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTGGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

England (3) VBD (Dorset), VB14 (Dorset), VB88 (Dorset); Wales (1) VB18 (Gower)

Haplotype 11 (2)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAGG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Sweden (2) VB112, VB113

Haplotype 12 (1)

CTTAGCCTTCTCATCCATTGTTCACATAACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCTTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Switzerland (1) GB j1 (DQ186031)

Haplotype 13 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATATCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Denmark (1) GB j15 (DQ186045)

Haplotype 14 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTCCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTCGGAACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Estonia (1) GB j19 (DQ186042)

Haplotype 15 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTATTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAT
TGTGAGGTGGCTTCTCCATCAATGACCCGACTCTAACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Poland (1) GB j27 (DQ186059)

Haplotype 16 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAT
TGTGAGGTGGCTTCTCCATCAATGACCCGACTCTAACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Slovakia (1) GB j28 (DQ186060)

Haplotype 17 (1)

CCTAGCCTTCTCATCCATTGTCCACATCACCCGGGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTATTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTACTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAATTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAAATATCATTTTGAGCAGCAACAGTAATC
ACTAACCTCCTAACAGCAGTCCCATACCTTGGGACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAACGATCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Italy (1) GB j35 (DQ186066)

Haplotype 18 (1)

CTTAGCCTTCTCATCCATTGTCCACATCACCCGGGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTACTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAATTACCCTCATAGCAACAGCTTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACTAACCTCCTAACAGCAGTCCCATACCTTGGGACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAATGATCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Italy (1) GB j37 (DQ186070)

Haplotype 19 (1)

CCTAGCCTTCTCATCCATTGTCCACATCACCCGGGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTATTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTACTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAATTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGGCAGATATCATTTTGAGCAGCAACAGTAATC
ACTAACCTCCTAACAGCAGTCCCATACCTTGGGACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAACGATCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT
Slovenia (1) GB j38 (DQ186072)

Haplotype 20 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGAGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGTAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT
Wales (1) GB j8 Wales A (DQ186039)

Haplotype 21 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGAGGACAAATATCATTCTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT
France (1) GBFrance (AY321091)

Haplotype 22 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TCTGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTTGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

Russia (1) GB Russia (Sachalin) (AJ275728)

Haplotype 23 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGGCAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

England (1) VB130 (New forest, England)

Haplotype 24 (1)

CTTAGCCTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ACTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTAGTCACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGGGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTAACAGCAATCCCATACCTTGGGACCACCTTAACCACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATATCCTTC
TCCTTCACAACGAAGGCT

England (1) VB155Nott (Nottingham, England)

Haplotype 25 (1)

CTTAGCTTTCTCATCCATTGTCCACATCTCCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTATCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTACTTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGAGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTGACAGCAATCCCATACCTTGGAACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATGTCCTTC
TCCTTCACAACGAAGGCT

Croatia (1) VB153C

Haplotype 26 (4)

CTTAGCTTTCTCATCCATTGTTCACATCACCCGAGATGTCCCATACGGTTGA
ATTATACAAAACTCACACGCCATCGGCGCATCCTTGTTTTTCATATGTACCT
ACACACACATTGCACGAGGCCTCTACTATGGGTCCTATTTAAATAAAGAAG
TATGACTATCAGGCACTACCCTCCTACTTACCCTCATAGCAACAGCCTTCTT
CGGCTATGTTCTTCCATGAGGACAGATATCATTTTGAGCAGCAACAGTAATC
ACCAACCTCCTGACAGCAATCCCATACCTTGGAACCACCTTAACTACCTGAC
TGTGAGGTGGCTTCTCCATCAATGACCCAACTCTGACCCGATTCTTCGCCCT
TCACTTCATCCTCCCATTCACTATTATCTCAATATCCTCCATCCATGTCCTTC
TCCTTCACAACGAAGGCT

Croatia (4) VB150C, VB151C, VB152C, VB153C

Coronella austriaca

(141bp cyt b; 50sequences; 20 Haplotypes; 1 unique to this project)

CA= Project sequence GB= Gen Bank sequence

Haplotype 1 (13)

TCCATATTCTTCATCTGCATTTATATCCATATCGCACGCGGCTTATACTACGG
GTCGTACTTAAATAAAAACGTCTGACTTTCAGGAACTACACTACTAATTATT
CTAATAGCGACAGCCTTTTTCGGCTATGTACTACCC

Austria (1) GBA1 (EU022651); England (11) GBGB1 (EU022648), CA1, CA3, CA4, CA6, CA7, CAD-STO, CA9, CA15, CA19, CA21-Surrey; France (1) GBFR2 (EU022650)

Haplotype 2 (1)

TCCATATTCTTCATCTGCATTTACATCCATATCGCACGTGGCTTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTATCAGGGACCACTCTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGGACTACCC

Spain (1) GBSP1 (EU022665)

Haplotype 3 (6)

TCCATATTCTTCATCTGCATTTACATCCATATCGCACGCGGGTTATACTATGG
GTCATACCTAAATAAAAACGTATGGCTTTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Croatia (3) CA38, CA39, CA43; Italy (1) GBI1 (EU022646); Netherlands (2) CA40 CA41

Haplotype 4 (2)

TCCATGTTCTTCATCTGCATTTACGTCCATATCGCACGTGGCTTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTATCAGGGACCACTCTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Spain (2) GBSP2 (EU022666), GBSP3 (EU022667)

Haplotype 5 (1)

TCCATATTCTTCATCTGCATTTACATCCATATCGCACGTGGCTTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTATCAGGGACCACTCTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Portugal (1) GBPO1 (EU022669)

Haplotype 6 (7)

TCCATATTTTTCATCTGCATTTACATCCACATCGCACGTGGCTTATACTATGG
GTCATACCTAAATAAAAACGTCTGACTCTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Spain (7) GBSP10 (EU022655), GBSP11 (EU022656), GBSP12 (EU022657), GBSP13 (EU022658), GBSP14 (EU022659), GBSP15 (EU022660), GBSP16 (EU022661)

Haplotype 7 (1)

TCCATATTTTTCATCTGCATTTACATCCACATCGCACGTGGCTTATACTATGG
GTCATACCTAAATAAAAACGTCTGACTCTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTGCTACCC

France (1) GBFR3 (EU022654)

Haplotype 8 (1)

TCCATGTTCTTTATCTGCATTTACATCCACATCGCACGTGGATTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTTTCAGGAACCACACTGCTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Greece (1) GBG1 (EU022647)

Haplotype 9 (1)

TCCATATTCTTCATCTGCATTTATATCCATATCGCACGCGGCTTATACTACGG
GTCGTACTTAAATAAAAACGTCTGACTTTCAGGAACTACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

France (1) GBFR1 (EU022649)

Haplotype 10 (1)

TCCATATTCTTCATCTGCATTTACATCCACATCGCACGTGGATTATACTACG
GGTCGTACCTAAATAAAAACGTTTGACTTTCAGGAACCACACTACTAATTAT
TCTAATAGCAACAGCCTTTTTCGGCTATGTCCTACCA

Turkey (1) GBturkey (AY486930)

Haplotype 11 (1)

TCCATATTCTTCATCTGCATCTATATCCATGTCGCACGTGGCTTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTTTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTGCTACCC

Italy (1) GBI2 (EU022670)

Haplotype 12 (2)

TCCATATTTTTCATCTGCATTTATATCCACATCGCACGTGGCTTATACTACGG
GTCATACCTAAATAAAAACGTCTGACTCTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Spain (2) GBSP8 (EU022652), GBSP 9 (EU022653)

Haplotype 13 (1)

TCCATATTCTTCATCTGCATCTATATCCATGTCGCACGTGGCTTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTTTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Italy (1) GBI3 (EU022671)

Haplotype 14 (1)

TCAATATTCTTTATCTGCATTTACATCCATATCGCACGTGGTCTATACTACGG
GTCATACCTAAATAAAAACGTCTGACTTTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTGCTACCC

Spain (1) GBSP7 (EU022673)

Haplotype 15 (1)

TCAATATTCTTTATCTGCATTTACATCCATATCGCACGTGGCCTATACTACGG
GTCATACCTAAATAAAAACGTCTGACTTTCAGGAACCGCACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGTTATGTGCTACCC

Spain (1) GBSP4 (EU022675)

Haplotype 16 (1)

TCAATATTCTTTATCTGCATTTACACCCATATCGCACGTGGCCTATACTACG
GGTCGTACCTAAATAAAAACGTCTGACTTTCAGGAACCACACTACTAATTAT
TCTAATAGCAACAGCCTTTTTCGGCTATGTGCTACCC

Spain (1) GBSP5 (EU022674)

Haplotype 17 (4)

TCCATATTCTTCATCTGTATTTACATCCATATCGCACGCGGGTTATACTATGG
ATCCTACCTAAATAAAAACGTCTGACTTTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTGCCC

Russia (4) GBR1 (EU022642), GBR2 (EU022643), GBR3 (EU022644), GBR4 (EU022645)

Haplotype 18 (1)

TCCATGTTCTTCATCTGCATTTACATCCATATCGCACGTGGCTTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTATCAGGGACCACCCTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Portugal (1) GBPO4 (EU022664)

Haplotype 19 (3)

TCCATGTTCTTCATCTGCATTTACATCCATATCGCACGTGGCTTATACTATGG
GTCGTACCTAAATAAAAACGTCTGACTATCAGGGACCACTCTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTACTACCC

Portugal (3) GBPO2 (EU022662), GBPO3 (EU022663), GBPO5 (EU022668)

Haplotype 20 (1)

TCAATATTCTTTATCTGCATTTACATCCATATCGCACGTGGTCTATACTACGG
GTCGTACCTAAATAAAAACGTCTGACTTTCAGGAACCACACTACTAATTATT
CTAATAGCAACAGCCTTTTTCGGCTATGTGCTACCC

Spain (1) GBSP6 (EU022672)

Appendix III

Appendix III presents ariel photographs of the Donor sites (outlined in yellow) and Receptor sites (outlined in red) used for data collection in Chapter 4.

Figure A3.1 Ariel photograph of Donor site 1 (outlined in yellow) used for data collection in Chapter 4

Figure A3.2 Ariel photograph of Donor site 2 (outlined in yellow) used for data collection in Chapter 4.

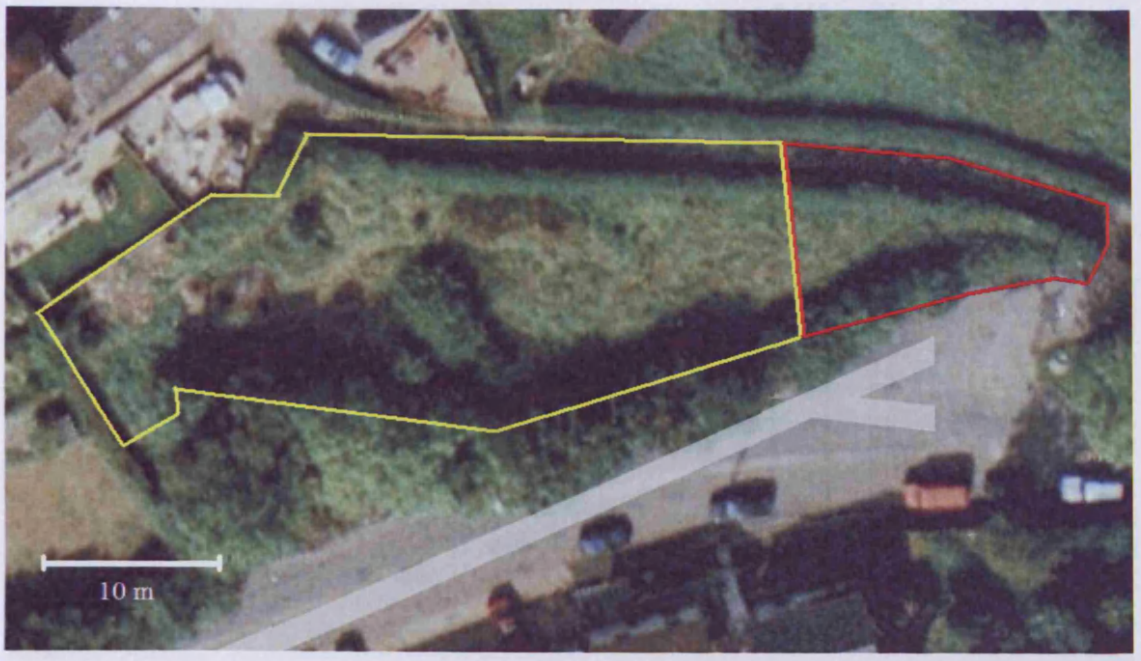


Figure A3.3 Ariel photograph of Donor site 3 (outlined in yellow) and Receptor site 3 (outlined in red) used for data collection in Chapter 4.

Figure A3.4 Ariel photograph of Receptor site 1 (outlined in red) used for data collection in Chapter 4.

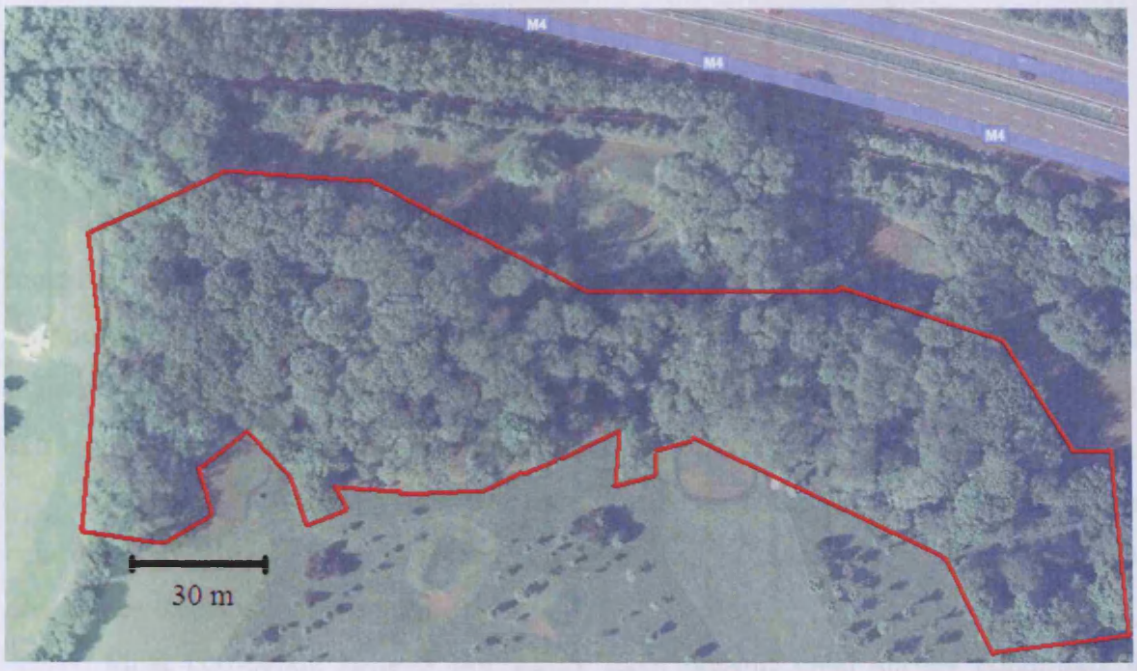


Figure A3.5 Ariel photograph Receptor site 2 (outlined in red) used for data collection in Chapter 4.

Appendix IV

Ideal weather conditions for slow worm capture

Slow worms are ectothermic animals reliant on ambient environmental temperatures to regulate their body temperature. In addition, slow worms are thigmotherms preferring to raise body temperatures through conduction rather than radiation. For this reason slow worms can be encountered occupying roofing felt refugia at a higher frequency than the native heliothermic reptiles.

Site visits to recover animals should only be undertaken under ideal weather conditions. However, there is currently a great deal of ambiguity when defining what constitutes 'ideal weather conditions'.

Most ecologists believe hot sunny days present ideal survey conditions for reptiles and will abandon capture attempts at the first sign of rain. However, rain can present excellent opportunities to recover reptiles, especially slow worms. Firstly, slow worms will attempt to stay dry and reduce temperature loss by sheltering under roofing felt refugia during light rain. In addition, the lizard's main mollusc prey becomes increasingly active under such conditions escalating recovery of hunting reptiles.

Slow worms aim to maintain an average body temperature of 23°C and hence on days in excess of this temperature there is a decreased rate of animal recovery. Also, when summer temperature exceeds ideal body temperatures for a prolonged period, slow worms will retire underground for extended periods and will, on occasion, undertake aestivation. Consideration of such behaviour is necessary prior to reporting a site free of reptile activity.

Finally, absence of evidence is not evidence of absence as native British reptiles remain highly cryptic within their environment. In conclusion, an ideal survey day would be conducted in late spring or early autumn during ambient temperatures of around 20°C in over cast dry conditions.

Appendix V

Artificial hibernacula construction

To ensure reptile welfare, sufficient over-wintering facilities had to be constructed at each of the receptor sites. On completion of receptor site surveys, routes were cleared to allow light plant (3.5-16 tonne mini diggers) access to sites.

Firstly, areas were identified at each site that were suitable for hibernacula construction. This was qualified by available construction dimensions, elevation, percentage vegetation cover, height above the water table, available sunlight and aspect with regards to southerly direction. All hibernacula requirements were evaluated onsite with the height of water table assessed from hydrology data and onsite scrapes.

Figure A4.1 The author supervising a 16 tonne digger as it excavates the base of the hibernacula at the receptor site.

Reprofiling and compaction of the receptor site's north facing bank had left a section of the receptor side devoid of vegetation and other features attractive to reptiles. A 16 tonne digger was able to excavate a ~10 m x 3 m hibernacula foundation strip to a depth of 1 m (see Figure A4.1). The entire site was examined for potential filler materials with additional rocks and rubble imported as necessary.

Figure A4.2 The author directs the placement of tree roots at the south facing edge of the hibernacula entrance.

Figure A4.3 The hibernacula foundation is filled with rocks (left) to a height of 1-2 m and then insulated with a 1 m layer of recycled branches and roots (right).

An access tunnel was facilitated by the placement of substantial recycled tree root systems at the south facing edge of the structure (see Figure A4.2). This tunnel connected to the centre of the structure leading approximately 1 m outward from the south side of the hibernacula. Diggers assisted in filling the excavated areas to a height of ~2 m from their base. Piling rocks and rubble to this height creates thermal pockets

suitable for reptile brumation. The rock layer was then covered with tree roots and recycled wood in the form of cut branches and brash. A blanket layer of bracken was laid on top of the wood to protect the structure from filling with soil (see Figure A4.3).

Figure A4.4 Completed hibernacula showing exposed root system access tunnels.

Approximately 1 m of soil was piled on the bracken layer to encapsulate the entire structure in earth. This soil was recovered from the excavated area and hence would assist in the vegetation of the hibernacula from the local seed bank it contained. The soil was gently compacted using the bucket of the digger to maintain structural integrity. Root systems were deliberately left exposed to allow natural passage through to the heart of the over wintering facility (see Figure A4.4).

Figure A4.5 Receptor site enhancement through the introduction of stone and log piles.

Further enhancement of the site was carried out through the inclusion of stone and log piles (see Figure A4.5). Both of these kinds of structures are attractive to reptiles with the latter an important feature for insects. This ensures the receptor site will encourage sufficient prey resources for the translocated insectivorous reptiles.

Figure A4.6 The finished enhanced site showing both hibernacula (to the right of the author), stone and log piles and arisings distributed across the site.

Finally, arisings recovered from the clearance of the bank prior to reprofiling were distributed over the entire site to maximise the vegetation of the area the following spring (see Figure A4.6). Over time the hibernacula will also vegetate and become relatively cryptic within the study site environment.

Appendix VI

Photographing slow worms

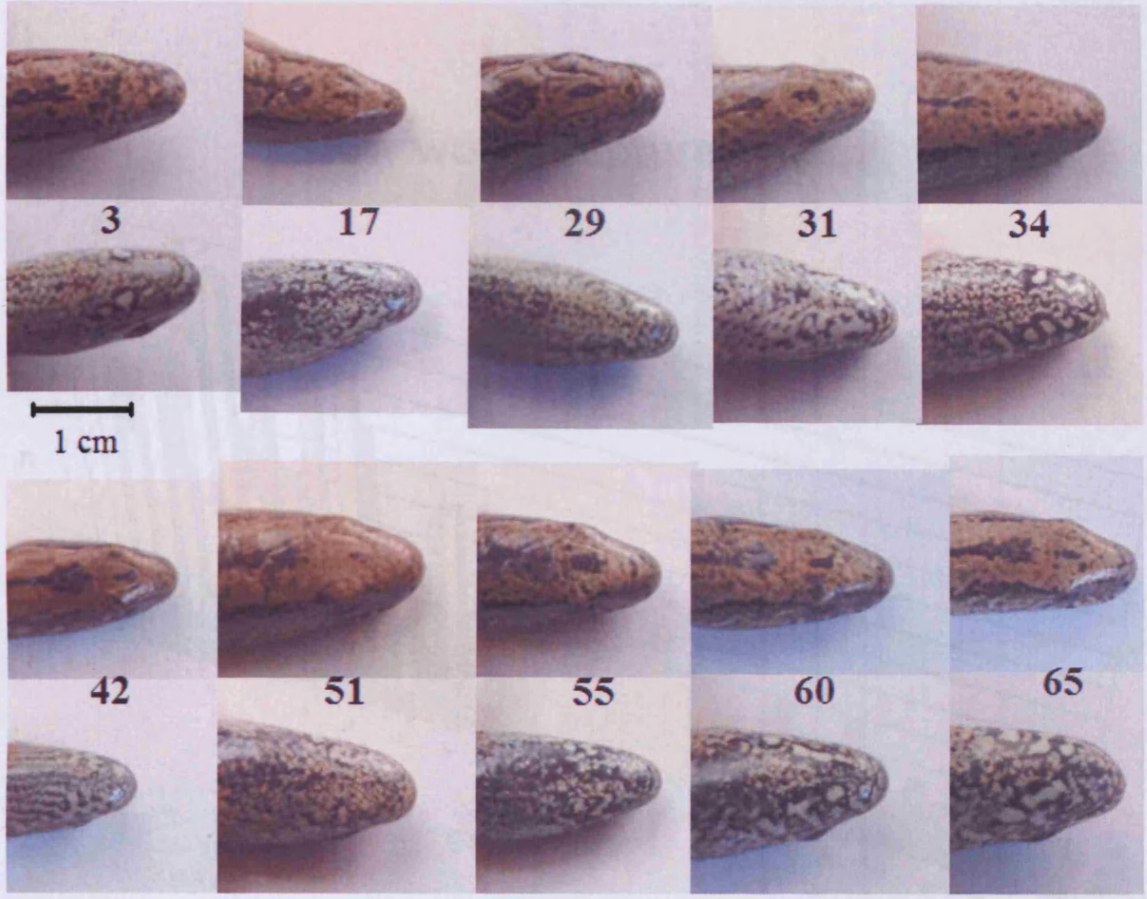
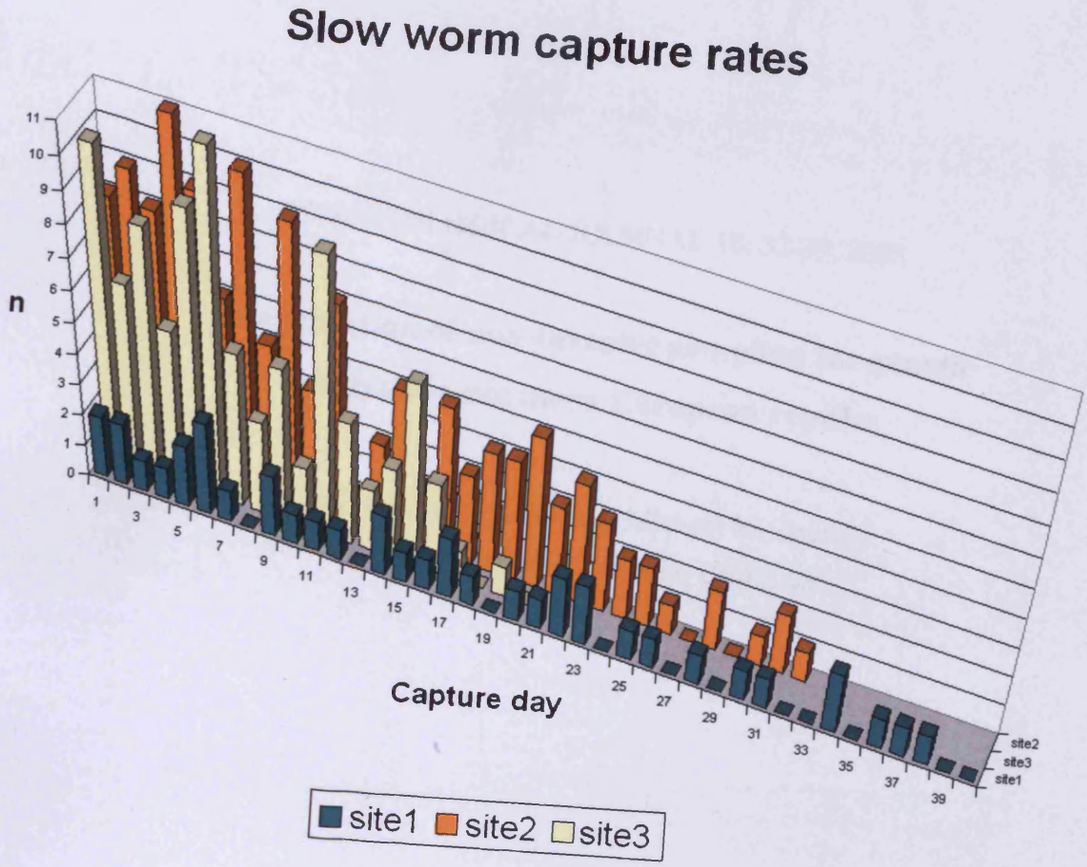


Figure A6.1 Dorsal (rows 1 and 3) and ventral (rows 2 and 4) photographs of adult female slow worms illustrating diversity in head markings. Photographs taken on 6 mega pixel Nikon camera using defused lighting.

Photographs were taken of both ventral and dorsal surfaces of each slow worm head to enable individual non-invasive identification. Photographs were taken on a 6 mega pixel Nikon camera which produced sufficient definition to differentiate between all collected lizards. Field photographs were taken in defused lighting conditions to avoid the glare of direct sunlight on reflective reptile scales. Such direct sunlight can mask fine discriminative markings essential for individual slow worm identification. Defused lighting was generally produced artificially using tissue paper screening of artificial light sources with photographs taken inside of vehicles. Alternatively, at remote access sites, photographs were taken whilst shielding the animal from direct sunlight through the position of the photographer. As technologies have advanced and become more affordable, projects are now being undertaken using 10 mega pixel images.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
site1	2	2	1	1	2	3	1	0	2	1	1	1	0	2	1	1	2	1	0	1	1	2	2	0	1	1	0	1	0	1	1	0	0	2	0	1	1	1	0	0
site2	8	9	8	11	9	6	10	5	9	4	7	1	3	5	2	5	3	4	4	5	3	4	3	2	2	1	0	2	0	1	2	1								
site3	10	6	8	5	9	11	5	3	5	2	9	4	2	3	6	3	1	0	1	0																				

Figure A7.1 Slow worm capture rates from Donor site 1 (Teal), Donor site 2 (Orange) and Donor site 3 (Cream) conducted over a 40, 32 and 20 day period respectively.

Publications

HERPETOLOGICAL JOURNAL 18: 32-39, 2008

An evaluation of non-invasive sampling for genetic analysis in northern European reptiles

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RPETOLOGICAL JOURNAL 18: 32-39, 2008

An evaluation of non-invasive sampling for genetic analysis in northern European reptiles

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Genetic studies of native herpetofauna populations are important for the conservation of European biodiversity, but previous studies have been largely dependent on invasive sample collection. Here we explore the efficiency of non-invasive sampling (NIS) for molecular studies and review the various potential sources of such samples. Snakes produce a multitude of by-products, such as sloughed skin, faeces and eggs or embryos, that, along with road kills, predated specimens and museum samples, could potentially be used in molecular studies. We describe a new method for obtaining snake faeces in the field and, using mitochondrial cytochrome *b* primers, we successfully amplified 500 and 758 bp sequences from a variety of tissues collected by NIS. The availability and degradation of such material differed greatly, and both DNA extraction and PCR success appeared dependent upon sample origin and storage. Nevertheless, for the first time we demonstrate that faecal, egg and foetal tissues, as well as sloughed skin and carcasses, represent valuable NIS source material permitting genetic studies with minimal disturbance to the individual and its population.

Key words: Coronella austriaca, mitochondrial DNA, *Natrix natrix*, snakes, *Vipera berus*

INTRODUCTION

The study of indigenous herpetofauna in the field is important for conservation and our understanding of reptile ecology in a changing landscape. Increasingly, prime reptile habitat in northern Europe is being modified, destroyed or fragmented (Beebee & Griffiths, 2000), with reptiles having to adapt to changes instigated by anthropogenic expansion. In general, translocation success rates for amphibians and reptiles are lower than those for mammals and birds (Griffith et al., 1989; Dodd & Seigel, 1991; Platenberg & Griffiths, 1999; Reinert & Rupert, 1999), and yet translocation exercises rarely employ genetic data. This is perhaps surprising, given the now seminal study of Madsen et al. (1999), who demonstrated unequivocally that the introduction of new genotypes into a severely inbred and isolated population of *Vipera berus* not only halted its precipitous decline towards extinction but resulted in dramatic population expansion. However, molecular ecological approaches are now more commonly used in the conservation of herpetofauna (e.g. Ciofi & Bruford, 1999; Madsen et al., 1999; Leaché & Reeder, 2002; Morrison & Scott, 2002) and cryptic snake taxa are being re-assigned following genetic analysis (Puorto et al., 2001; Burbrink, 2002; Wüster et al., 2002). Molecular characterization of individuals may also resolve novel insights into how genetic variation is partitioned within and among populations (e.g. Carlsson et al., 2004).

Invasively obtained material has tended to be the source for herpetological genetic studies to date (Keogh, 1998; Feldman & Spicer, 2002; Voris et al., 2002) but this is now less acceptable with the wide availability of commercial non-invasive sampling (NIS) DNA extraction kits. These offer affordable, alternative methods for molecular studies with minimal disturbance to the animal and its population (Morin & Woodruff, 1996). Below we summarize the various sources of NIS material that could potentially be utilized for molecular studies, concentrating on our target taxa, the three species indigenous to northern Europe: the adder (*Vipera berus*), the grass snake (*Natrix natrix*) and the smooth snake (*Coronella austriaca*).

1) Sloughed skin

Sloughs can be found fragmented or whole around hibernation sites, often entwined within gorse, bracken and other coarsely textured plants, and beneath tins or other such cover. With warm weather conditions, sloughs dry quickly in the field and can be folded into an envelope or sample bag on collection and stored dry.

2) Aborted embryo, egg, road kill and museum samples

Muscle is available for DNA extraction from semi-predated and stillborn carcasses, road kills and museum samples (Dallas et al., 2003). Stillborn offspring from both the adder and the smooth snake can be collected in the vicinity of hibernation sites. Grass snake eggs, laid from late June to July, are found in compost and manure heaps, decaying tree stumps, woodchip piles and even rotting seaweed. Around 30% of eggs fail to hatch in the autumn or are infertile (Beebee & Griffiths, 2000), therefore non-viable unfertilized eggs should only be collected after this period, being identifiable by their discoloured state. Although museums are a potential source of animal tissue, traditional specimen fixatives (commonly formalin) are optimized for morphological study, which degrades DNA (Chaw et al., 1980; Pääbo, 1989; Chang & Loew, 1994). Extracting DNA from formalin-fixed material is possible but laborious and PCR amplification success rates are low (Serth et al., 2000). The analysis of tissue stored in forma-

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Table 1. DNA extraction and PCR amplification success of 500 bp cytochrome *b* mtDNA products from non-invasively collected British snake samples (A.E.P.T. = Ancient Ethanol Preserved Tissue).

Species	Tissue type	No. of samples	Preservation method	No. of extracts	No. of PCRs	Positive PCR
Coronella austriaca	Slough	5	Dried	9	17	5
	Foetus	2	Ethanol	2	2	2
	Carcass	2	Ethanol	2	2	2
	Road kill	3	Ethanol	2	7	3
Natrix natrix	A.E.P.T	3	Ethanol (one stored for 50–100 y)	9	26	3
	Faeces	1	Ethanol	1	2	0
	Faeces	1	Frozen	1	2	1
	Slough	5	Dried (one fresh)	6	8	5
	Egg	2	Frozen	3	4	2
	Carcass	2	Ethanol	2	2	2
	Road kill	3	Frozen	4	5	3
Vipera berus	Faeces	1	Ethanol	1	2	0
	Faeces	1	Frozen	2	3	1
	Slough	5	Dried (one 2 y old)	8	15	5
	Foetus	2	Ethanol	2	2	2
	Carcass	2	Ethanol	2	2	2
	Road kill	2	Frozen	3	4	2
	Road kill	1	Ethanol	1	1	1

lin pH <7 for longer than 12 months should be restricted to analysis of short (<100–200 bp) DNA fragments (Bucklin & Allen, 2003).

3) Faeces

Faecal samples typically contain low quantities of degraded target DNA (Gerloff et al., 1995; Taberlet et al., 1999), but have proved to be a valuable source of DNA from avian and mammalian samples (e.g. Robertson et al., 1999; Taberlet et al., 1999; Regnaut et al., 2006) and should also be useful in herpetological studies. Snake faeces are challenging to obtain in the field non-invasively but can potentially be found throughout the snakes' active period from late April to October. As faecal material contains a range of micro-organisms and is particularly prone to deterioration by endogenous nucleases, the highest quality DNA is found in freshly collected faeces (Taberlet et al., 1999; Wehausen et al., 2004). Faecal matter gathered in the field should be immediately sealed and cooled in a collection bag. This can either be frozen at –20 °C or stored in ethanol (>95% molecular grade), RNALater (Ambion) or silica gel (Nsubuga et al., 2004) at 4 °C.

4) Cloacal and buccal swabs

Cloacal and/or buccal swabbing is a rapid, inexpensive and potentially easy to implement field method for obtaining reptile DNA samples (Miller, 2006). However, in addition to the delicate bone structure of British reptiles, there are obvious hazards associated with buccal swabbing from both venomous and non-venomous reptiles. Therefore, for both these reasons this procedure is not recommended for British snakes.

5) Teeth and bone

Teeth and bone samples, from semi-predated and still-born carcasses, shed teeth, road kill and museum samples, might yield DNA of sufficient quality for certain studies, but because of the time involved in sample preparation and the number of replicates required (see Wandeler et al., 2003; Rohland et al., 2004), they should only be considered in the absence of other more suitable tissues.

This study aimed to demonstrate the utility of a range of non-invasively collected samples for mitochondrial DNA PCR in the three native UK snake species.

MATERIALS AND METHODS

Origin of samples

Table I shows the preservation method of the samples collected during the current study. An additional 20 sloughs and 20 carcasses from *Vipera berus* were collected 1–30 days prior to DNA extraction. Field collected carcasses, road kills and foetal samples were directly frozen at –20°C or preserved in 95% ethanol. Ancient ethanol preserved tissue (A.E.P.T.; collected pre-1907 to 1969) consisted of museum samples donated by the National Museum of Wales, Cardiff. Non-viable grass snake eggs, located in compost heaps, were preserved at –20°C. Sloughed skins, mostly collected at the entrance of identified adder domains, were preserved dry at room temperature for up to two years. An additional fresh slough was obtained from an adder observed in the process of ecdysis. Snake faeces (associated with sloughs)

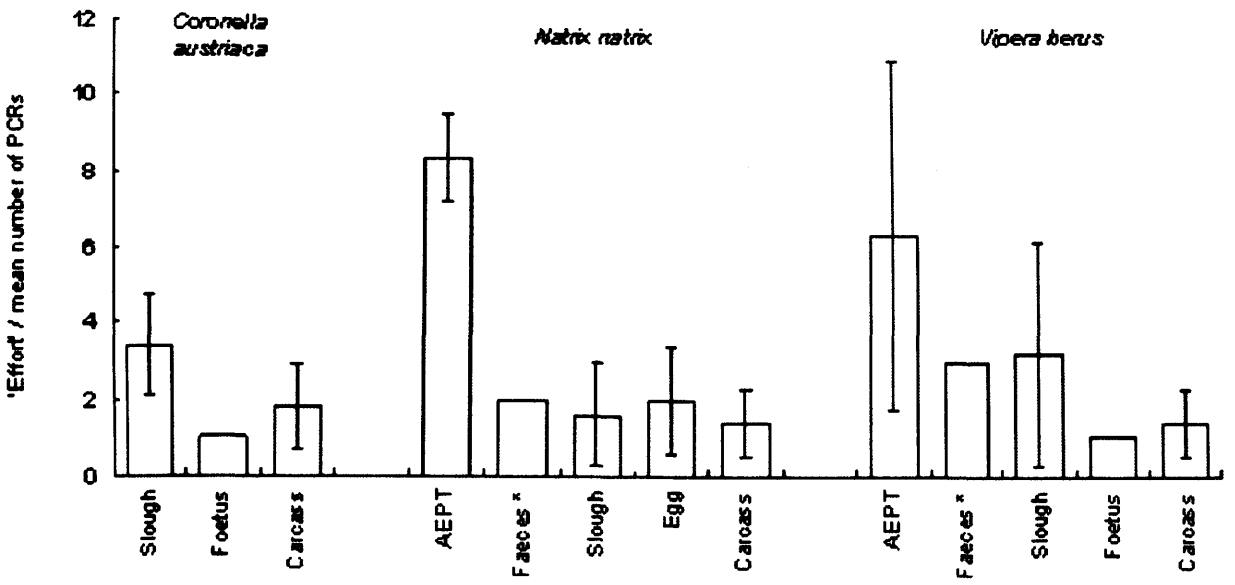

Fig. 1. Mean number (±SE) of PCR reactions required to generate a positive 500 bp partial cytochrome *b* gene amplification product from non-invasive snake sample material. Road kill and other carcasses are grouped together. *Error bars are not shown for the faecal samples as for both species only one of the two PCRs successfully amplified.

collected from a range of UK sites were either immediately frozen at –20°C or preserved in 95% ethanol. A 10 µl blood sample, obtained by caudal extraction from an adder and stored in 90 µl of Seutin's buffer (Seutin et al., 1991) at room temperature, was collected as a positive PCR control.

DNA extraction

Sloughed skin required a rehydration step to remove impurities prior to DNA extraction. A fragment (1–2 cm^2) of slough was placed in a 1.5 ml eppendorf tube containing 1 ml of double distilled (dd) water at 55 °C in a rocking incubator. After 4–6 h the water was removed and a further 1 ml of dd water added to each sample prior to incubation at 55 °C for a further 8–12 h. DNA extraction was performed on these rehydrated samples, egg yolks (approximately 0.2 cm^3), NIS muscle (1 cm^3) and blood (5 µl in Seutin's buffer) following the manufacturer's protocols for Qiagen DNeasy® (Cat. # 69504) tissue extraction kit. Faecal material (1 cm^3) was extracted following the manufacturer's protocol for the QIAamp® DNA stool mini kit (Cat. # 51504). DNA extraction was not attempted from teeth or bone in the current study but relevant protocols and commercial kits are available (see QIAamp® DNA minikit protocol). A maximum of three extraction attempts were performed for each sample, with second and third extractions only prepared on failure to successfully amplify a product after three PCR attempts from the previous extraction.

Partial cytochrome b gene amplification and sequencing

Two different snake PCR primer sets were used that generated approximately 500 and 758 bp amplicons. The first primer pair consisted of a forward primer (UKsnakecyto_F, 5' CAACATCAACTTAGCCTTCTC 3') adapted from cytochrome *b* primer, 703bot (Pook et al., 2000) and a reverse primer (UKsnakecyto_R, 5' GTGGAATGGGATTTTATCG 3') designed from an alignment of partial cytochrome *b* gene from *Vipera berus* (GenBank accession number AJ275728) and *Natrix natrix* (AF471059). The second primer set, 5' TCAAACATCTCAACCTGATGAAA 3' and 5' GGCAAATAGGAAGTATCATTCTG 3', were previously used by Pook et al. (2000) to generate a 758 bp cyt *b* fragment. Primer set 1 was tested on all tissue samples, whereas set 2 was only tested on the additional 20 sloughs and 20 carcasses from *Vipera berus*. Each PCR was performed in 25 ml comprising 1 ml of DNA, 1 × Invitrogen buffer (200 mM Tris-HCL, pH 8.4, 500 mM KCl), 3 mM $MgCl_2$, 250 mM of each dNTP, 0.5 mM of each primer and 1 U of Invitrogen *Taq* polymerase. DNA amplification was performed at 96 °C for 4 min and then 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min with a final extension cycle of 72 °C for 3 min in an Applied Biosystems GeneAmp® PCR system 9700 thermocycler. Both negative (dd water) and positive (adder blood) controls were included with each PCR. PCR success was determined by running products on an agarose gel. Fragment length of PCR product was determined by interpolation using a 100 bp ladder.

PCR products were purified using the GeneClean® Turbo for PCR kit. For each DNA sequencing reaction, 3 µl of PCR product, 2.5 µl Better Buffer (Webscientific Ltd, Cat. # 3BB-10), 0.5 µl ABI Big Dye Vs. 3 and 2.4 pmol of primer was made up to a final volume of 11 µl volume with deionised dd water. Sequencing of the isopropanol purified products was performed in both forward and reverse directions. Sequencing PCR entailed a step of 94 °C for 90 s followed by 25 cycles of 96 °C for 15 s, 50 °C for 10

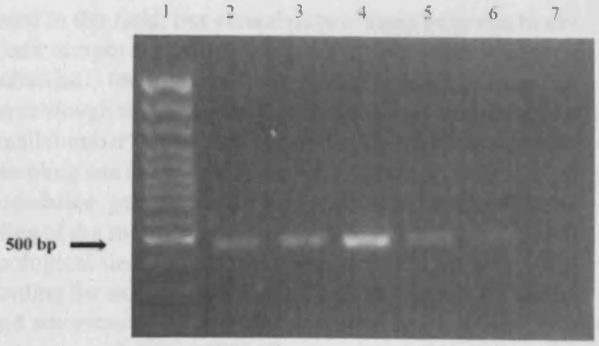

Fig. 2. Gel electophoresis of partial cytochrome *b* amplification products from *Vipera berus* and *Natrix natrix* non-invasively collected tissues and blood. Lane 1) 100 bp DNA size ladder; 2) blood; 3) sloughed skin; 4) carcass (road kill); 5) Ancient Ethanol Preserved Tissue (museum sample); 6) egg; 7) faeces.

s and 60°C for 4 min. Samples were run on an ABI 3100 DNA semi-automated DNA analyser (Perkin Elmer) and sequences were aligned using Sequencer™ and corrected by eye.

RESULTS

Faecal sample collection

Although potentially accessible, snake faeces are challenging to identify and rarely located in the field. During the course of this study, we developed a simple semi-invasive method of faeces collection following the observation that snakes often defecate in response to direct disturbance or handling. In a series of trials, individual snakes were carefully scooped from the ground in an upright motion whilst the tail was quickly transferred into a small plastic collection bag. As adders are venomous and have particularly delicate cervical vertebrae, extreme care is necessary when securing a hold on these animals and the mid-body should also be supported while the snake is held aloft and the tail placed into the collecting bag. However, unless suitably qualified we strongly advise the use of restraint tubing to secure the adder allowing safer handling. Repeated sampling (n=100) revealed that the majority of British snakes handled in this fashion would defecate in the bag, usually within 20 s of capture. This procedure was most reliable with grass snakes (about 95% defecation) compared to smooth snakes (about 75%) and adders (about 70%). These animals could then be released after photography, measurement and other details had been recorded. The sample bag was quickly cooled *in situ* before storage at –20 °C.

DNA extraction and amplification

Table 1 displays the success rates of extraction and amplification of the 500 bp mtDNA products from non-invasively collected sample material. These tissue types were sampled for all three British snake species with the exception of faeces and ancient ethanol preserved tissue (A.E.P.T.) for *Coronella austriaca*, which were not available in the current study. All different types of tissue samples eventually amplified and sequenced, but with varying rates of success both between tissue types and species (Fig. 1).

As predicted, the highest success rates for DNA extractions and amplification were obtained from recently preserved muscle samples, namely fresh foetus and other carcasses including road kill. Theses samples consistently produced the strongest intensity 500 bp fragment (based on comparison to 100 bp ladder luminosity) with an amplification success of 84% (*V. berus* 90%; *N. natrix* 86%; *C. austriacas* 81%). There was no obvious difference between ethanol and frozen samples, but sample sizes were low. High amplification success (70%) was also obtained using a second primer set that consistently generated 758 bp of *cyt b* sequence from 20 *V. berus* carcasses. Three DNA extractions from eggs generated faint 500 bp bands in two out of four PCRs (Table 1). Dried sloughs, including a sample stored for two years, amplified strong 500 bp products with an overall 61% amplification success rate (*V. berus* 64%; *N. natrix* 85%; *C. austriacas* 40%), although the brightness of this product was more intense from the fresh slough (Fig. 2). Surprisingly, 758 bp fragments were also obtained from 10 of 20 *V. berus* dried sloughs. From faeces, 500 bp fragments were amplified from one of five samples for both *V. berus* and *N. natrix*, but products were weak in intensity and the resulting sequences were of extremely poor quality. A.E.P.T. snake samples required up to three extractions before positive amplifications were achieved in five of the six samples tested (Table 1), with only 41% and 11% amplification success rate for *V. berus* and *N. natrix*, respectively.

Sequences of 500 bp with very few or no ambiguous bases were obtained from all tissue types, apart from faeces. The 758 bp *cyt b* fragment was successfully sequenced from *V. berus* carcasses and sloughs; sequencing of this larger amplicon was not attempted from other tissues types. Interspecific sequence homology allowed alignment of partial *cyt* b sequences from all three species and yet sufficient base substitution existed to reliably determine and identify sequences at species level. BLAST searches revealed that all sequences generated during the current study matched with GenBank sequences of adder, grass snake or smooth snake.

DISCUSSION

This is the first study to demonstrate the feasibility of DNA amplification from snake faeces, egg and foetal material, as well as from other non-invasive samples including slough skin and non-invasively sampled (NIS) muscle. Although snake faeces have previously been used to identify the morphological remains of specific prey items (e.g. in the black rat snake; Weatherhead et al., 2003), their utility as an NIS material for DNA extraction from snakes had not previously been investigated. The most reliable NIS sources in this study were muscle tissue and slough skin, with ancient tissue samples being the least reliable. Some snake by-products can be easily lo-

cated in the field, but consideration must be given to exclude temporal duplication of samples from the same individual, for instance multiple skin sections from the same slough that have broken up and dispersed. If only a small number of samples are analysed, such non-random sampling can skew data and give a false representation of population genetic diversity. However, repeated sampling of the same individual over time can provide useful ecological data regarding movement and lifespan. Recording the exact location of collected samples is critical and advances in geographic information system (GIS) technology (Salem, 2003) allow patterns of genetic structure to be analysed in a geographical context (e.g. Kidd & Ritchie, 2006). It is also important to record the age of the sample and method of preservation. PCR from template DNA extracted from degraded tissue is problematic, due not only to general DNA degradation but also to the presence of inhibiting factors (Kohn & Wayne, 1997; Wehausen et al., 2004). In addition, using universal (highly conserved) mitochondrial PCR primers, there is an increased likelihood of inadvertently amplifying non-target organism DNA. Furthermore, even if DNA does amplify, decayed nuclear DNA is more commonly associated with genotyping errors, such as allelic dropout and false alleles (Taberlet et al., 1999).

In the current study, we also developed a new method for faeces collection from wild-caught snakes. In the field, British snakes can be secured and scooped vertically from the ground, initiating a defecation defence response. The faeces can be simultaneously collected and appropriately preserved for subsequent DNA extraction. There appeared to be a relationship between stool consistency and successful sample collection: grass snakes (that have loose stools) are more likely to defecate when handled that either smooth snakes (with intermediate stools) or adders (firmer stools). Such variation in faecal consistency is related to diet, with grass snakes preferring amphibians and fish, smooth snakes eating mostly reptiles and rarely small mammals, and adders consuming mostly small mammals. Typically, snakes with loose stools defecate more regularly than those with firm stools and so our observations are not surprising, but we demonstrate how this natural response can be exploited for semi-NIS.

Faecal samples have previously been an overlooked source of reptile DNA. However, faeces are routinely used to genotype protected species (e.g. Bayes et al., 2000; Goossens et al., 2000; Garnier et al., 2001; Chih-Ming et al., 2004). Although DNA from faecal samples is degraded, microsatellite analyses and sequencing of short amplicons is usually possible, but DNA fragments of greater than 500 bp are difficult to sequence, as observed in the current study. More in-depth studies are required to assess the maximum size of amplicons than can be sequenced from snake faeces. In addition, storage methods for snake faeces should be optimized, as has been done for large mammals. For example, Roeder et al. (2004) described a two-step method of preservation whereby gorilla faecal samples stored in ethanol for 24–36 h were subsequently transferred into silica for optimal DNA preservation.

The most reliable source of non-invasive sample material was muscle tissue from carcasses (road killed and semi-predated specimens), recent museum samples and foetal tissue. Roads provide excellent basking opportunities for snakes (under low-traffic conditions) as they heat up quickly, maintaining temperature throughout the day and into the cooler evening (Ashley & Robinson, 1996; Shine et al., 2004). Unfortunately, snakes are often not quick enough to evade vehicles, exhibiting momentary immobilization in response to traffic (Andrews & Gibbons, 2005), but no agency records snake road kill statistics for the UK, despite the fact that animal carcasses do provide high quality genetic data for a multitude of studies including phylogeography and phylogenetics (Keogh, 1998; Doyon et al., 2003; Piertney et al., 2005).

DNA from grass snake eggs was successfully extracted and amplified, but success rates could not be assessed during the current study due to the small sample size. Unhatched eggs should only be collected late in summer/autumn after all viable eggs have hatched, but such samples are likely to be contaminated by microbial PCR inhibitors (Fernando et al., 2003). Avian eggshell membrane is established as a non-invasive DNA source (Fernando et al., 2003; Strausberger & Ashley, 2001), allowing genotyping of an identified egg-laying female without disturbance; however, this is the first study to show that yolk tissue can also be used to identify British reptiles such as the grass snake or sand lizard. We have not yet tested whether DNA can be successfully extracted from the membranous egg shells of grass snakes.

Of the non-invasive materials tested in this study, sloughed skin was the easiest to collect and store directly from the field. DNA extraction from slough does require an initial rehydration step, but this is still a simple and quick method. The lower yield of DNA (compared to muscle) and its potentially fragmented condition can make amplification above 500 bp intermittent, especially in older samples. However, with relatively fresh sloughs it is possible to amplify DNA fragments in excess of 750 bp, although previous studies indicate the ability to amplify such products would diminish with time (Fetzner, 1999). Sloughed skins are often subjected to moisture, UV and microbial damage before collection, which reduces DNA quality. Generally, it is recommended that studies based on dry, room-temperature-stored sloughs collected over a year (in various states of decay) should not aim to target sequences over 700 bp (e.g. Ursenbacher et al., 2006). The inclusion of slough as a source of DNA is becoming more frequent for snakes (Vidal et al., 2000; Burbrink 2002; Clark et al., 2003) and other animals (e.g. Fetzner, 1999; Sigler et al., 2002; Valsecchi et al., 1998).

Ancient tissue sources were amongst the least viable non-invasively collected source tissues for reptiles. This could be due to degradation of DNA (Thomas et al., 2005; Krause et al., 2006) or contamination of the sample storage medium, e.g. in the event of evaporation, alcohol preserved sample bottles being "topped up" from neighbouring bottles containing related sample material. Although a single band may apparently be amplified, it is common to produce a recombinant or multiple sequence

comprising two or more individuals. Depending on storage media and sample age, extractions from preserved museum samples often prove labour-intensive (Pääbo, 1989; Serth et al., 2000).

Non-invasively collected British herpetofaunal samples reliably yielded DNA of sufficient quality and quantity for sequencing; in fact the current study is a conservative estimate of the value of snake NIS tissues for genetic studies as we intentionally targeted relatively long DNA fragments (500 and 758 bp). Populations and species of an endangered, threatened or highly protected nature, such as the British sand lizard or smooth snake, can now be studied non-invasively, producing viable data and valuable insight into the ecology of these reclusive animals.

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